Directed Alteration of the D1 Polypeptide of Photosystem II: Evidence That Tyrosine-161 Is the Redox Component, Z, Connecting the Oxygen-Evolving Complex to the Primary Electron Donor, P680

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ABSTRACT: In photosystem II, electrons are sequentially extracted from water at a site containing Mn atoms and transferred through an intermediate carrier (Z) to the photooxidized reaction-center chlorophyll ($P680^+$). Two polypeptides, D1 and D2, coordinate the primary photoreactants of the reaction center. Recently Debus et al. [Debus, R. J., Barry, B. A., Babcock, G. T., & McIntosh, L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 427–430], have suggested that Z is a tyrosine residue located at position 161 of the D1 protein. To test this proposal, we have engineered a strain of the cyanobacterium *Synechocystis* PCC 6803 to produce a D1 polypeptide in which Tyr-161 has been replaced by phenylalanine. Wild-type *Synechocystis* PCC 6803 contains three nonidentical copies of the *psbA* gene which encode the D1 polypeptide. In the mutant strain, two copies were deleted by replacement with antibiotic-resistance genes, and site-directed mutations were constructed in a cloned portion of the remaining gene (*psbA*-3), carrying a third antibiotic-resistance gene downstream. Transformants were selected for antibiotic resistance and then screened for a photoautotrophy-minus phenotype. The mutant genotype was verified by complementation tests and by amplification and sequencing of genomic DNA. Cells of the mutant cannot evolve oxygen and, unlike the wild type, are unable to stabilize, with high efficiency, the charge-separated state in the presence of hydroxylamine and DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea]. Analyses by optical and EPR spectroscopy of reaction centers purified from this mutant indicate that Z can no longer be photooxidized and, instead, a chlorophyll cation radical, Chl^+, is produced in the light. In the wild type, charge recombination between Z^+ and the reduced primary quinone electron acceptor Q_A^- occurs with a $t_{1/2}$ of 80 ms. In the mutant, charge recombination between Chl^+ and Q_A^- occurs with a $t_{1/2}$ of 1 ms. From these observations, we conclude that Z is indeed Tyr-161 of the D1 polypeptide.

Photosystem II (PSII)[1] possesses a number of redox-active components which enable it to catalyze the oxidation of water and the reduction of plastoquinone. Although many of these cofactors have been characterized biophysically, it is only recently that a picture has emerged of the protein framework which anchors them in the complex. Two polypeptides, D1 and D2, are now thought to bind all the species required for charge transfer across the membrane (Nanba & Satoh, 1987). Little is known about their three-dimensional structure. They do, however, show some similarities in primary structure to the L and M subunits of the reaction centers of purple bacteria [reviewed in Michel and Deisenhofer (1988)]. This homology, plus knowledge of the structure of these reaction centers from *Rhodopseudomonas viridis* (Deisenhofer et al., 1985) and *Rhodobacter sphaeroides* (Allen et al., 1988; Chang et al., 1986), has been used to predict the folding pattern of the D1 and D2 heterodimer within the membrane and also to propose functional roles for individual amino acids (Trebst, 1986; Michel & Deisenhofer, 1988).

Of particular interest in this study is the redox-active component, known as Z, which acts between the primary electron donor chlorophyll (P680) of PSII and the Mn cluster that extracts electrons from water. Several lines of evidence have suggested that Z and another component of the oxidizing side of PSII, known as D, are tyrosine residues (Barry & Babcock, 1987; Gerken et al., 1988; Debus et al., 1988a). Substitution of specific tyrosine residues with amino acids of similar structure, but different redox properties (e.g., phenylalanine), should provide direct evidence as to their identity. Debus et al. (1988a) and also Vermaas et al. (1988) were able to engineer mutants of the cyanobacterium *Synechocystis* PCC 6803 (*Synechocystis* 6803), in which the Tyr-160 of the D2 polypeptide was changed to a phenylalanine (i.e., D2-Y160F). They were able to demonstrate that these mutants lacked the EPR signal associated with the oxidized free radical form of D. Furthermore, Debus et al. (1988a) hypothesized that Z would be the Tyr-161 residue of the D1 polypeptide. Recently, a mutant of *Synechocystis* 6803, in which this tyrosine was changed to a phenylalanine (i.e., D1-Y161F), was obtained and shown to have impaired electron donation to $P680^+$ (Debus et al., 1988b). In this paper, we describe an independently constructed D1-Y161F mutant of *Synechocystis* 6803 and

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[1] Abbreviations: Chl, chlorophyll; D1, a polypeptide component of the PSII reaction center, encoded by the *psbA* gene; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; F_{max}, maximum fluorescence yield, with all Q_A reduced; F_0, initial fluorescence yield, with all Q_A oxidized; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; P680, a chlorophyll molecule (or pair of chlorophyll molecules), which acts as the primary electron donor of the PSII reaction center; PSII, photosystem II; Q_A, primary quinone electron acceptor; Q_B, secondary quinone electron acceptor; $Td_{1,2}$, a transformed strain of *Synechocystis* 6803, in which *psbA*-1 and *psbA*-2 have been deleted (see also wild type*); wild type*, the $Td_{1,2}$ strain of *Synechocystis* 6803 [since it contains only one copy of the *psbA* gene (i.e., *psbA*-3), it serves as the reference wild-type strain used during biophysical characterization of the D1-Y161F and D1-Y161 stop mutants].

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present a detailed biophysical characterization of this mutant [an abstract concerning preliminary aspects of this work has been published (Diner et al., 1988)].

Synechocystis 6803 is a unicellular cyanobacterium which possesses a natural DNA uptake system that allows it to be readily transformed via integration of exogenous DNA into its chromosome by homologous recombination (Williams, 1988). Additionally, this organism can grow photoheterotrophically when provided with glucose, allowing for recovery of mutants lacking PSII activity. *Synechocystis* 6803 contains three copies of the *psbA* gene which encode the D1 polypeptide (Jansson et al., 1987). Our strategy for introducing alterations into the D1 polypeptide of the PSII reaction center complex involved deletion of two of the *psbA* genes and crossing in mutations into the third copy with a plasmid construction in which an antibiotic-resistance gene had been placed downstream to permit selection for transformants. Our detection of transformants which possessed the desired mutations in *psbA* relied on the assumption that these alterations would impair PSII activity (i.e., result in a $PSII^-$ phenotype).

Experimental Procedures

Growth and Manipulation of Cyanobacterial Strains. Wild-type *Synechocystis* 6803 and the mutant strains were grown either in liquid media or on agar plates under constant illumination as described in Rippka et al. (1979) and Williams (1988). Glucose, at 5 mM, was included in all media except when photoautotrophic growth was being assayed (i.e., PSII activity). Transformations and isolation of DNA from *Synechocystis* 6803 were performed as described by Williams (1988). The mutant strains were maintained on media supplemented with appropriate antibiotics (25 μg/mL kanamycin, 5 μg/mL chloramphenicol, and 20 μg/mL spectinomycin). An inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), at 10 μM was added when it was necessary to eliminate the influence of PSII activity on growth rates (i.e., when introducing mutations into the Tyr-161 codon of *psbA*-3, see below). All transformants were maintained on selective media for at least 1 month prior to further manipulation to allow time for segregation of the multiple chromosomes of *Synechocystis* 6803 (Williams, 1988).

Manipulations of DNA and Analyses of DNA Sequence Data. Manipulations of DNA, such as cloning and Southern blot analysis, were performed according to standard protocols (Maniatis et al., 1982). Amplification of *Synechocystis* 6803 genomic DNA was accomplished by the polymerase chain reaction (PCR) method using *Taq* DNA polymerase (Saika et al., 1988) and a commercially available kit (from Perkin Elmer Cetus, Norwalk, CT). Two 24-bp oligonucleotides (G+C contents of 56 and 58%) which flank a 530-bp region of *psbA*-3 containing the Tyr-161 codon were made on a Coder 300 DNA synthesizer (Du Pont, Wilmington, DE). The thermal cycling routine for PCR consisted of a 1-min denaturation step at 94 °C followed by annealing and extension at 62 °C for 5 min (Kim & Smithies, 1988). This was repeated 27 times, with a final extension time of 10 min at 64 °C. The amplified DNA was cloned by cutting with the restriction enzymes *Taq*I and *Kpn*I (see Figure 1) and ligating into a *Cla*I/*Kpn*I cut Bluescript vector (Stratagene Cloning Systems, La Jolla, CA). Computer-assisted analyses of DNA sequence data utilized the software package of the Genetics Computer Group of the University of Wisconsin (Devereux et al., 1984).

Deletion of psbA-1 and psbA-2. Wild-type *Synechocystis* 6803 contains three nonidentical copies of the *psbA* gene (termed *psbA*-1, *psbA*-2, and *psbA*-3) (Jansson et al., 1987).

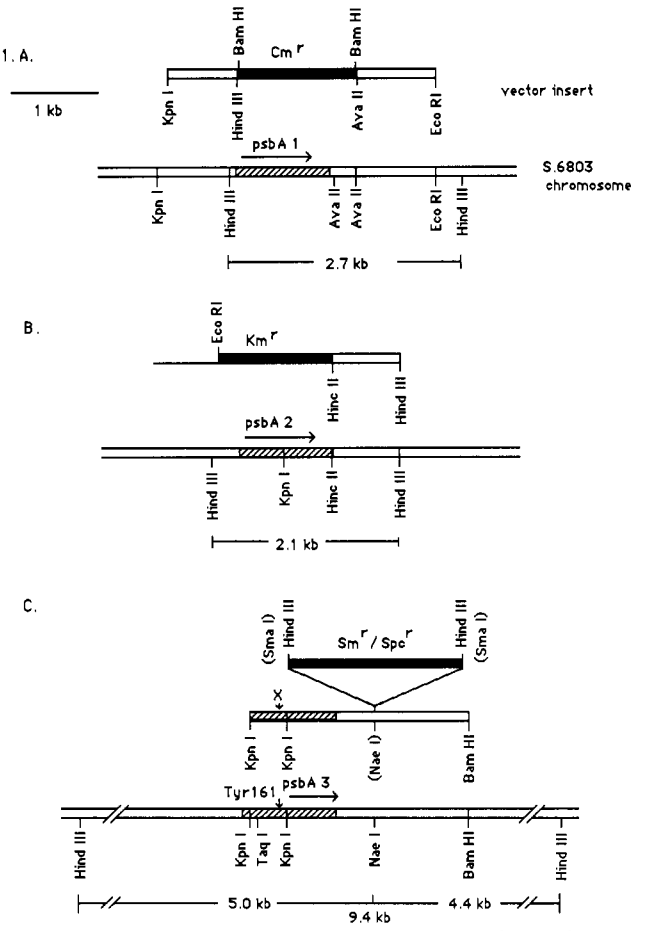

FIGURE 1: Restriction maps of the three *psbA* genes of *Synechocystis* 6803 and the plasmid constructions used to delete or to introduce directed modifications into these genes. The chromosomal DNA is represented as open-ended bars with the locations of the *psbA* genes shown as striped areas. The insert DNA within the plasmid constructions designed to modify the *psbA* loci are shown above the corresponding chromosomal DNA. Regions of these plasmids containing chromosomal DNA (i.e., regions where homologous recombination can occur) are indicated as open or striped bars, and the relevant restriction sites are indicated. The genes conferring resistance to the various antibiotics are shown as solid bars. (A) The scheme used for deletion of *psbA*-1 and its replacement with the Cm^R gene. (B) The *psbA*-2 gene was deleted from the chromosome by ectopic mutagenesis as described in the text. The solid line to the left of the Km^R gene represents vector DNA. Southern blot analysis indicates that, in addition to recombination occurring in the region of homologous DNA, heterologous recombination occurred between the vector DNA and the chromosome at a site upstream of *psbA*-2, resulting in a deletion of this gene. (C) Directed modifications of the *psbA*-3 Tyr-161 codon (indicated with an X) were carried into the gene with the construction shown here. Further details are provided in the text.

A plasmid containing 3.2 kb of *Synechocystis* 6803 genomic DNA, including all of *psbA*-1, and another plasmid containing 1.3 kb of genomic DNA including 0.5 kb of the 3′ end of the coding region of *psbA*-2 were kindly provided by J. G. K. Williams (Du Pont, Wilmington, DE). For deletion of *psbA*-1, the entire coding region of the gene was replaced with a 1.3-kb *Bam*HI fragment (originally from pACYC 184; Chang & Cohen, 1978) which encodes resistance to chloramphenicol (Cm). This was accomplished by excising the *psbA*-1 gene at the *Hin*dIII and *Ava*II restriction sites, indicated in Figure 1A, and then by using two synthetic oligonucleotides to engineer a new *Bam*HI restriction site for insertion of the Cm-resistance gene. The resulting construct was used to transform *Synechocystis* 6803 to Cm resistance through homologous recombination. Treatment of $\sim 3 \times 10^7$ cells with 2 μg of

plasmid DNA yielded over 4000 Cm^R transformants. Southern blot analysis of one such clone (Td_1) confirmed that all of *psbA*-1 had been deleted (data not shown; but see Figure 2).

DNA clones were not available which contained the 5′ end of *psbA*-2; therefore, an alternative method was used to delete this gene (see Figure 1B). The clone containing 0.5 kb of the 3′ end of *psbA*-2 and 0.8 kb of downstream DNA was digested with *Eco*RI (present in the multiple cloning region of the pUC19 vector; Yanisch-Perron et al., 1985) and *Hin*cII to remove all but the final six base pairs of the *psbA*-2 coding region. It was replaced with a 1.2-kb, *Eco*RI/*Hin*cII, kanamycin- (Km) resistance gene cassette (originally from pUC4K; Vieira & Messing, 1982). Treatment of $\sim 1 \times 10^9$ cells of *Synechocystis* 6803 with 34 μg of DNA of the final plasmid construction yielded seven Km^R colonies. Six of these clones were also ampicillin (Amp) resistant (at 10 μg/mL). The pUC19 plasmid vector carries the Amp^R gene, and Southern blot analyses of genomic DNA from these clones revealed that the entire plasmid construction had integrated at the *psbA*-2 locus via a single crossover event (data not shown). The remaining clone (Td_2) was sensitive to Amp, suggesting that, in addition to homologous recombination in the *psbA*-2 downstream flanking DNA, a nonhomologous recombination event had occurred between the vector prior to, or within, the Amp^R gene and the chromosome [i.e., ectopic mutagenesis; see Buzby et al. (1985) and Golden (1988)]. The result is the replacement of a portion of the *Synechocystis* 6803 chromosome, containing the *psbA*-2 gene, with the Km^R gene plus an undefined (but probably less than 1.8 kb) portion of the pUC19 plasmid. Transformation of this strain with the plasmid construction shown in Figure 1A, resulted in the recovery of many Km^R/Cm^R colonies in which both *psbA*-1 and *psbA*-2 had been deleted. Figure 2 shows a Southern blot of *Hin*dIII-digested genomic DNA which has been probed with radiolabeled *psbA* gene from *Amaranthus hybridus* (Hirschberg & McIntosh, 1983). It can be seen that in the Km^R/Cm^R strain ($Td_{1,2}$; Figure 2, lane 2), two of the *psbA* hybridizing bands have been deleted.

Mutagenesis of psbA-3. Plasmid libraries of $Td_{1,2}$ genomic DNA (lacking *psbA*-1 and *psbA*-2) were screened with the *A. hybridus psbA* gene as a probe, and two positive clones containing inserts of 0.4 kb (*Kpn*I/*Kpn*I) and 2.1 kb (*Kpn*I/*Bam*HI) were recovered. These two clones were sequenced with the dideoxynucleotide chain-termination method (Sanger et al., 1977) and a commercial kit (Sequenase) from U.S. Biochemical Corp., Cleveland, OH. Comparison to the published sequence for *psbA*-1 of *Synechocystis* 6803 (Osiewacz & McIntosh, 1987) revealed that the *Kpn*I/*Kpn*I clone contained 438 bp from near the 5′ end of the gene (positions 99–537 of the *psbA*-1 coding region), including the codon for Tyr-161 (centered at position 482). The other fragment contained the rest of the 3′ end of the gene and 1.7 kb of flanking DNA. In order to select for transformed cells, a 2.0-kb *Sma*I fragment of pHP45-Ω (Prentki & Kirsch, 1984) which encodes resistance to spectinomycin (Spc) was inserted into a *Nae*I site 460 bp downstream of the *psbA*-3 coding sequence (Figure 1C) in the *Kpn*I/*Bam*HI fragment.

Conversion of the *psbA*-3 Tyr-161 codon, TAC, to TTC (Phe), or to TAG (stop), was accomplished by oligonucleotide directed mutagenesis employing the enrichment method described by Kunkel et al. (1987). The protocols outlined in a commercial kit obtained from Bio-Rad Laboratories (Richmond, CA) were followed. The *Kpn*I/*Kpn*I fragment was subcloned into M13 for the generation of single-stranded template DNA, and the oligonucleotides ACCAATGGG*GAA*GATCAAGAA (Tyr to Phe) and ACCAATGGG*CTA*GATCAAGAA (Tyr to stop) were used for introduction of directed changes. Clones containing the mutated DNA were identified by sequencing the region of interest. Those which were selected for transformation of *Synechocystis* 6803 strains were sequenced over the entire length of the *Kpn*I/*Kpn*I insert to verify that only the intended alterations had been introduced. The clone used to introduce the stop signal contained the desired TAC (Tyr) to TAG (stop) codon conversion. In this case, the G was unintentionally inserted between the original A and C rather than replacing the C (see Figure 3C). The mutated fragments were cloned into the *Kpn*I site of the *Kpn*I/*Bam*HI fragment, containing the Spc^R gene inserted downstream of the *psbA*-3 coding region (see Figure 1C), and the orientation of the DNA in the final constructions was verified. Cells of the $Td_{1,2}$ strain were incubated with the appropriate plasmid DNA, and Spc^R transformants were selected. These colonies were then screened for a $PSII^-$ phenotype (i.e., inability to grow photoautotrophically).

Preparation of PSII Particles. PSII reaction center particles were prepared from cells grown in liquid medium. All subsequent procedures were carried out at 4 °C or on ice. The cells were collected by centrifugation (8300*g*, r_{av} = 85 mm, for 5 min), washed with solution A (20 mM MES, pH 6.5, 20 mM $CaCl_2$, 10 mM $MgCl_2$), and suspended in solution B (solution A containing 0.5 M mannitol). They were broken by a pulsed glass bead treatment (0.2-mm beads with 10 pulses of 30 s and 2-min cooling intervals) in a Bead Beater (Biospec Products, Bartlesville, OK) which was cooled by ice–water. The broken cells were filtered through Miracloth to remove the beads and centrifuged (1100*g*, r_{av} = 80 mm, for 6 min). The supernatant was spun (125000*g*, r_{av} = 69.8 mm, for 30 min) to pellet the thylakoid membranes. These were homogenized in solution C (solution B containing 20% glycerol) and diluted to a final Chl concentration of about 1 mg/mL. The membranes were extracted with 1% dodecyl β-D-maltoside (30-min stirring in the dark), followed by a centrifugation step (184000*g*, r_{av} = 65.7 mm, for 90 min) to remove nonsolubilized material. The supernatant fraction was layered onto sucrose density gradients (8–32% in solution B containing 0.04% dodecyl β-D-maltoside) and spun for 14 h at 150000*g*, r_{av} = 66.6 mm. The upper of two green bands was dialyzed against solution B and concentrated in an Amicon ultrafiltration cell fitted with a YM100 membrane. PSI and PSII particles were separated on an anion-exchange column (Mono Q HR 10/10, Pharmacia) in a Hewlett-Packard HPLC apparatus (Model 1090M), according to a modification of the procedure of Dekker et al. (1988). Details of this procedure will be described in a forthcoming paper.

The numbers of chlorophylls per PSII reaction center were determined by measuring the absorbance change, at 320 nm, corresponding to the reduction of primary quinone electron acceptor, Q_A ($\Delta\epsilon$ = 12.5 mM^{-1} cm^{-1}). Wild-type PSII core preparations were preincubated with 10 μM $K_3Fe(CN)_6$ in 20 mM HEPES, pH 7.5, to oxidize Q_A^-. NH_2OH (2 mM) was added in the dark and the ΔA_{320nm} measured following a series of 15 saturating actinic flashes. The addition of DCMU prior to illumination had no effect on the ΔA_{320nm}, consistent with the absence of secondary electron quinone acceptor, Q_B. In the case of the D1-Y161F mutant PSII core preparations, the Chl/Q_A was determined by measuring the ΔA at 20 μs, associated with the reduction of Q_A at 325 nm ($\Delta\epsilon$ = 13 mM^{-1} cm^{-1}). These determinations gave values of 45–50 Chl/Q_A in both preparations.

Optical Spectroscopy. Light-induced absorbance changes were measured on PSII core preparations with a flash-detection spectrophotometer, similar to that originally described by Joliot et al. (1980). Saturating actinic laser flashes, provided by a Candela SLL250 dye laser using Sulforhodamine 640 (λ_{max} = 645 nm), were followed by a series of detecting flashes provided by a xenon flash (EG&G, FX199U) passed through a Jobin-Yvon HL300 monochromator. The sample and reference photodiodes (UV-444BQ, EG&G) were protected by either Corion Solar Blind Filters for measurements in the ultraviolet or Corning 4-96 filters for measurements in the visible.

Fluorescence Yield Measurements. Flash-induced increases in the fluorescence yield of Chl in whole cells were measured in the flash-detection spectrophotometer using a xenon actinic flash (EG&G, FX199) filtered by an infrared reflecting filter (MTO Athervex TA2) and a Corning 4-96. The fluorescence yield was measured with probe flashes at 422 nm. The sample photodiode was protected by a set of blocking filters—Schott LF550, Ulano Rubylith, Corning 2-64, and a Kodak Wratten 70 which together transmitted wavelengths of >670 nm.

Electron Spin Resonance Spectroscopy. X-band EPR measurements at low temperatures were performed on a JEOL ME-3X spectrometer interfaced to a DEC MINC-23 computer and equipped with an Oxford ESR-900 liquid helium cryostat. EPR measurements at room temperature were performed on a Varian E-9 spectrometer.

Results

Construction of the Mutants. In order to examine the effects of specific mutations in *psbA*, we first deleted two of the three copies of this gene (i.e., *psbA*-1 and *psbA*-2). This strain is capable of photoautotrophic growth, demonstrating that *psbA*-3 can be expressed and its product can function in PSII reaction centers [see also Jansson et al. (1987)]. The desired mutations in *psbA*-3 were incorporated into the genome of *Synechocystis* 6803 by transforming this double-deletion strain with the construction shown in Figure 1C. Spc^R colonies were presumed to have resulted from a double-crossover event, one or either side of the original *Nae*I site, so that a proportion now contained the desired codon change depending upon whether recombination had occurred upstream or downstream of the codon for Tyr-161. After growth for 1 month on media containing spectinomycin, glucose, and DCMU, the resulting colonies were streaked onto plates lacking the glucose and DCMU (the same colonies were also transferred to new media containing spectinomycin, glucose, and DCMU for further use). Approximately 12% of the Spc^R colonies resulting from transformation with Tyr-to-stop and Tyr-to-Phe constructions were incapable of photoautotrophic growth, consistent with an impaired *psbA* gene product. That the cause of this lesion was present in the 0.44-kb *Kpn*I fragment containing the Tyr-161 codon was confirmed by transformation of the $PSII^-$ strains with wild-type and mutant forms of this fragment. The wild-type form restored photoautotrophic growth at ~100 times the level of the mutant forms, indicating that the alteration responsible for the $PSII^-$ phenotype was at or near the Tyr-161 codon.

A Southern blot analysis of genomic DNA isolated from a $PSII^+/Spc^R$ strain and from $PSII^-/Spc^R$ strains resulting from transformation with both the Tyr-to-stop and Tyr-to-Phe constructions was performed (Figure 2). Since there are *Hin*dIII restriction sites associated with the Spc^R gene, it was anticipated that in these mutants the *psbA*-3 gene would now be present in a 5.0-kb *Hin*dIII fragment rather than in the 9.4-kb fragment found in the wild-type strain (see Figure 1C).

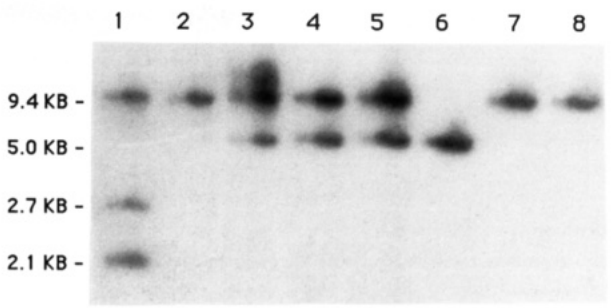

FIGURE 2: Southern blot analysis of DNA from wild-type *Synechocystis* 6803 and the strains containing alterations in the *psbA* genes. Total genomic DNAs were digested with *Hin*dIII and the fragments separated by agarose gel electrophoresis, transferred to a nylon membrane (Gene Screen Plus, NEN Research Products, Boston, MA), and hybridized with a radiolabeled *psbA* gene from *A. hybridus*. The size, in kb, of the *psbA*-hybridizing fragments are indicated to the left of the autoradiogram. Lane 1: Wild-type *Synechocystis* 6803, showing the three *psbA* *Hin*dIII fragments (*psbA*-1 = 2.7 kb, *psbA*-2 = 2.1 kb, and *psbA*-3 = 9.4 kb). Lane 2: The double-deletion strain ($Td_{1,2}$), in which *psbA*-1 and *psbA*-2 have been replaced by the Cm^R and Km^R genes, respectively. Lanes 3, 4, and 5: Examples of Spc^R transformants, from cells maintained on 25 μg/mL Spc, showing the original 9.4-kb band and the new 5.0-kb band (see text for discussion). Lane 3, $PSII^+$; lane 4, $PSII^-$ (D1-Y161F); lane 5, $PSII^-$ (D1-Y161stop). Lanes 6 and 7: DNA fragments from the putative D1-Y161F strain, size fractionated by agarose gel electrophoresis and rerun on this gel, showing the separation of the two *psbA*-hybridizing bands prior to PCR amplification and cloning (see text and Figure 3D,E). Lane 6, 5.0-kb zone; lane 7, 9.4-kb zone. Lane 8: A $PSII^-/Spc^S$ strain (D1-Y161F) showing the single *psbA* hybridizing band at 9.4 kb.

However, both mutants gave a mixture of these bands, indicating the presence of both types of *psbA*-3 locus. Cyanobacteria similar to *Synechocystis* 6803 have been shown to contain multiple copies of their genome (Mann & Carr, 1974; Golden et al., 1986), so a mixture of *psbA*-3 loci could be tolerated. Despite growing these mutants in up to 100 μg/mL Spc, we failed to segregate strains which showed just the 5.0-kb *Hin*dIII band upon Southern analysis. This indicated that there was a selectional pressure to maintain some of the genomic copies of *psbA*-3 gene without the Spc^R gene downstream. This is not due to any influence of PSII activity, since the inhibitor, DCMU, was continually present during growth of the cells. Subsequent sequencing experiments have shown that the *Nae*I site into which the Spc^R gene was inserted lies within an open reading frame starting some 155 bp downstream of the *psbA*-3 stop codon. This open reading frame shows strong sequence homology to the *dnaE* gene of *Bacillus subtilis* (Wang & Doi, 1986) which encodes a DNA primase (data not shown). It seems reasonable to assume that the open reading frame downstream of *psbA*-3 also encodes an essential protein, possibly a primase, thus explaining the maintenance of a partial diploid state during Spc^R selection [see also Golden et al. (1986)]. Gene conversion (e.g., double-crossover events between identical regions of the chromosomes) would explain the ability to isolate a $PSII^-$ phenotype indicative of the *psbA*-3 mutations while having heterogeneity at the Spc^R gene insertion site downstream. When Spc is omitted from the growth medium, $PSII^-/Spc^S$ strains were readily obtained, which upon Southern blot analysis possessed a pattern identical with the parental double-deletion strain (Figure 2, lane 8). These strains could also be transformed to a $PSII^+$ phenotype by the wild-type 0.44-kb *Kpn*I fragment at a 100-fold higher rate than the mutant form of this fragment.

In order to verify that *psbA*-3 in these strains contains the desired mutations, the region of genomic DNA containing the Tyr-161 codon was amplified and sequenced. Figure 3 shows

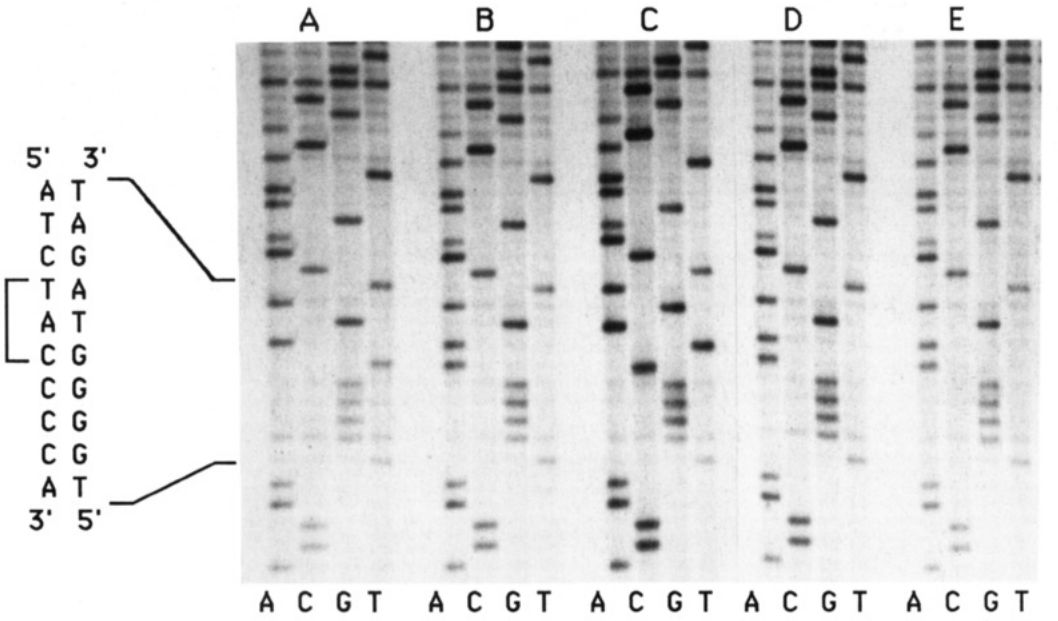

FIGURE 3: Sequencing gel autoradiogram showing the region of the *psbA*-3 genes of *Synechocystis* 6803 containing the Tyr-161 codon (the data from the anticoding strand are shown). (A) The $PSII^+/Spc^R$ strain showing the original wild-type sequence (indicated to the left of the autoradiogram, along with the complementary coding strand sequence, the Tyr-161 codon is marked with a bracket). (B) The $PSII^-/Spc^R$ (D1-Y161F) mutant, showing the single base change which results in the conversion of the TAC Tyr codon to a TTC Phe codon. (C) The $PSII^-/Spc^R$ (D1-Y161stop) strain showing the insertion of a single base which converts the TAC Tyr codon to a TAG stop codon. (D and E) Sequences derived from PCR-amplified DNA from the two, electrophoretically separated, *psbA*-hybridizing bands shown in Figure 2, lanes 6 and 7, respectively. Both show the sequence of the D1-Y161F mutation.

a portion of the autoradiograph of a sequencing gel which includes the Tyr-161 codon of *psbA*-3. Sequence data are shown for DNA from a $PSII^+/Spc^R$ clone, which retains the wild-type *psbA*-3 sequence (lane A), and examples of the $PSII^-/Spc^R$ strains obtained from transformation with the Tyr-to-Phe (lane B) and Tyr-to-stop (lane C) constructions. It can be seen that the TAC sequence of the Tyr codon in *psbA*-3 has been altered to TTC and to TAG (with the G as an insertion), as expected. As a further test that the *psbA* genes present on the 5.0- and 9.4-kb *Hin*dIII fragments (Figure 2, lane 4) were identical, we used agarose gel electrophoresis to separate these bands (see Figure 2, lanes 6 and 7) and then amplified and sequenced the region containing putative mutations. Figure 3, lanes D and E, shows the sequence data, and it is clear that the *psbA*-3 gene associated with both bands contains the TAC to TTC alteration. For each of the sequences analyzed, no base changes, other than those indicated above, were detected in this region of DNA. All of these data indicate that we have indeed isolated mutants of *Synechocystis* 6803 which contain a homogeneous population of the *psbA*-3 gene into which we have introduced either a Phe codon or a stop signal in place of Tyr-161 codon present in the wild-type gene. These strains will now be referred to as D1-Y161F and D1-Y161 stop, to indicate these alterations to the D1 protein. For biophysical characterizations, the double-deletion strain, containing only *psbA*-3, serves as the reference wild-type strain (termed wild type*) to eliminate possible D1 polypeptide heterogeneity arising from differential expression of the *psbA* genes.

Flash-Induced Increase in Fluorescence Yield. Photoreduction of Q_A in whole cells is readily followed by the measurement of the relative fluorescence yield of Chl during illumination in the presence of the inhibitor, DCMU, which blocks oxidation of Q_A^- by Q_B. Q_A is initially present in the oxidized state in the dark and is photoreduced upon illumination. As there is little indication of energy transfer (Joliot & Joliot, 1964) between PSII reaction centers in *Synechocystis* 6803, the fluorescence yield is an approximate linear indicator of the redox state of Q_A. The fluorescence yield is low (F_0 level) with Q_A fully oxidized and high (F_{max} level) with the acceptor fully reduced (Duysens & Sweers, 1963).

If the D1-Y161F mutation were indeed to have eliminated secondary donor Z, then one would expect charge recombination between the donor and acceptor sides to occur more rapidly, the primary donor being unable to transfer its oxidizing equivalent to Z. One indicator of such a situation would be to look at the increase in fluorescence yield in the presence of the artificial donor, hydroxylamine (Bennoun & Joliot, 1969), and DCMU. Following light-induced charge separation, DCMU would block oxidation of Q_A^- by Q_B, while NH_2OH would compete with Q_A^- for reduction of the oxidized donor. NH_2OH at a concentration greater than 1 mM would also inactivate the oxygen-evolving complex (Cheniae & Martin, 1971), leaving Z as the terminal electron donor to the PSII reaction center. At a given concentration of NH_2OH, the faster the charge recombination between Q_A^- and the oxidized donor, the less the oxidation of NH_2OH and the lower the yield of stable Q_A^- resulting from the charge separation.

Synechocystis 6803 wild-type* (containing only *psbA*-3) and mutant cells (D1-Y161F and D1-Y161stop) were first incubated with 0.5 mM *p*-benzoquinone in the dark for 10 min. Pretreatment with this oxidant was found to be necessary for the complete oxidation of the acceptor side prior to the addition of inhibitor (Wollman, 1978). The cells were then made 20 μM in DCMU and 0, 2, or 10 mM in NH_2OH and illuminated by a series of 15 saturating light flashes (18 Hz). The wild-type* cells show a rapid increase in the fluorescence yield in the presence of 10 mM NH_2OH (Figure 4), attaining the maximum yield within three flashes. Doubling the concentration of NH_2OH has no effect on the flash yields in wild-type* cells (not shown). In the case of the D1-Y161F mutant, the fluorescence yield increases slowly with each flash in the absence of added NH_2OH. The yield per flash is increased upon addition of 2 mM NH_2OH and further increased by raising the concentration to 10 mM. Ten millimolar NH_2OH is the same concentration used for the wild-type* cells, showing

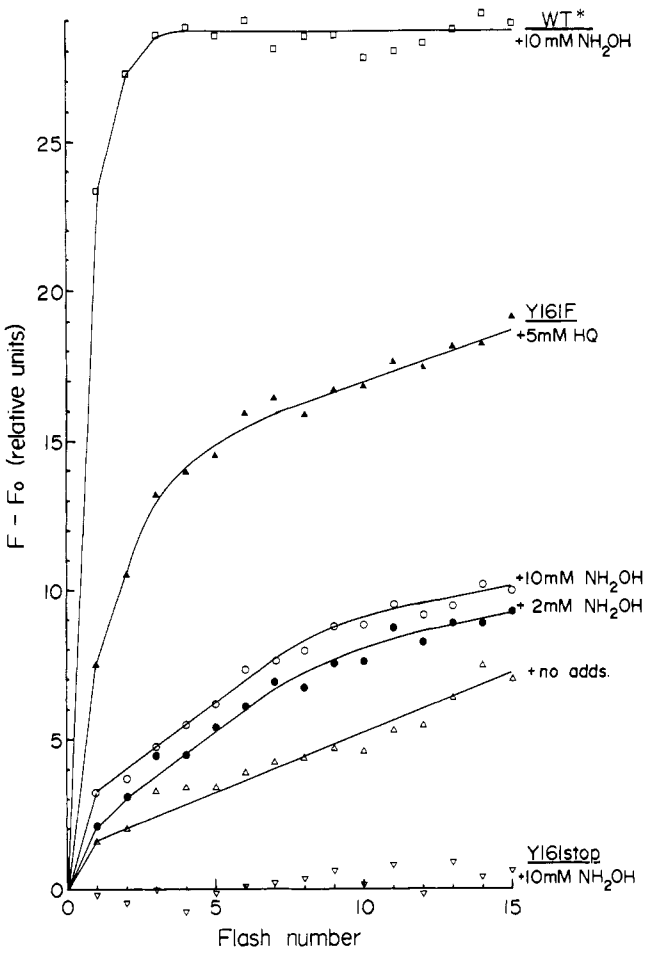

FIGURE 4: Increase in the fluorescence yield of chlorophyll in mutant and wild-type* (i.e., the strain in which *psbA*-1 and *psbA*-2 have been deleted) *Synechocystis* cells during a series of 15 saturating actinic flashes (18 Hz). The fluorescence yield was detected at 5 ms after the actinic flash. The cells of wild-type*, D1-Y161F, and D1-Y161stop strains were suspended in BG-11 medium at 20 μg of Chl/mL. TES buffer (50 mM, pH 8.2) was added in the experiments involving hydroxylamine. The cells were pretreated for 10 min with 0.5 mM *p*-benzoquinone and then made 20 μM in DCMU and 0 (△), 2 (●), or 10 mM (○, □) in NH_2OH or 5 mM in hydroquinone (▲) and incubated for an additional 10 min. (□) Wild type*; (△, ●, ○, ▲) Y161F; (▽) Y161stop. The ratios of $(F_{max} - F_0)/F_0$ for the wild type* and Y161F are respectively 0.8 and 0.5.

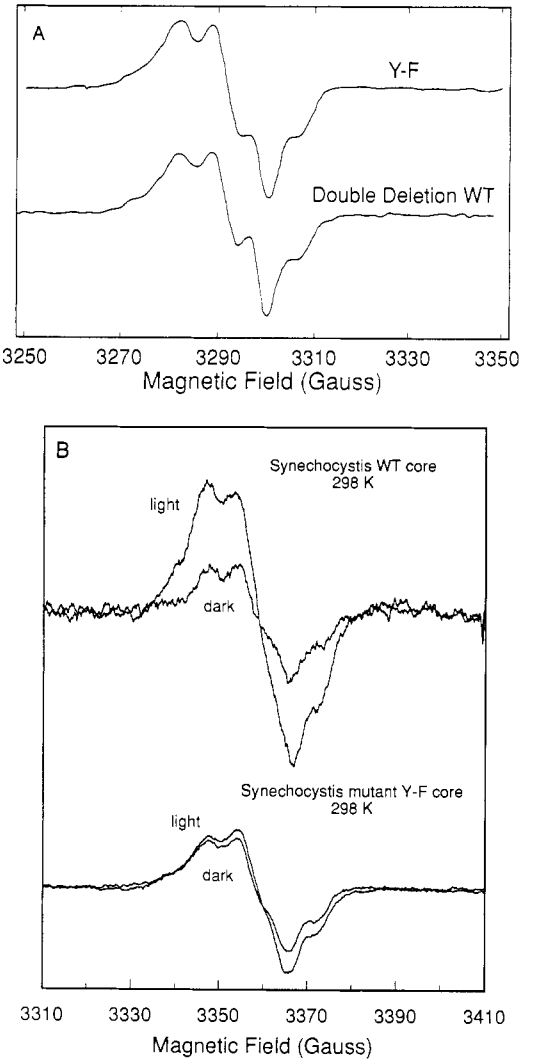

FIGURE 5: EPR spectra of PSII core preparations from *Synechocystis* 6803 D1-Y161F and wild-type* strains at 672 and 200 μM Chl, respectively. (A) Spectra of dark-adapted PSII preparations, in the presence of 15 μM $K_3Fe(CN)_6$, recorded at 15 K. EPR conditions: microwave frequency, 9.2 GHz; microwave power, 80 nW; field modulation amplitude, 4 G; field modulation frequency, 100 kHz. The spectrum of the wild-type* sample was multiplied by a factor 3.36 to compensate for the 3.36 times lower Chl concentration. (B) Room-temperature spectra of the same preparations, in the presence of 300 μM $K_3Fe(CN)_6$, in saturating light and following 4–8 min of dark. EPR conditions: microwave frequency, 9.4 GHz; microwave power, 40 mW; field modulation amplitude, 4 G; field modulation frequency, 100 kHz. The spectra for the wild-type* and D1-Y161F mutant preparations were collected by using 8-min scans with a 3-s time constant and 4-min scans with a 1-s time constant, respectively. The spectra were corrected for the difference in scan rates and, as in (A), are shown normalized to the same Chl concentration.

that the flash yield of Q_A^- is greatly reduced in the mutant. NH_2OH is, therefore, a poor donor in the mutant, producing only a modest flash-induced increase in fluorescence yield compared to *p*-benzoquinone and DCMU alone. The fluorescence rise observed without the addition of reductant is attributed primarily to electron donation by the hydroquinone which is generated in situ from added *p*-benzoquinone and in part from the oxidation of donor D. Donation by hydroquinone in the mutant, also observed by Debus et al. (1988b), is confirmed by the much higher flash yield of Q_A^- upon the addition of 5 mM hydroquinone. The D1-Y161stop mutant, which cannot assemble photoactive PSII reaction centers, shows no increase in fluorescence yield during flash illumination and has an elevated initial fluorescence yield.

These experiments show that the flash-induced increase in fluorescence yield arises solely from PSII and that the D1-Y161F mutation results in a lowered flash yield for the stable reduction of Q_A, consistent with the loss of Z.

Detection of D^+ and Z^+ by EPR in PSII Core Preparations. PSII core preparations were isolated from the wild-type* strain, described above, and from the D1-Y161F mutant. These contain 45–50 Chl/reaction center, on the basis of photoreduction of Q_A. They have no secondary acceptor Q_B. The EPR spectra (Figure 5A), measured in the dark at 15 K, are normalized to the same Chl, and therefore reaction center, concentration by adjusting the gain. These show typical Signal II spectra arising from donor, D^+ (Babcock & Sauer, 1973). They are equivalent in amplitude, indicating that the Y161F mutation in polypeptide D1 does not affect the presence or concentration of this oxidized donor. Figure 5B shows EPR spectra taken at room temperature during illumination with saturating light and then during the ensuing dark period. Ferricyanide (300 μM) needed to be added to ensure a maximum amplitude of light-induced Signal II in saturating light. The wild-type* preparation shows the dark D^+ signal. Signal II is slightly more than doubled in saturating light. This increase arises from Z^+ and a small contribution from D^+ not

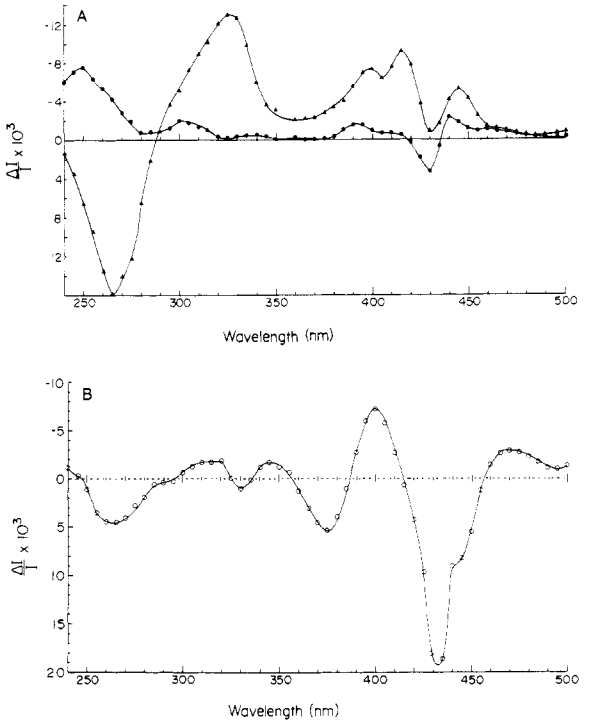

FIGURE 6: Saturating flash-induced absorbance changes measured at 0.5 ms from 240 to 500 nm in the same PSII core preparations as in Figure 5. (A) Wild type*. The $\Delta I/I$ of $Q_A^- - Q_A$ was measured at 270 ms after saturating laser flash excitation of WT* PSII core preparations (20 μM Chl) in the presence of 20 μM benzidine, 10 μM ferricyanide, and 20 mM HEPES, pH 7.5. Shown (▲) is the spectrum multiplied by a factor of 1.37 to give the amplitude at 0.5 ms after the actinic flash (see text). The $\Delta I/I(Q_A^- - Q_A)$ spectrum at 0.5 ms (▲) was subtracted from the total $\Delta I/I$ spectrum at 0.5 ms, measured in the same core preparation (20 μM, Chl) in 20 mM MES, pH 5.9, containing 500 nM $K_3Fe(CN)_6$. (●) Total $\Delta I/I$(0.5 ms) minus $\Delta I/I(Q_A^- - Q_A)$ at 0.5 ms. (B) D1-Y161F. The spectrum shown is the total $\Delta I/I$ spectrum at 0.5 ms measured in the D1-Y161F mutant core preparations (20 μM Chl) in 20 mM MES, pH 5.9, containing 500 nm $K_3Fe(CN)_6$ minus 0.85 times the $Q_A^- - Q_A$ spectrum at 0.5 ms (▲) of (A) (see text).

entirely oxidized in the dark. Z^+ and D^+ are each assumed to contribute one spin per reaction center (Babcock, 1987). A similar experiment performed on the D1-Y161F mutant shows only a very small increase in the Signal II amplitude, which we attribute to the oxidation of D alone. Z oxidation is, therefore, not observed in the mutant.

Optical Absorbance Changes following Flash Illumination of PSII Core Preparations. The wild-type* core preparation used above was given single saturating laser flashes in the presence of 20 μM benzidine and 10 μM ferricyanide at pH 7.5. This artificial donor–acceptor couple is capable of existing in solution such that ferricyanide can reoxidize Q_A^- and benzidine can reduce the oxidized donor, $P680^+$, following charge separation. Under these conditions, a light – dark difference spectrum, recorded 270 ms after the actinic flash, represents only the $Q_A^- - Q_A$ difference spectrum, as Z^+ is completely reduced by this time ($t_{1/2} \approx 3$ ms). Control experiments with hydroxylamine confirmed that oxidized benzidine did not contribute to this spectrum (Diner & de Vitry, 1984). Figure 6A shows this spectrum of $Q_A^- - Q_A$ normalized to its full amplitude at 0.5 ms after a saturating actinic flash, a factor of 1.37 times higher than the $\Delta I/I$ at 270 ms. This factor was determined in the presence of benzidine and ferricyanide by measuring, at 0.5 and 270 ms, the $\Delta I/I$ at 320 nm, an isosbestic point for the spectrum $Z^+ - Z$. This factor is consistent with the maximum $\Delta I/I_{320nm}$ of $Q_A^- - Q_A$ measured 50 ms after the last of 15 saturating flashes in the

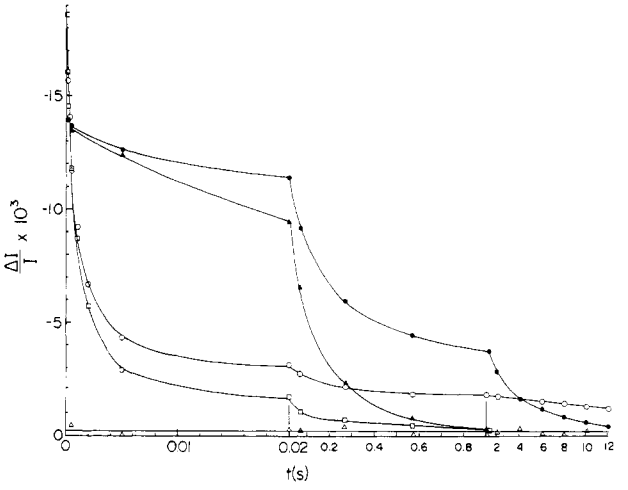

FIGURE 7: Kinetics of relaxation of $\Delta I/I$ following a saturating actinic flash measured at 240 (▲, △), 320 (●, ○), and 430 (□) nm in PSII core preparations isolated from the wild-type* (▲, ●) and mutant D1-Y161F (△, ○, □) strains, under the same conditions as in Figure 6B. These absorbance changes are essentially those of $Z^+ - Z$ (▲), $Q_A^- - Q_A$ (●, ○), and $Chl^+ - Chl$ (□), respectively. The signals shown for $\Delta I/I$ (430 nm, □), in the D1-Y161F mutant and for $\Delta I/I$ (240 nm, ▲) in the wild type* were multiplied by –0.5 and +2.5, respectively, to allow them to be plotted on a more convenient scale. The $t_{1/2}$ for the $Z^+Q_A^-$ and the $Chl^+Q_A^-$ recombination are 80 and 1 ms, respectively. The earliest time points measured in these experiments were at 10 μs for Y161F and 150 μs for the wild type*.

presence of 2 mM NH_2OH, pH 7.5. The amplitude and shape of the $Q_A^- - Q_A$ difference spectrum is independent of pH between 5.9 and 7.5 (van Gorkom, 1974). The $\Delta I/I_{320nm}$, 150 μs after the actinic flash, showed equivalent amplitudes at these pHs. The spectrum of $Q_A^- - Q_A$, at 0.5 ms after the actinic flash [(▲) Figure 6A], was subtracted from the total $\Delta I/I$ measured at 0.5 ms, in the presence of 500 nM ferricyanide alone, pH 5.9. One obtains the spectrum shown by (●) in Figure 6A. This spectrum is that of $Z^+ - Z$, previously identified by Diner and de Vitry (1984) and Dekker et al. (1984). It is characterized by peaks in absorption at 250 and 300 nm and by local electrochromic shifts of reaction-center pigments in the blue. Therefore, at 0.5 ms after the actinic flash, Z^+ and Q_A^- are the only redox components contributing to the flash-induced difference spectrum.

Absorbance changes at 0.5 ms after the actinic flash were measured in the D1-Y161F mutant centers, as well, in the presence of 500 nM ferricyanide, pH 5.9. The contribution of $Q_A^- - Q_A$ to the $\Delta I/I$ at 0.5 ms was determined by measuring the decay of the $\Delta I/I$ at 325 nm, attributed solely to the oxidation of Q_A^-. This reoxidation was estimated to be 15% between 10 μs and 0.5 ms. Therefore, 0.85 times the $\Delta I/I(Q_A^- - Q_A)$ of Figure 6 (▲) was subtracted from the total $\Delta I/I$ (0.5 ms), measured in the mutant, to give the spectrum indicated by (○) in Figure 6B. This spectrum shows none of the characteristic features of the $Z^+ - Z$ spectrum. It shows instead large absorbance changes in the blue which are typical of the generation of a Chl cation radical ($Chl^+ - Chl$) (Borg et al., 1970; Davis et al., 1979), similar to that attributed to $P680^+ - P680$ (Pulles et al., 1976; Gerken et al., 1988). It is clear that, in the mutant, secondary electron transfer from Z is no longer observed. The terminal electron donor in the mutant is most likely P680.

The kinetics of recombination of the oxidized donors and the reduced acceptors were followed at characteristic wavelengths following a single saturating flash. These are shown in Figure 7, in the absence of any artificial electron donor in order to allow charge recombination to occur. Ferricyanide

was added at a concentration of 500 nM to ensure the oxidation of Q_A^- in the dark prior to flash illumination. Oxidation of Q_A^- by 500 nM ferricyanide is much slower than the recombination rate following a flash in both wild-type* and mutant reaction centers. The $\Delta I/I$ for $Q_A^- - Q_A$, $Z^+ - Z$, and $Chl^+ - Chl$ were measured at 320, 240, and 430 nm, respectively. The latter two wavelengths are isosbestic for $Q_A^- - Q_A$; the first is isosbestic for $Z^+ - Z$ and nearly so for $Chl^+ - Chl$ (which contributes ~15% to the total $\Delta I/I$ at this wavelength). Z^+ disappears with a $t_{1/2}$ of 80 ms along with 75% of Q_A^- in wild-type* centers. It would appear then that 75% of the centers recombine betweeen Z^+ and Q_A^-. The remaining 25% of the centers have probably either lost Z^+ through reduction by a more distant donor or did not generate it because of competition with a parallel pathway for electron donation to $P680^+$. The 25% of Q_A^- remaining is reoxidized slowly either by slow recombination or by reoxidation by ferricyanide. In the D1-Y161F mutant, Chl^+ and 90% of Q_A^- disappear in parallel with a $t_{1/2}$ of only 1 ms.

Discussion

We have created a strain of *Synechocystis* 6803 which contains a single copy of the *psbA* gene and have introduced specific mutations into this gene such that an altered D1 protein has been produced. A complicating factor has been the presence of an open reading frame downstream which apparently encodes an essential polypeptide. The availability of the DNA sequence of this region will allow for the insertion of resistance markers at a different site which will not interrupt this gene. Apparently, the *psbA*-3 gene and this open reading frame are not cotranscribed. The cyanobacterium *Synechococcus* PCC 7942 also contains three *psbA* genes. Golden et al. (1986) found that two of the genes encoded identical polypeptides, while the predicted polypeptide of the third copy differed from the other two by 25 out of the predicted 360 amino acids. The case of *Synechocystis* 6803 is likely to be very similar, on the basis of the published sequence for *psbA*-1 and our partial sequence data for *psbA*-2 and *psbA*-3 (data not shown). Debus et al. (1988b) have independently constructed a D1-Y161F mutation in *Synechocystis* 6803, by deleting most of *psbA*-1 and *psbA*-3 and carrying the mutation into *psbA*-2. They demonstrated that the *psbA*-2 gene product can be expressed in *Synechocystis* 6803. The function of three copies of *psbA* in *Synechocystis* 6803 remains unclear; however, for the purpose of molecular dissection of D1 activity, *psbA*-2 and *psbA*-3 appear to be equivalent.

The following arguments led Debus et al. (1988b) and our group to target D1-Tyr-161 as the possible location of donor Z: (a) The Signal IId spectrum of D^+ has been shown to arise from an oxidized tyrosine free radical (Barry & Babcock, 1987). (b) The EPR spectra of Signal IId (D^+) (Babcock & Sauer, 1973) and Signal IIf (Babcock & Sauer, 1975) are identical, indicating that they are the same chemical species. (c) The optical difference spectrum, first identified for $Z^+ - Z$, under conditions where one observes EPR Signal IIf (i.e., O_2-evolving complex inactive) (Diner & de Vitry, 1984; Dekker et al., 1984), is identical with that observed under conditions where one observes EPR Signal IIvf (i.e., O_2-evolving complex intact) (Gerken et al., 1988). The same redox component is probably responsible for both of these signals, which differ only in their oxidation and reduction kinetics. (d) Replacement of D2-Tyr-160 with Phe results in the loss of D^+ (Debus et al., 1988a; Vermaas et al., 1988). (e) D1-Tyr-161 is located in a position symmetrical to that of D2-Tyr-160.

Debus et al. (1988b) have recently shown, in studies on whole cells of a D1-Y161F mutant isolated in parallel with ourselves, (a) that the ability to evolve oxygen is lost, (b) that the quantum yield of Q_A reduction is reduced in continuous light in the presence of DCMU, and (c) that the amplitude and spectrum of D^+ are unaffected by the mutation. They show that electron transfer on the PSII donor side has been disrupted in the mutant but do not establish the step at which electron transfer is blocked. In this paper, we extend these observations with measurements on highly purified PSII core preparations which allow direct EPR and optical spectroscopy measurements on the redox components functioning on the donor side of the photosystem.

We show here that replacement of D1-Tyr-161 with Phe results in the loss of Z oxidation in the light. The absence of Z oxidation was determined by EPR and optical spectroscopic techniques conducted at room temperature. Saturating light gave rise to only a small increase in Signal II amplitude in the mutant (factor of 1.3) as compared to the wild type* (factor of 2.4). We attribute the small increase in Signal II in the mutant to the oxidation of D in 21% of the centers. This compares favorably with the increase in Signal II amplitude in wild-type* centers upon saturating illumination, where 17% of the centers would show oxidation of D and 100% oxidation of Z. When the light is turned off, Signal II shows decay components on the minutes time scale. This presumably arises from reduction of D^+ by ferrocyanide in the experiment of Figure 5B.

A single saturating light flash given to wild-type* reaction centers generated Z^+ in at least 75% of the centers, measured within the time resolution of the experiment of Figure 7 (500 μs). This estimate is based on the extinction coefficient of $\Delta A(Z^+ - Z)$ at the ultraviolet peak (7.3 mM^{-1} cm^{-1}) (Dekker et al., 1984) and on the fraction of Q_A^- which back-reacts with Z^+. In the mutant centers, the absorbance changes measured at 0.5 ms, after subtraction of the contribution of $\Delta I/I(Q_A^- - Q_A)$, showed no indication of Z^+ oxidation in the ultraviolet region of the spectrum. The absorbance changes, measured in the blue and ultraviolet, are indicative, instead, of the formation of a Chl cation radical (Borg et al., 1970; Davis et al., 1979) and resemble spectra previously attributed to $P680^+ - P680$ (Pulles et al., 1976; Gerken et al., 1988). The absorbance change measured at 430 nm is consistent with ~90% of the centers showing $P680^+$ at 10 μs, using an extinction coefficient at 430 nm of −46 mM^{-1} cm^{-1} as in deoxycholate-derived PSII particles (van Gorkom, 1976) and 50 Chl/PSII reaction center. As there should be little correction necessary for particle flattening (Pulles, 1978), this estimate is consistent with the observation that 90% of Q_A^- decays with the same kinetics as Chl^+.

The simplest model would, therefore, attribute the Chl cation radical to $P680^+$. It has been proposed, however, that upon illumination at cryogenic temperature, under conditions where Z and cytochrome *b*-559 oxidation are blocked, another Chl is oxidized having a somewhat broader EPR line shape (ΔH = 10–11 G) from that of $P680^+$ (ΔH = 8 G) (Visser et al., 1977). The conditions prevailing in the D1-Y161F mutant at room temperature in the presence of ferricyanide (Figure 6B) are probably analogous to these. The presence of an oxidizable accessory Chl has been invoked by Thompson and Brudvig (1988) as a possible redox bridge to cytochrome *b*-559. This model has received some support by a recent report that cytochrome *b*-559, a probable heterodimer, would have its heme located on the stromal side of the thylakoid membrane, the opposite side to that of $P680^+$ (Tae et al., 1988). At the moment we cannot exclude the possibility that what we are seeing is such an oxidized accessory Chl.

At least 90% of the centers in the D1-Y161F mutant show photooxidation of Chl, with Chl^+ disappearing by back-reaction with Q_A^- in 1 ms. This observation implies that there is little or no oxidation of phenylalanine-161. The midpoint potential of Phe^+/Phe is probably too high for phenylalanine to act as an electron donor.

There is no evidence in the room-temperature, saturating-light, EPR spectrum of the mutant (Figure 5B) of a Chl^+ free radical. It is likely that the $Chl^+Q_A^-$ recombination rate in the mutant is too fast ($t_{1/2}$ = 1 ms), compared to the excitatory light intensity, to accumulate any appreciable Chl^+ in the steady state. This rate is considerably slower than that previously reported ($t_{1/2}$ = 150–300 μs) and attributed to $P680^+Q_A^-$ recombination in various particle preparations and Tris-, low-pH-, and hydroxylamine-treated chloroplasts (Haveman & Mathis, 1976; Reinman et al., 1981). The difference between the present case and these earlier measurements is the presence of Z^+ in the latter, which could accelerate the recombination rate. However, it has been reported that $P680^+Q_A^-$ recombination occurs with a half-time of 800 μs in PSII particles isolated from *Phormidium laminosum*, another cyanobacterium, where Z^+ should have been present (Reinman et al., 1981). This slower recombination rate may be characteristic of cyanobacterial reaction centers. The 1-ms half-time for recombination explains why, in the D1-Y161F mutant, a lower flash yield is observed for the reduction of Q_A in the presence of NH_2OH and DCMU as compared to that observed in the wild-type* strain (Figure 4). The low yield and the dependence on the hydroxylamine concentration stems from the much faster reduction of the oxidized donor through recombination with Q_A^- than through reduction by NH_2OH. The low flash yield of Q_A reduction in the mutant, despite the presence of D (D^+), implies either that D^+ is not reduced by 10 mM NH_2OH in 10 min or that D is a poor donor to $P680^+$.

Hydroquinone [see also Debus et al. (1988b)] is a much better donor to Chl^+ at 5 mM than is NH_2OH at 10 mM, resulting in a higher yield of Q_A^- per flash (Figure 4) with this donor. Catechol also worked as a donor but gave no more than half the yield of Q_A^- per flash observed for hydroquinone at the same concentration. Diaminodurene, *p*-phenylenediamine, and benzidine did not work well as donors in whole cells, probably because of their positive charge and consequent slow diffusion across membranes.

Charge recombination between $Z^+Q_A^-$ and $Chl^+Q_A^-$ shows respectively $t_{1/2}$ of 80 and 1 ms. We have determined, by kinetic measurements at 435 nm, that electron transfer from Z to $P680^+$ occurs with a $t_{1/2}$ of 17 μs at pH 5.9 (not shown). The rate of equilibration of the reaction $ZP680^+ = Z^+P680$ is determined by the sum of the forward and reverse rate constants and is consequently much faster than the 80-ms $Z^+Q_A^-$ recombination observed in the wild-type* centers. If we make the reasonable assumption that recombination in the mutant and wild type* occurs through the $P680^+Q_A^-$ couple, then the equilibrium constant for $ZP680^+ = Z^+P680$ should be 80 at pH 5.9. This gives an $E_{m,5.9}$ for Z^+/Z 110 mV below that of Chl^+/Chl. Assuming an $E_{m,5.9}$ for $P680^+/P680$ approximately equal to the $E_{m,7}$ of 1.1 V, estimated by Klimov et al. (1979), one estimates the $E_{m,5.9}$ for Z^+/Z at around 1 V. This midpoint potential is in approximate agreement with an estimate at pH 7 of 0.85 V for $Tyr^•/Tyr$ (Jovanovic et al., 1986), particularly as the latter E_m should show a −60 mV/ΔpH unit pH dependence. An $E_{m,5.9}$ of 1 V for Z^+/Z is also consistent with the 890 mV required for the oxidation of water to O_2 at this pH. If the Chl^+ species detected here were to be an accessory Chl, then the Z^+/Z midpoint potential would need to be shifted downward accordingly.

Debus et al. (1988a) have presented evidence for attributing donor D to tyrosine-160 located on polypeptide D2. The identity of the Z^+ and D^+ EPR spectra argue that Z is also a tyrosine. The biophysical evidence we present here demonstrates that replacement with phenylalanine of D1-Tyr-161, symmetric to D2-Tyr-160, results in the inability to generate Z^+. A Chl cation radical and rapid charge recombination are observed in the mutant following a light flash, whereas Z^+ and an 80-fold slower charge recombination are observed in wild type* under similar conditions. The mutant consequently shows inefficient photoreduction of Q_A in the presence of DCMU and an artificial electron donor. These observations provide compelling evidence in favor of identifying D1-Tyr-161 with Z. The loss of Z oxidation and the inability to evolve oxygen in the mutant argue that D1-Tyr-161 is the only route by which oxidizing equivalents are transferred from the primary donor to the oxygen-evolving complex.

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