ABSTRACT

Michelle L. Kosovac. *SYNECHOCYSTIS* 6803 PHOTOSYSTEM II PROTEINS CP43 AND D2: THE ROLE OF THE N-TERMINAL THREONINE RESIDUE AS PROBED BY OLIGONUCLEOTIDE SITE-DIRECTED MUTAGENESIS. (Under the direction of Cindy Putnam-Evans, Ph.D.) Department of Biology, September, 1997.

This project examined the CP43 and D2 proteins of the Photosystem II (PSII) complex using a cyanobacterial model, *Synechocystis* 6803, which highly resembles the PSII complex of higher plants. It has been established that the N-terminal threonine residues of both CP43 and D2 are phosphorylated in higher plants, yet it is not clear what role(s) this phosphorylation may assume in Photosystem II function. The primary objective of this research was to examine how the alteration of the proposed phosphorylation site at the N-terminus of these two proteins affects the function and structure of the PSII complex in cyanobacteria. Using oligonucleotide site-directed mutagenesis, the N-terminal threonine codon in the genes encoding CP43 and D2 was converted to either alanine or aspartate. Plasmids bearing site-directed mutations were re-introduced into *Synechocystis* 6803 along with an antibiotic resistance marker. Putative mutants were examined for antibiotic resistance and possible loss of photoautotrophic growth. Altering the N-terminal threonine residue to an alanine in CP43 had little effect on the growth rates or oxygen evolution rates. However, alteration of the N-terminal threonine residue to aspartate in CP43 caused a major defect in PSII function, resulting in the loss of photoautotrophic growth as well as the inability of the PSII complex to evolve oxygen.

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SYNECHOCYSTIS 6803 PHOTOSYSTEM II PROTEINS CP43 AND D2: THE ROLE OF THE N-TERMINAL THREONINE RESIDUE AS PROBED BY OLIGONUCLEOTIDE SITE-DIRECTED MUTAGENESIS

by
Michelle L. Kosovac

APPROVED BY:

DIRECTOR OF THESIS _____
Cindy Putnam-Evans, Ph.D.

COMMITTEE MEMBER _____
Mary A. Farwell, Ph.D.

COMMITTEE MEMBER _____
Jean- Luc Scemama, Ph.D.

COMMITTEE MEMBER _____
Sandra K. Armstrong, Ph.D.

CHAIR OF THE DEPARTMENT OF BIOLOGY

_____
Charles E. Bland, Ph.D.

DEAN OF THE GRADUATE SCHOOL

_____
Thomas L. Feldbush, Ph.D.

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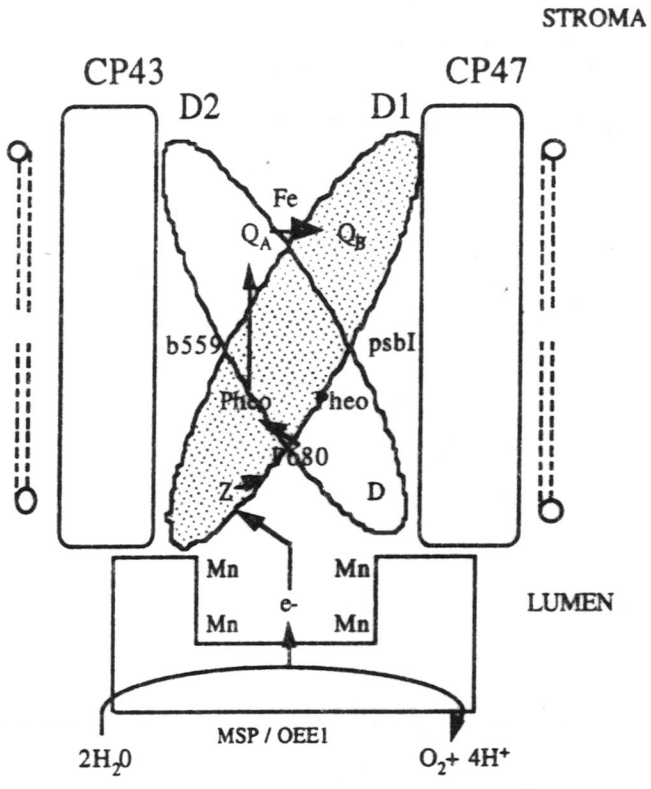
INTRODUCTION

Photosynthesis is the process whereby light energy is converted to chemical energy, resulting in the synthesis of carbohydrate and the evolution of oxygen (Pakrasi and Vermaas 1992). The Photosystem II (PSII) pigment-protein complex (Figure 1) resides within the appressed regions of the thylakoid membranes of chloroplasts and cyanobacteria and is involved in the initial stages of photosynthesis i.e.; the light induced transfer of electrons from water to plastoquinone (Debus et al. 1988). The sequence of events is as follows. First, the light harvesting array absorbs photons and transfers energy to the PSII reaction center. The light harvesting array is the intrinsic chlorophyll a and b light harvesting complex (LHC) in higher plants. In cyanobacteria and red algae, the light harvesting array is the extrinsic phycobilisome complex (Glazer 1983), which is organized into hollow rods containing phycocyanin and allophycocyanin pigments (Shen and Vermaas 1994). The photons of light absorbed excite the chlorophyll-a dimer, p680, of the PSII reaction center and initiate electron transfer. This results in the transfer of an electron from p680 to the primary PSII electron acceptor, pheophytin, and primary charge separation between the reaction center chlorophylls (Noren et al. 1991). Next, electron transport continues to the first stable electron acceptor Q_A and then to the second plastoquinone, Q_B. As a result, plastoquinone is reduced with the formation of plastoquinol. A tyrosine radical, "Z," tyrosine residue 161 of the D1 protein, is responsible for transferring electrons from the site of water oxidation and reducing the photooxidized reaction center, $p680^+$. Four manganese ions are responsible for obtaining the oxidizing equivalents necessary to split water for the final evolution of molecular oxygen (Debus et al. 1988).

Model of PSII From Cyanobacteria



M. Baltscheffsky (ed.), (1990)*Current Research in Photosynthesis* Vol I, 231-238.

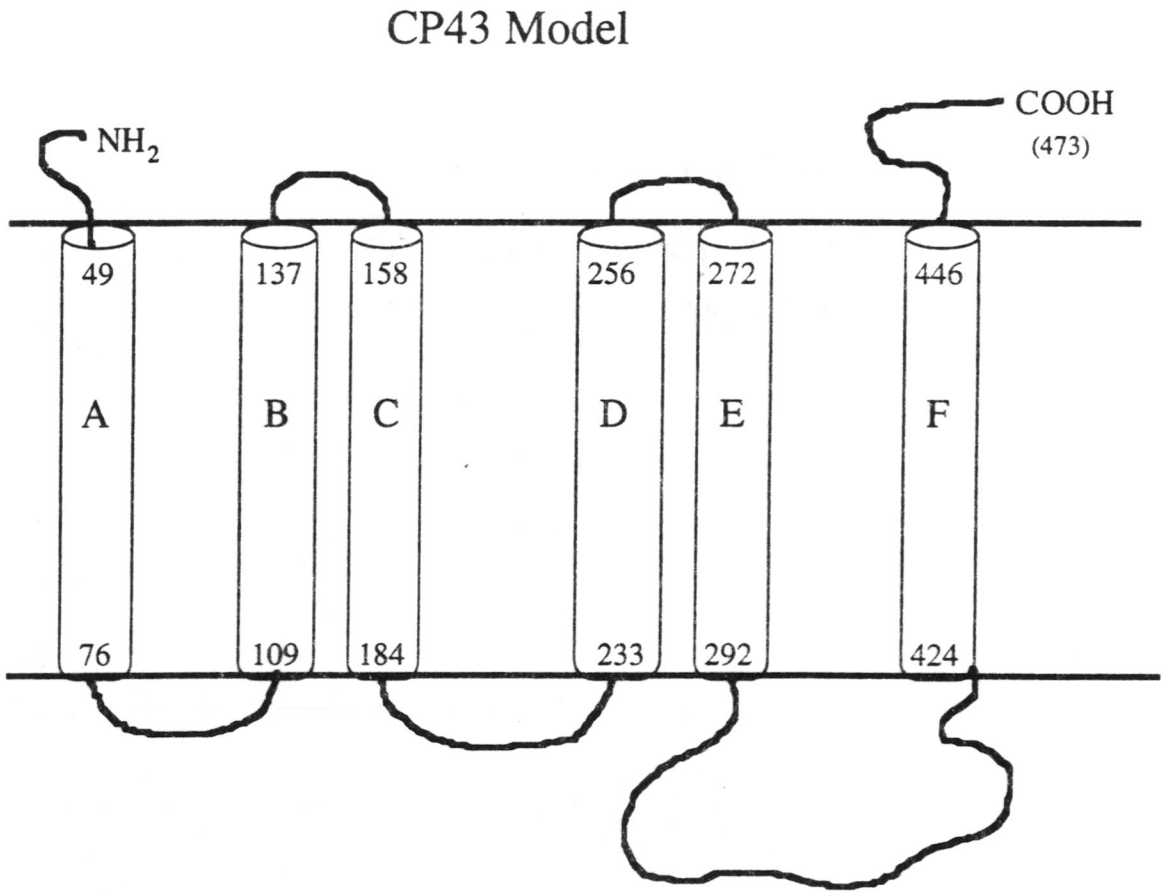
Figure 1. Structural representation of the Photosystem II complex.

A comparison of the PSII protein complexes of cyanobacteria and higher plants has revealed a high degree of similarity in both structure and function (Arntzen and Pakrasi 1986). The PSII complex consists of both integral membrane proteins that form the core of the complex and additional peripheral protein subunits (Barber 1987). The intrinsic thylakoid membrane proteins forming the reaction center core include two antennae chlorophyll-a binding proteins, CP43 and CP47, the reaction center D1 and D2 proteins, the α and β subunits of cytochrome b_{559}, and a 4 kDa *psb*I gene product (Ikeuchi and Inoue 1988). These seven polypeptides are necessary to form the minimal complex capable of oxygen evolution (Burnap and Sherman 1991; Bricker 1992). It is the D1 and D2 integral membrane proteins, forming the heterodimer complex of the reaction center, that are responsible for the binding of the p680 chlorophyll dimer, pheophytin, Q_A, and Q_B (Barber 1987; Frankel and Bricker 1989). Cytochrome b_{559} is necessary for the stable assembly of the D1/D2 heterodimer (Ikeuchi et al. 1985). Both CP43 and CP47 are responsible for the transfer of excitation energy from the light harvesting complex or the phycobilisomes to p680 (Pakrasi et al. 1992). Three extrinsic protein components associated with the oxygen evolution complex include: the 24 kDa and 17 kDa proteins of higher plants, and a 33 kDa manganese stabilizing protein which is present in higher plants as well as cyanobacteria. These extrinsic polypeptides, located on the lumenal surface of thylakoid membranes, are involved in photosynthetic oxygen evolution (Shen and Inoue 1993). Oxygen evolution from PSII additionally requires the presence of ionic cofactors including calcium, four protein bound manganese ions, and from one to several chloride ions (Lindberg et al. 1993; Sinclair 1984; Theg et al. 1984; Debus 1992).

Two major chlorophyll binding proteins, CP43 and CP47 are essential for the functioning of the PSII complex. The *psb*C gene product and a primary focus of this research, CP43 functions primarily as an internal chlorophyll-a light harvesting antennae in

photosystem II, transferring light energy to the PSII reaction center D1-D2-b_{559} (Bassi et al. 1987). In addition, CP43 has a critical role in the stabilization and function of PSII as demonstrated by the introduction of both nuclear and chloroplast site-directed, insertional, and deletion mutations within the *psb*C gene of *Chlamydomonas reinhardtii*. These mutants, which were unable to accumulate CP43, contained reduced levels of the CP47, D1, and D2 proteins in the PSII complex (Rochaix et al. 1989). Carpenter et al. (1990), Rogner et al. (1991), and Kuhn & Vermaas (1993), showed the critical role of CP43 in the stability of PSII by creating deletion mutations in the *psb*C gene in *Synechocystis* 6803. Mutants that lack the *psb*C gene entirely, constructed by Rogner et al. (1991) and Carpenter et al. (1990), contain only 10% of the wild type reaction center complex.

Like other PSII polypeptides, the CP43 protein is well conserved with the cyanobacterial CP43 showing 77% homology to that of higher plants (Erickson and Rochaix 1992). The CP43 protein (Figure 2) consists of six membrane spanning α-helices (Bricker 1990) connected by a number of small hydrophilic loops. These membrane spanning regions contain histidine chlorophyll ligands which are located near the stromal and lumenal surfaces of the protein (Sayre and Wrobel-Boerner 1994). There are approximately 20-25 chlorophylls bound to CP43, a majority of which are bound to these histidine residues (Gounaris et al. 1990). Studies by Ghanotakis et al. (1989) have shown that CP43 can be removed from the PSII complex without removal of CP47, the other chlorophyll-a light harvesting antennae of PSII. Therefore, CP43 is more closely associated with the PSII reaction center complex than CP47. Removal of CP43 allows for only partial chain electron transport in complexes that only contain CP47 attached to the reaction center. Analysis of spinach CP43 by Michel et al. (1988) has revealed that after post-translational removal of the two N-terminal residues, methionine and glutamate, the first residue of this protein is N-acetyl-phosphothreonine, which is exposed to the stromal,



Sayer, R.T. and Wrobel-Boerner, E.A. (1994) *Photosynth. Res.*

Figure 2. Transmembrane spanning model of the CP43 protein of Photosystem II.

hydrophilic side of the thylakoid membrane.

The second focus of this research, the PSII *psb*D gene product ,D2, comprises the heart of the reaction center in the form of a heterodimer with the D1 protein (Debus et al. 1988). Two copies of the *psb*D gene are present in cyanobacteria, *psb*DI and *psb*DII (Michel et al. 1988). In higher plants as well as cyanobacteria, the last fourteen nucleotides of the *psb*DI gene overlap with the first five codons of the *psb*C gene (Erickson and Rochaix 1992). In purple bacteria, Nanba and Satoh (1987) showed the existence of a high degree of similarity in amino acid sequences between the L and M subunits of the purple bacteria reaction center and the D1 and D2 proteins of higher plants. This suggested that D1 and D2 together form the PSII reaction center along with cytochrome b_{559}, a necessary component for the stabilization of the D1/D2 heterodimer. Like CP43, the N-terminal residue of D2 is N-acetyl-phosphothreonine and occurs on the stromal face of the thylakoid membrane (Michel et al. 1988). The cyanobacterial D2 protein is also highly conserved showing 86% homology to higher plant D2 (Erickson and Rochaix 1992).

Proposed Roles of Phosphorylation in PSII Function

Bennett (1977) was the first to document the phosphorylation of thylakoid membrane proteins *in vivo* in ^{32}P labeled pea leaves and *in vitro* in pea chloroplasts. In this study, a number of chloroplast phosphoproteins were identified from 7-70 kDa. It was confirmed that two of these proteins, a 9 kDa protein and the 26 kDa LHCII protein, were both phosphorylated at the N-terminal threonine residue. Thus far, the majority of PSII phosphorylation research has concentrated on higher plant proteins, including LHCII, D1, D2, CP43, and the 9 kDa *psb*H gene product (Harrison and Allen 1991). Allen et al. (1981), Horton et al. (1981), and Allen and Horton (1981), determined that the LHCII complex of higher plants is phosphorylated under plastoquinone reducing conditions by a

redox controlled protein kinase, resulting in its dissociation from PSII. This process involves a light induced transition of the photosystem from state-1 to state-2 where PSII is preferentially excited, resulting in a transfer of energy to photosystem I. The site of this phosphorylation, according to Bennett and co-workers (1987), is the N-terminal threonine residue of LHCII.

Phosphorylation of integral membrane proteins has also been reported. Aro et al. (1992), showed that phosphorylation of the D1 protein in higher plants occurs at the N-terminal threonine residue at the stromal face of the membrane. A study by Michel et al. (1988), using mass spectrometry on spinach phosphopeptides, revealed that both the CP43 and D2 proteins are phosphorylated at the N-terminal threonine as well. One suggested function for CP43 phosphorylation in higher plants is providing negative charge for the repulsion of phospho-LHC II from PSII during a state-2 transition (Ikeuchi et al. 1987; Michel et al. 1988). A study by Koivuniemi et al. (1995), in which γ- ^{32}P labeled spinach thylakoids were subjected to high light intensities, examined the effect of phosphorylation on heterodimeric D2 protein. In this study, the phosphorylated form of the D2 protein exhibited a much slower rate of degradation compared to the non-phosphorylated form. Additionally, D1 and D2 phosphorylation has been suggested to function in the stable assembly of the PSII complex (Allen 1992). Similar to the LHCII, these integral membrane PSII proteins are phosphorylated by a light-dependent, redox regulated kinase (Allen 1992). It is unclear whether this kinase is the same as the one responsible for the phospo-LHCII. Bennett (1991) found that conditions which affected the phosphorylation of the LHCII complex showed little if any effect on the phosphorylation of PSII core proteins. Thus, these phosphorylation events could be regulated either by two independent kinases or by a single kinase that reacts with different substrate specificities.

There is far less information cited in the literature on phosphopeptides of lower plants and cyanobacteria. Research performed using the green algae, *Chlamydomonas reinhardtii,* revealed that both CP43 and D2 proteins are phosphorylated (Delepelaire and Wollman 1985; Ikeuchi et al. 1987). In contrast, phosphorylation of CP43 and D2 has not been unequivocally demonstrated in cyanobacteria. In *Synechococcus*, phosphorylated bands of 13, 15, and 19 kDa have been observed in cells illuminated under PSII light (Allen 1992 and Sanders et al. 1989). Only the identity of the 19 kDa protein, a phycobilisome component, is known. Other phosphorylated bands were observed that correspond in molecular size to CP43 and D2 (Harrison et al. 1991); however, these were never confirmed. The presence of conserved threonine residues in CP43 and D2 of *Synechocystis* 6803, suggests the possible phosphorylation of these proteins as well (Golden and Stearns 1988).

In addition to the proposed roles of phosphorylation of the CP43 and D2 proteins, a relationship has been established between protein phosphorylation and protection from photoinhibition of photosynthesis (Aro et al. 1993). During photoinhibition, plants, algae, and cyanobacteria are exposed to strong illumination resulting in the degradation of PSII proteins and inhibition of electron transport (Ohad et al. 1995). This is due to either the overreduction of the acceptor side of PSII, resulting in the formation of degradative oxygen radicals (Jergerschold et al. 1990; Blubaught et al. 1991), or accumulation of oxidizing radicals on the donor side (Kyle et al. 1984).

The integral membrane D1 protein is the best characterized among the PSII proteins involved in photoinhibition (Michel et al. 1988; Elich et al. 1992). In higher plants D1 is damaged during photoinhibition of electron transfer, and recovery of a functional PSII requires resynthesis of this protein (Ohad et al. 1985). Once damaged, the D1 protein is degraded by a serine type proteinase of the PSII complex, which initiates the transfer of the

PSII complex from the stacked to unstacked regions of the thylakoids where new D1 protein is incorporated (Aro et al. 1993). High light intensities inducing this photoinhibition of the D1 in higher plants also induce maximum phosphorylation of the D1 protein (Aro et al. 1993a) which results in a slower degradation rate than in the non-phosphorylated form (Aro et al. 1992 and Kettunen et al. 1992). This mechanism allows for protection of D1 from degradation until the PSII repair system can cope with the rate of photoinhibition (Rintamaki et al. 1994a). Phosphorylation of the D1 protein has not yet been reported in cyanobacterial membranes or in green algae (Kanervo et al. 1993).

Similar to the well characterized D1 protein, the D2 and CP43 proteins of higher plants also undergo light induced degradation. A study by Yamamoto and Akasaka (1995), showed that the CP43 and D2 proteins in tris-washed spinach thylakoid membranes were all degraded by reactive radicals upon illumination with strong, white, photoinhibitory light (5000 μmoles photons/m^{-2}/s^{-1}). As a result, three degradation products of CP43 were formed, as well as extensive crosslinks between the CP43, CP47, and D1 proteins. The degradation of CP43 during photoinhibition caused the entire PSII complex to become unstable and disorganized, leading to accelerated disintegration of the PSII complex. Schuster et al. (1988) demonstrated that under high light intensities, the D2 protein of *Chlamydomonas reinhardtii* turns over faster than other proteins with the exception of the D1 protein. Results from translational fusion of the two *psb*D genes of the cyanobacterium *Synechococcus* 7942 with the *LacZ* gene of *E. coli* indicated the function of duplicate *psb*D genes under high light intensities. Under photoinhibitory conditions, the *psb*DII gene functions to maintain optimum levels of functional PSII, thus resulting in a higher incorporation of the D2 polypeptide in the thylakoid membranes (Bustos and Golden 1992). A later *in vitro* study by Koivuniemei et al. (1995) in which isolated pumpkin thylakoids were exposed to photoinhibitory conditions and then subjected to SDS-PAGE,

showed that the degradation of the D2 protein under high light intensities is regulated by reversible phosphorylation.

Research Problem

At this time, very little is known about the functional aspects of CP43 and D2 protein phosphorylation. Mutagenesis studies provide a direct method for revealing the possible role of any particular amino acid in the regulation of PSII. Even though phosphorylation of CP43 has not been unequivocally demonstrated in cyanobacteria, we chose to alter the threonine residues in question as a direct test of the function. In this project, the PSII proteins CP43 and D2 from *Synechocystis* 6803 were altered to test the functional significance of N-terminal threonine residues on PSII structure and function. Mutants lacking N-terminal threonine residues were constructed via oligonucleotide-directed mutagenesis, and mutagenic gene sequences were verified via automated DNA sequencing. Upon verification, these mutations were transformed back into a *psb*DI/C/DII⁻ deletion strain of *Synechocystis* 6803. Analysis of the photosynthetic characteristics of the resulting mutants was then undertaken.

Synechocystis 6803, a cyanobacterium, is an ideal model system for the study of the PSII complex. *Synechocystis* is easily manipulated genetically and naturally transformable strains are readily available. In addition, it may be grown on glucose-containing media in the absence of a functional PSII complex allowing for the isolation of mutant colonies with impaired PSII function (Dzelzkalns and Bogorad 1988).

MATERIALS AND METHODS

Synechocystis Cultures

Synechocystis 6803 was grown on BG-11 media in liquid culture (Williams, 1988) at 30° C with a light intensity of 25 μmoles photons/m^{-2}/sec^{-1}, with shaking. The *psb*DI/C/DII$^-$ deletion strain (kindly supplied by Dr. Vermaas, University of Arizona) was grown in BG-11 liquid media, 5 mM glucose, 10 μg/ml chloramphenicol, and 10 μg/ml spectinomycin. The CP43 and D2 control strains, as well as our control strain (prepared by transformation of wild-type *Synechocystis* with the plasmid pDICK) and the *psb*DII$^-$ strain (from Dr. Vermaas, University of Arizona), were grown in BG-11 liquid media supplemented with 5 mM glucose and 10 μg/ml of kanamycin. Stock plate cultures were maintained on BG-11 media supplemented with 1.5% agar, 10 mM TES/KOH, pH 8.2 and 0.3% sodium thiosulfate. All constructed mutants were maintained on BG-11 media with 5 mM glucose, 10 μM DCMU and kanamycin at 10 μg/ml.

Site-Directed Mutagenesis

To test the function of phosphorylation/dephosphorylation of the CP43 and D2 proteins in PSII of *Synechocystis* 6803, the N-terminal phosphorylated threonine residues were replaced with an aspartate residue, which conserved the negative charge of a phosphorylated threonine, and a non-phosphorylatable alanine residue, which removed the charged residue. A site-directed mutagenesis kit from Bio-Rad, using the method of Kunkel (1985), was used to construct the N-terminal mutations (Figure 3). This method is based on strong selection against the non-mutagenized strand of double stranded DNA. The DNA is first synthesized in a dut$^-$ ung$^-$ mutant *E. coli* bacterium. This bacterium substitutes uracils for thymines as a result of the *dut* mutation. The *ung* mutation allows

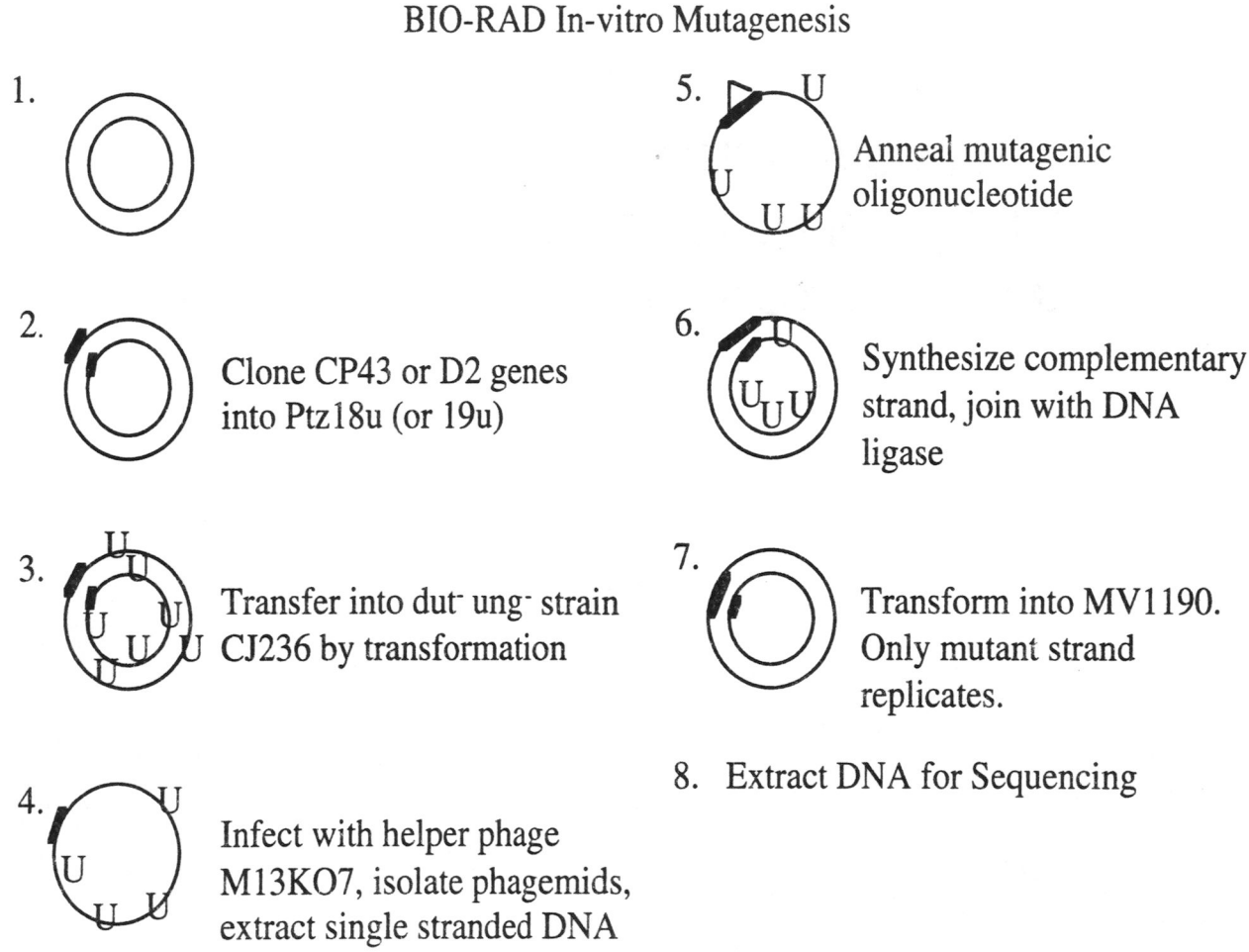


Figure 3. Steps involved in the BIO-RAD site-directed mutagenesis procedure.

stable incorporation of uracils due to the inactivation of uracil N- glycosylase. The uracil containing strand is used as a template for annealing of the mutagenic oligonucleotide. The resulting double stranded DNA is then transformed into a bacterium with a proficient uracil N-glycosylase, resulting in the excision of the uracil containing strand and its replacement resulting in the incorporation of the desired mutation into both strands of the plasmid.

Initial Subcloning of the D2 DNA Fragment. For mutagenesis of the *psb*DI gene, a 1.2 kb *SfiI/BssH*II fragment (Figure 4) from the plasmid pDICK (from Dr. Vermaas, University of Arizona) was amplified by the polymerase chain reaction (PCR). PCR primers (Gibco BRL), with incorporated *Bam*HI and *Eco*RI restriction endonuclease sites, were designed for placement of the amplified fragment into the multiple cloning site of the phagemid pTZ18U (Bio-Rad Laboratories). These primers corresponded to nucleotides 302-1506. PCR was performed in a 100 µl reaction containing 10 ng of DNA, 20 pmol of each primer, 20 mM Tris HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 1 mM dNTP's, and 2 units of Taq DNA polymerase (Gibco BRL). The thermal cycle routine consisted of the following steps: 2 min. of denaturation at 94°C, 1 min. annealing at 55°C, and 3 min. elongation at 72°C for a total of 24 cycles. Once amplified, the 1.2 kb PCR product was ligated into a vector designed for the cloning of PCR products, pGEM-T, which was utilized to transform *E. coli* JM109 (Promega Corporation). Standard and control ligation reactions were performed using 1 µl of T4 DNA Ligase 10x Buffer, 1 µl of pGEM-T Vector (50 ng), 1 µl of T4 DNA Ligase, 7 µl of PCR product (or 2 µl of PGEM-T Vector Control) and dH_20 to a final volume of 10 µl. The ligations were incubated at 15°C overnight and were then heated for 10 minutes at 70°C. To transform the ligated PCR product, 50 µl of JM109 competent cells (Promega) were added to 2 µl of each ligation reaction. The reaction tubes were gently mixed, incubated on ice for 20

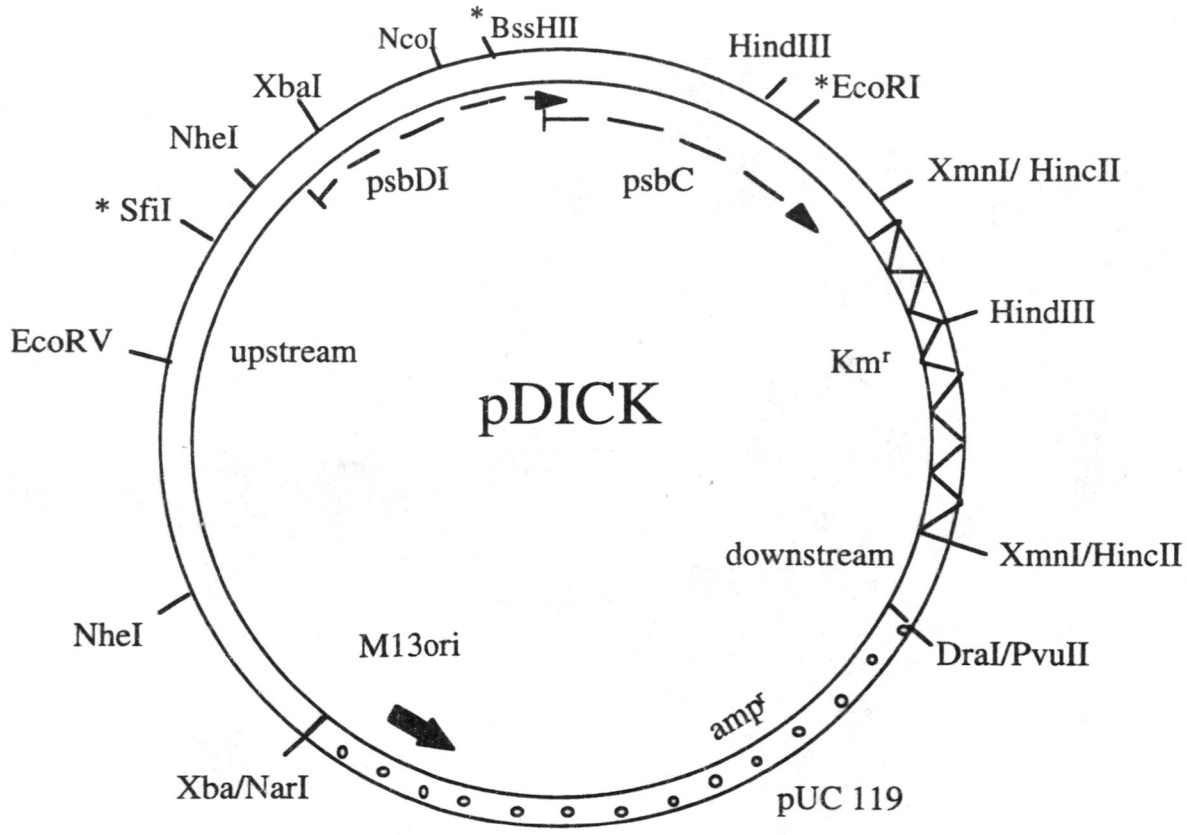


Figure 4. 10 kb pDICK construct containing the overlapping *psb*DI and *psb*C genes. Restriction sites used for subcloning the *psb*DI and *psb*C fragments have been designated with a *.

minutes, and were heat shocked for 45 seconds at 42°C. The transformations were placed on ice for two minutes and 400 µl of luria broth (LB) was added to each tube. The tubes were gently inverted and were incubated for 1 hour at 37°C. The transformations were incubated overnight at 37°C on LB plates containing 50 µg/ml ampicillin, 100 mM IPTG, and 80 µg/ml X-Gal.

Several recombinant transformants were subcultured overnight at 37°C in 5 ml of LB containing 50 µg/ml ampicillin. Plasmid DNA was isolated from the overnight cultures using the Promega Wizard Plus Minipreps DNA Purification System (Promega). For plasmid isolation, 3 ml of cells were pelleted at 14,000 rpm for 1-2 minutes and were resuspended in 200 µl each of Cell Resuspension Solution (50 mM Tris, pH 7.5; 10 mM EDTA; 100 µg/ml Rnase A), Cell Lysis Solution (0.2 M NaOH and 1% SDS), and Neutralization Solution (1.32 M potassium acetate). After centrifugation for 5 minutes at 14,000 rpm, the supernatants were combined with 1 ml of DNA Purification Resin. For each miniprep, the cleared lysate was filtered through a Wizard Minicolumn, attached to a 3 ml syringe, by gently pushing the slurry through the minicolumn. After filtration, 2 ml of Column Wash Solution (80 mM Potassium acetate; 8.3 mM Tris-HCl, pH 7.5, 40 µM EDTA, and 55% ethanol) was pipetted into the syringe and was gently pushed through the minicolumn. The minicolumn was removed from the syringe and was centrifuged at 14,000 rpm for 15 seconds to remove any residual wash solution. Plasmid DNA was eluted in 40 µl of 70°C dH_2O by an additional 20 second spin at 14,000 rpm.

A restriction endonuclease digest was performed to verify the presence of the 1.2 kb *psb*DI insert using 4 µl (0.5µg/µl) of DNA, 1 µl of 10x React 4 Buffer (Gibco BRL), 1 µl of *Spe*I (Gibco) and 4 µl of dH_20. To subclone the *psb*DI fragment into the multiple cloning site of the pTZ18U phagemid (Figure 5), both pGEM-T and pTZ18U were digested with *Bam*HI and *Eco*RI in React 3 Buffer (Gibco BRL). The double digests were

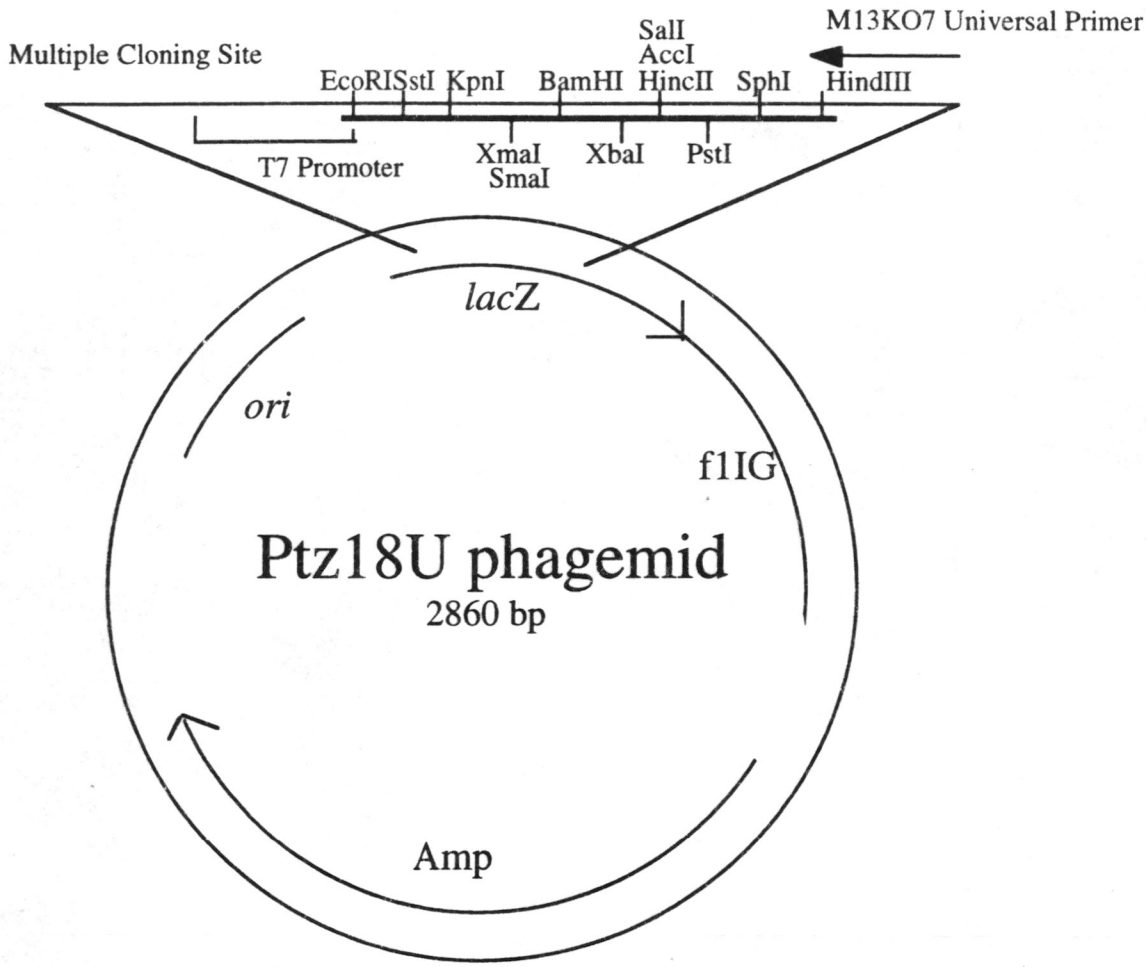


Figure 5. 2860 bp pTZ18U phagemid used for subcloning the *psb*DI and *psb*C genes prior to site directed mutagenesis.

electrophoresed on a 1% agarose gel, and the *psb*DI and pTZ18U fragments were excised for purification using the QIAquick Gel Extraction Kit (Promega). For DNA extraction, the gel slices were weighed, mixed with 3 volumes of QX1 Buffer, and were incubated at 50°C for 10 minutes. Each sample was loaded onto a QIAquick spin column (attached to a 2 ml collection tube) and was centrifuged for 60 seconds. After discarding the flow through, 75 µl of PE Buffer was added to the QIAquick column. Two successive 60 minute spins were performed to remove residual wash buffer, and the DNA was eluted from the column with 30 µl of dH_20.

To subclone the *psb*DI fragment into the multiple cloning site of the pTZ18U phagemid, a ligation reaction consisting of 64 ng of insert, 21 ng of pTZ18U (BioRad), 1.5 µl of T4 DNA Ligase (Gibco BRL), 4 µl of 5x Ligase Buffer (Gibco BRL), and 10.5 µl of dH_20, was prepared and incubated overnight at 20°C. Electrocompetent DH5α cells were prepared for transforming the pTZ18U-*psb*DI ligated product via electroporation as follows. One liter of LB broth was inoculated with 3 ml of an overnight DH5α culture and was grown to an OD_{550} of 0.5. Bacterial cells were pelleted in a GSA rotor at 6,000 rpm for 15 minutes, were washed twice with 500 ml of dH_20 and once with 20 ml of 10% glycerol. Competent DH5α cells were resuspended in 3 ml of 10% glycerol for storage at -70°C. Transformation by electroporation was performed using a Bio-Rad Gene Pulser (Bio-Rad Laboratories) as follows. An aliquot of DH5α cells (100 µl) was transferred to a chilled cuvette and was inoculated with 10 µl of the ligation reaction. The cell suspension was pulsed at 25 µF, 200 Ω, and 2.5 µV for approximately 2 seconds. Immediately after pulsing, 450 µl of SOC was added to the cells which were incubated at 37°C for 1 hour. The transformation was plated on an LB plate containing 50 µg/ml ampicillin and was incubated at 37°C overnight. Several transformants were selected and grown overnight at 37°C in LB supplemented with 50 µg/ml of ampicillin. The Promega

Wizard Miniprep Plasmid Isolation System was used to isolate pTZ18U plasmid containing the *psb*DI fragment. The DNA in the final step was resuspended in 40 µl dH_20. The pTZ18U-*psb*DI plasmid was digested with *BssH*II and *Sfi*I in React 2 buffer (Gibco), and was electrophoresed on a 1% agarose gel for verification of the construct.

Initial Subcloning of the CP43 Fragment. Prior to subcloning the N-terminal portion of *psb*C into the pTZ18U phagemid, a large scale plasmid isolation of pDICK (from Dr. Vermaas, University of Arizona) was performed. A single colony of pDICK in DH5α was grown overnight at 37°C in 20 ml of LB broth supplemented with 50 µg/ml of ampicillin. Using the "Midi" plasmid purification protocol (QIAGEN, Inc.), the bacteria was pelleted for 5 minutes at 10,000 rpm in an SS-34 rotor. The pellet was resuspended in 4 ml each of Resuspension Buffer P1 (50 mM Tris HCl, pH 8.0; 10mM EDTA; 100 µg/ml RnaseA) and Lysis Buffer P2 (200 mM NaOH, 1% SDS). Following a 5 minute incubation at room temperature, 4 ml of Neutralization Buffer P3 (3.0 M potassium acetate, pH 5.5) was added, and the mixture was incubated on ice for 15 minutes. After centrifugation at 30,000 x g for 20 minutes, the supernatant was placed onto a QIAGEN tip 100 column previously equilibrated with 4 ml of Equilibration Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% ethanol; 0.15% Triton X-100). Two 5 ml washes with Wash Buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% ethanol), were performed followed by elution with 4 ml of Elution Buffer QF (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% ethanol). The eluted DNA was precipitated with 0.7 volumes of isopropanol (2.8 µl), centrifuged at 12,000 x g for 30 minutes, and was resuspended in 100 µl of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The concentration of 0.75 µg/µl was determined spectrophotometrically.

N-terminal site-directed mutagenesis of the *psb*C gene was accomplished by restriction endonuclease digestion of pDICK, resulting in a 1.2 kb *Nco*I/*Eco*RI fragment (Figure 4). DNA polymerase Klenow (Gibco-BRL) was used to blunt-end the staggered *Nco*I termini prior to subcloning the fragment into *Sma*I/*Eco*RI cleaved pTZ18U phagemid. Briefly, 10 µl of pDICK plasmid (0.75 µg/µl), containing the entire *psb*C gene, was digested using 1 µl of *Nco*I, 1.5 µl of React 3 buffer (Gibco BRL) and 2.5 µl of dH_2O. The *Nco*I enzyme was heat inactivated at 65°C for 10 minutes, and 0.5 µl of 1 mM dNTP's and 0.5 µl (3 units) of DNA Polymerase Klenow (Gibco) were added. This reaction was incubated at 30°C for 15 minutes and finally at 75°C for 10 minutes to terminate the reaction. To complete the digestion, 1 µl of *Eco*RI and 1.5 µl of React buffer 3 (Gibco) was added to the reaction which was incubated at 37°C for 1 hour. The reaction was electrophoresed on a 1% agarose gel, and the 1.2 kb *Nco*I/*Eco*RI *psb*C fragment was excised using the QIAquick Gel Extraction Kit (Promega).

To subclone the *Nco*I/*Eco*RI 1.2 kb *psb*C fragment into the pTZ18U phagemid (Figure 5), pTZ18U was digested using 8 µl (1 ug/µl) of pTZ18U (BioRad), 1 µl of React 4 buffer, and 1 µl of *Sma*I (Gibco). The digest was electrophoresed on a 1% agarose gel. The desired fragment was excised using the QIAquick Gel Extraction Kit (Promega). Thirteen microliters (0.2 ug/µl) was digested using 0.5 µl of *Eco*RI and 1.5 µl of dH_20. Upon digestion, the *Sma*I/*Eco*RI cleaved pTZ18U fragment was removed from the gel and extracted using QIAquick (Promega) then set aside for ligation.

Ligation of pTZ18U-*psb*C was accomplished using 2 µl (20 ng) of *Sma*I/*Eco*RI cleaved pTZ18U, phagemid, 12 µl (60 ng) of *Eco*RI/*Nco*I cut pDICK, 4 µl of 5x Ligase buffer, 1.2 µl of T4 DNA ligase, and 0.8 µl of dH_20. After an overnight incubation at 14°C, 5 µl of the ligation reaction was used to transform 100 µl of DH5α cells (Gibco). A negative control reaction, not including DNA, was also performed. Both reactions were

incubated on ice for 30 minutes, heat shocked at 45°C for 90 seconds, and were added to 400 µl of LB. All reactions were incubated for 1 hour at 37°C with shaking and were grown overnight at 37°C on LB plates containing 50 µg/ml of ampicillin. Several colonies were subcultured overnight at 37°C in LB supplemented with 50 µg/ml of ampicillin. A "Midi" plasmid purification (QIAGEN) was performed as stated in the manufacturer's protocol to isolate pTZ18U-*psb*C plasmid. The DNA in the final step was resuspended in 40 µl of dH_20. The plasmid was then digested with *Bam*HI in React 3 buffer (Gibco), and was electrophoresed on a 1% agarose gel for verification of the pTZ18U-*psb*C construct.

Transformation of CJ236 with Insert-Carrying Phagemid. For selection against the non-mutated strand during mutagenesis, the pTZ18U-*psb*DI and pTZ18U-*psb*C phagemids (carrying ampicillin resistance genes) were first transformed into the *dut*$^-$ (dUTPase), *ung*$^-$ (uracil N-glycosylase) strain *E. coli* CJ236 (Bio-Rad). This strain incorporates residues stably into both strands of DNA due to the absence of the enzymes dUTPase and Uracil-N-glycosylase. For the transformation, 5 µl (10ng) of each phagemid was added to 100 µl of competent CJ236 cells. As a negative control, one reaction consisted of only CJ236 cells. All reactions were incubated under the following conditions: 30 minutes on ice, 90 seconds at 45°C, 2 minutes on ice, and 1 hour at 37°C. The transformed cells were then plated on LB plates containing 50 µg/ml ampicillin, and were incubated overnight at 37°C. Several colonies were subcultured overnight at 37°C, with shaking, in 5 ml of LB containing 30 µg/ml of chloramphenicol. pTZ18U-*psb*DI and pTZ18U-*psb*C plasmid, with uracil residues incorporated into both strands, was isolated utilizing the Promega Wizard Miniprep method. For verification of the phagemids in CJ236, restriction endonuclease digests were performed using *Nco*I and *Eco*RI (Gibco) to

digest the pTZ18U-*psb*C phagemid and *BssH*II and *Sfi*I (Gibco) to digest the pTZ18U-*psb*DI phagemid. All digests were electrophoresed on a 1% agarose gel.

Preparation of Uracil-Containing Phagemid DNA. Superinfection of CJ236 containing the pTZ18U-*psb*C and pTZ18U-*psb*DI uracil containing phagemids, with helper phage M13KO7 (Bio-Rad), produced phage particles containing single stranded DNA necessary for complementary strand synthesis. First, CJ236 cultures containing the verified pTZ18U-*psb*C and pTZ18U-*psb*DI constructs were streaked onto LB plates containing 30 μg/ml of chloramphenicol which were incubated overnight at 37°C. A single colony from each plate was subcultured overnight at 37°C in 20ml of LB supplemented with 15 μg/ml of chloramphenicol (Gibco). Next, 50 ml of 2xYT media, containing ampicillin and chloramphenicol, was inoculated with 1 ml of CJ236 overnight culture containing either pTZ18U-*psb*C or pTZ18U-*psb*DI. These cultures were grown at 37°C, with shaking, to an O.D. of 0.3 (approximately 4 hours = $1x10^7$ cfu/ml). Helper phage M13KO7 (Bio-Rad) was then added to a M.O.I. (multiplicity of infection) of 20 phage/cell. Following a 1 hour incubation with shaking at 37°C, kanamycin was added to a final concentration of 70 μg/ml. Incubation at 37°C was continued for 6 additional hours, then 30ml of each culture was centrifuged using an SS-34 rotor at 12,000 rpm for 15 minutes. The supernatants, containing the phagemid particles, were transferred to a fresh tube and were inoculated with 150 μg of RnaseA. The reaction was then incubated at room temperature for 30 minutes. Following incubation, 1/4 volume of 3.5 M ammonium acetate/ 20% PEG 6000 was added, and the reactions were incubated on ice for 30 minutes. Phagemids were collected by centrifugation at 12,000 rpm for 15 minutes. The supernatants were decanted and the phage pellets were resuspended in 200 μl of high salt buffer (300 mM NaCl, 100 mMTris, pH 8.0, 1 mM EDTA) and were stored at 4°C.

For purification of the single stranded DNA prior to its use as a template in *in vitro* mutagenesis reactions, the single stranded DNA was purified by extraction of isolated phage heads. The entire 200 µl of phagemid stock was extracted as follows: twice with an equal volume of phenol, once with an equal volume of PCI (1:1:1/48 phenol:chloroform:isoamyl alcohol) and once with an equal volume of chloroform/isoamyl alcohol. Each mixture was vortexed for 1 minute and was centrifuged briefly between extractions. To increase the yield of single stranded DNA, back extractions were performed using 100 µl of TE (10 mM Tris, pH 8.0, 1 mM EDTA). The aqueous phases were then pooled, combined with 1/10 volume of 7.8 M NaAc and 2.5 volumes of ethanol, and were incubated at -70°C for 30 minutes. Following centrifugation at 14,000 rpm for 15 minutes in a chilled microcentrifuge, the supernatants were removed. Single stranded pTZ18U-*psb*C and pTZ18U-*psb*DI uracil-containing DNA pellets were resuspended in 20 µl of TE. Several microliters were electrophoresed on a 1% agarose gel to determine the approximate concentration of single stranded DNA.

Design and Phosphorylation of Mutagenic Oligonucleotides. Mutagenic oligonucleotides (Table 1) were designed to replace the N-terminal threonine residues of CP43 and D2 with alanine and aspartate residues (Figure 6). Prior to use of the mutagenic primers in site-directed mutagenesis, the lyophilized oligonucleotides were phosphorylated using 200 pmoles of the mutagenic oligonucleotide, 5 µl of Forward RXN Buffer, 2.5 µl of 10 mM ATP (Gibco), and dH_20 to a final volume of 25 µl. After mixing, 10 units (1 µl) of T4 polynucleotide kinase was added to each reaction mixture which was incubated at 37°C for 45 minutes. Each primer phosphorylation reaction was terminated by an incubation at 65°C for 10 minutes, followed by the addition of 0.6 µl of 250 mM EDTA.

PRIMER DESIGNATION	MUTANT PRIMER SEQUENCE	SOURCE
MKALCP43	AT CGA AGT ATT AGA GAG AGC TAC CAC GGG GGA GA	Gibco BRL
MKASPCP43	AT CGA AGT ATT AGA GAG GTC TAC CAC GGG GGA GA	Gibco BRL
D2AL	AGA GGA TTT GCA TTT ATG GCT ATT GCA GTC GGA CGC	Gibco BRL
D2ASP	AGA GGA TTT GCA TTT ATG GAC ATT GCA GTC GGA CGC	Gibco BRL

Table 1. Mutagenic oligonucleotides used for CP43 and D2 Site-directed Mutagenesis. The site of each introduced mutation is underlined.

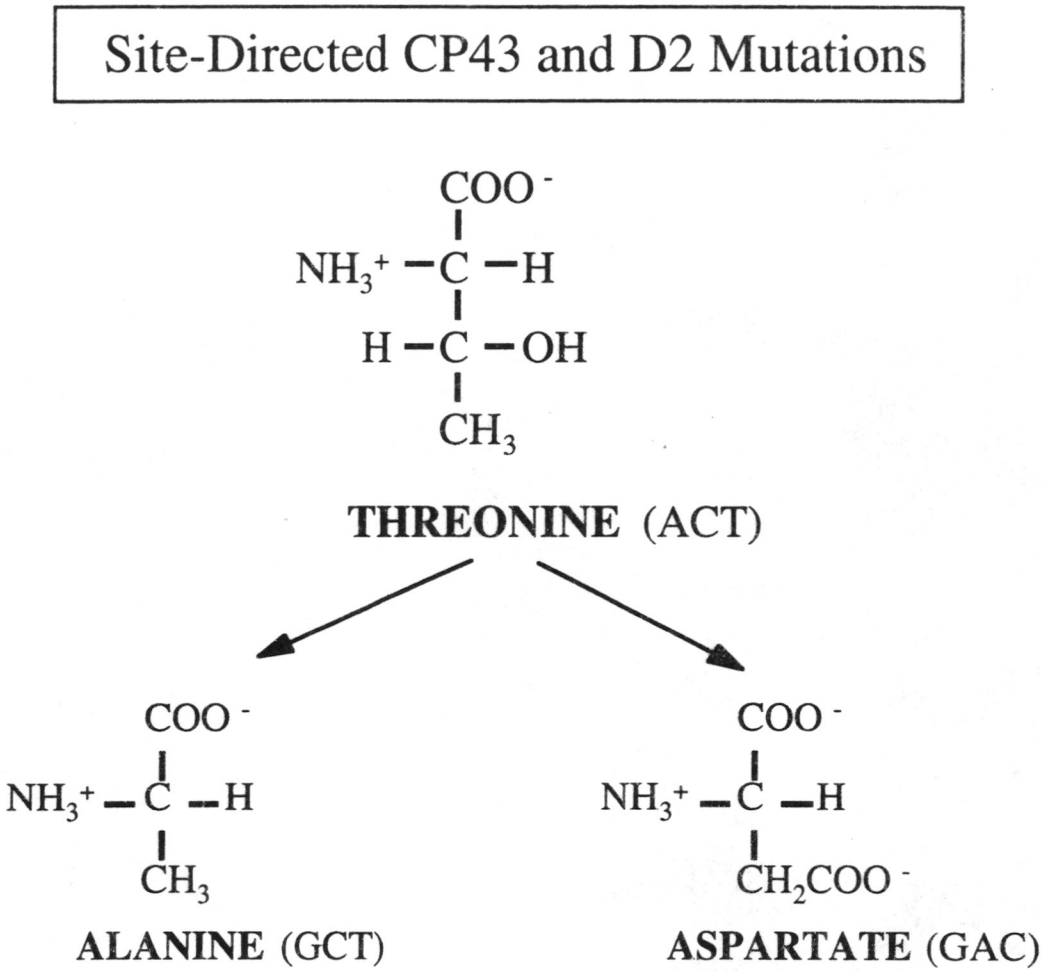


Figure 6. Structures of alanine and aspartate residues replacing the N-terminal threonine residues of the CP43 and D2 proteins of PSII.

Annealing of Mutagenic Oligonucleotides and Complementary Strand Synthesis. After phosphorylation, each mutagenic oligonucleotide was annealed to the single stranded, uracil-containing, pTZ18U-*psb*C or pTZ18U-*psb*DI DNA. Following the Bio-Rad protocol, a 20:1 molar ratio of primer to template was used in each annealing reaction consisting of 0.2 pmoles of uracil-containing DNA, 6 pmoles (1 µl) of mutagenic oligonucleotide, 1 µl of 10x annealing buffer (20 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 50 mM NaCl), and dH_20 to a final volume of 15 µl. A control reaction, consisting of all reactants except the primer, was performed to test for non-specific priming. All annealing reactions were placed in a 70°C water bath for 1 minute, were cooled at a rate of 1°C per minute to a final temperature of 30°C, and were placed on ice for complementary strand synthesis.

Complementary strand synthesis was accomplished by adding 1 µl of 10x synthesis buffer (0.4 mM of each dNTP, 0.75 mM ATP, 17.5 mM Tris-HCl (pH 7.4), 3.75 mM $MgCl_2$, 1.5 mM DTT), 1 µl (3 units) of T4 DNA ligase, and 1 µl (0.5 units) of T7 DNA polymerase (Bio-Rad) to each annealing reaction. After incubating each reaction on ice for 5 minutes, 25°C for 5 minutes, and 37°C for 30 minutes, 60 µl of TE stop buffer (10 mM Tris, pH 8.0, 10 mM EDTA) was added. The reactions were terminated by freezing at -20°C.

Transformation of MV1190 with Second Strand Synthesis Products. The second strand synthesis reaction products were used to transform *E. coli* MV1190 for producing pTZ18U-*psb*C and pTZ18U-*psb*DI plasmids with the mutation copied into both strands. This *E. coli* strain, consisting of a functional uracil N-glycosylase and dUTPase, excised the uracil containing, non-mutagenic strand, resulting in the replication of the mutagenized strand. Prior to transformation, competent MV1190 cells were prepared.

Following the Bio-Rad protocol, MV1190 cells were streaked onto a glucose minimal medium agar plate which was incubated overnight at 37°C. A single MV1190 colony was used to inoculate 20 ml of LB medium, which was incubated with shaking at 37°C overnight. Next, 250 ml of LB was inoculated with enough overnight culture to achieve an initial OD_{600} of 0.1. Once a final OD_{600} of 0.9 was achieved by shaking at 37°C, the bacterial culture was pelleted for 5 minutes at 5,000 rpm. After decanting the supernatants, the pellet was resuspended in 50 ml of cold 100 mM $CaCl_2$. Following a second spin, the cell pellet was resuspended in 10 ml of 100 mM $CaCl_2$, and then 100 ml of additional 100 mM $CaCl_2$ was added. After incubation on ice for 1 hour, the cells were harvested and resuspended in 12.5 ml of cold 85 mM $CaCl_2$. Competent MV1190 cells were stored at -70°C for future use.

Transformation of the competent MV1190 cells with the mutant pTZ18U-*psb*C and pTZ18U-*psb*DI second strand synthesis reactions was accomplished as follows. For each transformation, 0.3 ml of competent MV1190 cells were inoculated with 10 µl of each second strand synthesis reaction (3 µl of the control reaction), and were incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 3 minutes, and were placed on ice for 5 minutes. Each transformation reaction was supplemented with 1ml of LB, and was incubated with shaking at 37°C for 1 hour. Next, 100 µl of each transformation was plated onto an LB plate containing 50 µg/ml of ampicillin. All plates were incubated at 37°C overnight.

Several transformants were subcultured overnight at 37°C in 5 ml of LB containing 50 µg/ml of ampicillin. Putative pTZ18U-*psb*C and pTZ18U-*psb*DI double stranded mutant plasmid was isolated using the Promega Wizard Miniprep system mentioned previously (Promega). A restriction endonuclease digest was performed to determine the orientation of the pTZ18U-*psb*C construct using 5 µl (0.3 µg/µl) of DNA, 1 µl of React 2

buffer, 0.5 µl of *Hind*III (Gibco) and 3.5 µl of dH_20. The pTZ18U-*psb*DI construct was digested using 4 µl (0.5 µg/µl) of DNA, 1 µl of React 3, 1 µl of *Eco*RI (Gibco), and 4.5 µl of dH_20. All digests were electrophoresed on a 1% agarose gel.

Mutant Verification. To verify the putative N-terminal pTZ18U-*psb*C and pTZ18U-*psb*DI alanine and aspartate mutants, DNA sequencing was performed using the pUC M13 universal Forward and Reverse primers (Gibco-BRL). Sequencing reactions were set up utilizing the flourescence-based Prism Dye Terminator Cycle Sequencing Kit with Amplitaq DNA polymerase, FS (Applied Biosystems, Inc). As stated in the manufacturers protocol, each sequencing reaction consisted of 8.0 µl of terminator ready reaction mix, 1 µg of DNA, 3.2 pmoles of primer, and dH_20 to 20 µl. Automated DNA sequencing was performed on a 5% acrylamide/bis-acrylamide gel, and sequencing reactions were analyzed on the Applied Biosystems 373A DNA Sequencing System.

Subcloning of Mutations into pDICK. Both the *psb*C and *psb*DI gene fragments, containing the verified N-terminal alanine and aspartate mutations, were subcloned back into pDICK, which contains a selectable marker for kanamycin resistance in the 3' non-coding region of the *psb*DI/C operon. To subclone the *psb*C mutagenic fragments into pDICK, the pTZ18U-*psb*C and pDICK plasmids were digested using *BssH*II, *Eco*RI, and React 2 buffer (Gibco). Following electrophoresis on a 1% agarose gel, the 926 bp *BssH*II/*Eco*RI and 10 kb pDICK fragments were excised using QIAquick as stated in the manufacturer's protocol (Promega). Ligation of the *psb*C and pDICK fragments was achieved using 10 µl (25 ng) of *psb*C insert, 0.5 µl (90 ng)of pDICK vector, 1.5 µl of T4 DNA ligase, 4 µl of 5x ligation buffer, and 4 µl of dH_20.

For subcloning the *psb*DI mutagenic fragments into pDICK, the pTZ18U-*psb*DI and pDICK plasmids were digested using *Sfi*I, *BssH*II, and React 2 buffer (Gibco). After electrophoresis on a 1% agarose gel, the 1.2 kb *psb*DI and 10 kb pDICK fragments were excised using QIAquick (Promega). Ligation of the mutagenic *psb*DI and pDICK fragments was accomplished using 4 µl (32 ng) of *psb*DI insert, 5 µl (88ng) of pDICK vector, 1.5 µl of T4 DNA ligase, 4 µl of 5x ligation buffer (Gibco), and 5.5 µl of dH_20.

Following ligation, 100 µl of DH5α cells were transformed with 5 µl of each ligation reaction. The transformation reactions were incubated on ice for 30 minutes, and were heat shocked at 42°C for 2 minutes. Following an additional 2 minute incubation on ice, 400 µl of LB was added to each tube. The reactions were incubated at 37°C for 1 hour, and 200 µl of each transformation was plated on a LB plate containing 50 µg/ml of ampicillin. All transformation plates were incubated at 37°C overnight. For plasmid isolation, several colonies were subcultured overnight at 37°C in 5 ml of LB supplemented with ampicillin. Plasmid was isolated using the Promega Wizard Preps DNA Purification System, and restriction endonuclease digests were performed to confirm the presence of the mutagenic *psb*DI and *psb*C plasmids. To confirm the sequence of the mutations prior to transforming the *psb*DI/C/DII$^-$ deletion strain of *Synechocystis* 6803, theCP43 and D2 mutagenic plasmids were sequenced as above.

Synechocystis Transformation

The confirmed CP43pDICK and D2pDICK mutagenic plasmids were used to transform the *psb*DI/C/DII$^-$ deletion strain of *Synechocystis* 6803 (from Dr. Vermaas, University of Arizona), in which the coding region for *psb*DI, *psb*DII, and *psb*C was deleted. Transformants were selected on the basis of kanamycin resistance. Control strains were obtained by transformation of wild-type *Synechocystis* 6803 with non-

mutagenized pTZ18U-*psb*C and pTZ18U-*psb*DI. Using the procedure by Williams (1988), cells from a log-phase culture of *Synechocystis* 6803 *psb*DI/C/DII⁻ deletion strain were pelleted at 3000 rpm for 10 minutes and were resuspended in 1 ml of BG-11 liquid media. Mutant DNA's (2-3 μg) were added to 1 x 10^8 cells and were incubated for 5 hours at room temperature under 25 μE of light. The transformations were plated on solid BG-11 media containing 5 mM glucose (which allows for the growth of mutants in which the PSII complex has been affected) and 10 μM DCMU (inhibitor of PSII electron transport and slows growth of non-transformed colonies prior to the addition of antibiotics). After the two days necessary for the expression of the pDICK kanamycin resistance gene, 10 μg/ml of kanamycin was used to underlay the plates. *Synechocystis* colonies were then screened after four rounds of streaking individual colonies which is necessary for sorting multiple copies of its' genome.

Genomic DNA Sequencing

This technique was used to verify the incorporation of the induced mutations within the genome of *Synechocystis* 6803. Using the method of Williams (1988), genomic DNA from mutant colonies was isolated in the following manner. A total of 50 ml of cells from a mutant culture was pelleted, resuspended in 13 M NaI, incubated at 37°C for 20 minutes, and centrifuged at 10,000 rpm for 5 minutes. The resulting pellet was resuspended in 0.5 ml of a 50 mM Tris HCl (pH 8.5)/50mM NaCl/5 mM EDTA solution, and 0.5 ml of fresh 80 mg/ml lysozyme was added. After the addition of 20 μl of 10 mg/ml RNase, and incubation for 10 minutes at 37°C, 0.5 ml of 1 mg/ml Proteinase K was added. Following a second incubation at 37° C for 23 minutes, 0.5 ml of 10% N-lauroylsarcosine was added, and the mixture was incubated at 37°C for 20 minutes. Genomic DNA was extracted from the green lysate twice with PCI and once with chloroform. Each reaction

was centrifuged for 8 minutes at 8,000 rpm between extractions. The genomic DNA was precipitated with the addition of 1/10 volume of NaAc, 2 volumes of ethanol, and by an incubation at -70°C overnight. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 20 minutes, and was resuspended in 100 µl of TE.

Following the extraction of genomic DNA, the mutated *psb*C and *psb*DI gene portions were amplified using PCR. PCR was performed in a 50 µl reaction containing 10-50 ng of genomic DNA, 20 pmol of each primer, and 45 µl of PCR SuperMix (22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM $MgCl_2$, 220µM each of dGTP, dATP, dTTP, dCTP, and 22U recombinant Taq DNA Polymerase/ml (Gibco BRL). The thermal cycle routine consisted of the following steps: an initial 1 min. denaturation at 94°C, followed by 40 sec. of denaturation at 92°C, 40 sec. annealing at 45°C, and 1min. and 30 sec. elongation at 75°C for a total of 29 cycles.

Following the manufacturer's protocol, the mutant *psb*C and *psb*DI PCR fragments were ligated into the pGEM-T Vector System and were transformed into *E. coli* JM109 cells (Promega). All transformations were plated on LB plates containing 50 µg/ml of ampicillin, 100 mM IPTG and 0.08 mg/ml X-Gal. Several white colonies from each transformation were cultivated overnight with shaking at 37°C in LB supplemented with 50 µg/ml of ampicillin. Plasmid was isolated using the Promega Magic Minipreps DNA Purification System. To verify insert containing plasmids, restriction endonuclease digests were performed using SpeI and React 4 buffer (Gibco). Sequencing reactions were set up utilizing the ABI Prism Dye Terminator Cycle Sequencing Kit with Amplitaq DNA polymerase, FS. Automated DNA Sequencing was performed on the entire mutant *psb*C and *psb*DI gene fragments, using a series of sequencing primers (Table 2), on an Applied Biosystems 373A DNA Sequencing System.

PRIMER DESIGNATION	PRIMER SEQUENCE (GIBCO-BRL)
CP43PDICKF	5' ACTTTGTCTCCCAGGAGCTA 3'
CP43INTF	5' AACCCATGTATGAGCAG 3'
CP43-5F	5' ATAGGATCCGTGTATACGACACCTGGGC 3'
CP43PDICKR	5' AATATGGGGCAGGAGGA 3'
CP43INTR	5' GAAGATAATCGCCGGG 3'
CP43-5R	5' AACCGTTGGTCCCGCAC 3'
D2-1F	5' AGAGGATCCTTTGGCCCCTGTGGCCTGG 3'
D2-2F	5' TCTGTGCTATCCACGGT 3'
D2-3F	5' CAGTCGAAAGAGGATGG 3'
D2-4F	5' AATCGGTGGTTTGTGGC 3'
D2-1R	5' GTCCGACTGCAATAGTC 3'
D2-2R	5' ATCGAATTCGAGCCATCCAGGCGCGCATC 3'
D2-3R	5' AATCAGACCGAAGGCAC 3'
D2-4R	5' CTGCTTGGGTGGGTTCA 3'

Table 2. Oligonucleotide Primers Used in DyeDeoxy Sequencing of CP43 and D2 Mutants.

Analysis of Photosynthetic Characteristics of Mutants

Growth Characteristics. To test for possible loss of photoautotrophy and to compare the mutant growth rate with that of wild type *Synechocystis* 6803, mutants were grown in BG-11 media in the presence and absence of glucose. Growth cultures were set up as follows. First, cells of each *psb*C or *psb*DI mutant (in *psb*DI⁻/C⁻/DII⁻) were scraped from a stock plate and resuspended in 1ml of sterile BG-11. a strain in which wild-type *Synechocystis* had been transformed with the pDICK plasmid served as the control for site-directed mutants in CP43. The A_{730} of a 50 fold dilution of each resuspension was determined spectrophotometrically. For each growth culture, 100 ml of sterile BG-11 was inoculated with 1x 10^7 cells/ml of cells, 20 µl of 50 mg/ml kanamycin, and 45 ml of 20% glucose as needed. All growth experiments were performed in a 30°C Sherer growth chamber, under 25 µE of light, with shaking at 210 rpm. Both mutant and control cultures were removed from the growth chamber daily to record OD_{730} readings for 9 consecutive days.

Oxygen Evolution/ Electron Transport Assays. Steady state rates of oxygen evolution were measured from water to dichlorobenzoquinone (DCBQ) in whole cells using a Hansatech oxygen electrode. Mutant and control strains were prepared for the assay as follows. On day 6 of each growth experiment, 50 ml of cells were pelleted in an SS-34 rotor for 5 minutes at 10,000 rpm. After decanting the supernatants, the cell pellets were resuspended in 2 ml of BG-ll liquid media (without TES-KOH and thiosulfate, which interfere with the assay). To normalize the chlorophyll content of each sample, a spectrophotometric chlorophyll assay was performed by measuring the optical density of a fifty fold dilution of each sample at 678 nm, 710 nm, and 750 nm. The amount of chlorophyll per milliliter of culture was calculated using the following equation: ($[A_{678}$-

A_{750}] - [A_{710}-A_{750}]) x 14.96 x 50 = µg chlorophyll/ml (Williams 1988). Using the Hansatech Oxygen Electrode, oxygen evolution rates were measured using 10 µg chlorophyll/ml of cells, 0.01 ml of DCBQ, and BG-11 media to 1 ml. The final rate of oxygen evolution was calculated in µmoles of O_2/ mg chlorophyll/ hour.

RESULTS

Mutant Verification

To study the proposed role of the N-terminal threonine residues in CP43 and D2, the genes encoding these proteins were altered such that threonine was converted to either alanine or aspartate. These N-terminal pTZ18U-*psb*C and pTZ18U-*psb*DI alanine and aspartate mutants (T3A and T3D) were constructed using the mutagenic oligonucleotides listed in Table 1. Note that the codon at the site of mutagenesis is underlined. Figure 6 shows the amino acid structure of the wild type N-terminal *psb*C and *psb*DI threonine residues as well as the amino acid structures of the mutant alanine and aspartate residues. Both the plasmid constructs used for transformation of the mutant strains, and the genomic DNA of the resulting mutant strains were sequenced to verify the presence of the intended mutations. The introduced alanine and aspartate mutations were verified using a flourescence-based Prism DyeDeoxy Terminator Cycle Sequencing Kit with Amplitaq DNA polymerase, FS (Applied Biosystems, Inc., Foster City, CA). Primers (Gibco-BRL) used for sequencing the entire *psb*C and *psb*DI fragments are shown in Table 2. All sequencing reactions were analyzed on an Applied Biosystems 373A Stretch DNA Sequencing System. In both the *psb*C and *psb*DI genes, the original N-terminal threonine codon, ACT, was changed to GCT, which encodes alanine, and to GAC which encodes aspartate. These changes verify that the intended mutations were introduced.

It is necessary to mention that after sequencing the entire genomic *BssH*II/*Eco*RI fragment of *psb*C, no other mutations were found to be present aside from the intended alanine and aspartate mutations. However, when the entire genomic *Sfi*I/*BssH*II fragment of *psb*DI was sequenced, several other non-intended mutations were observed. Due to these additional mutations, further characterization of the *psb*DI mutants was not

undertaken. It would have been impossible to determine whether the phenotypes observed would be the result of the intended mutation, the non-intended mutations, or a combination thereof. It is assumed that these additional mutations occurred randomly during the process of homologous recombination during transformation of the deletion strain with the mutagenic *psb*DI constructs, since they were not present in the plasmid constructs. Therefore, the focus of the characterization studies was on the CP43 mutants.

Growth Characteristics

Photoautotrophic Growth of Control and Mutant Strains T3A and T3D. Figure 7 shows the results from averaging three independent growth experiments performed using control cells, and the CP43 alanine (T3A) and CP43 aspartate (T3D) mutant cells. The control strain was constructed by transforming wild-type *Synechocystis* with the pDICK plasmid which contains the overlapping *psb*DI and *psb*C genes (Figure 4) as well as a selective kanamycin resistance gene located 3' relative to the *psb*DI and *psb*C genes. For each growth culture, 100ml of sterile BG-11 was inoculated with 1x 10^7 cells/ml of *Synechocystis* cells and 20 µl of 50mg/ml kanamycin for expression of the pDICK insert containing plasmid. All growth experiments were performed in a 30°C Sherer growth chamber, under 25µmoles photons/m^{-2}/s^{-1} of light, with shaking at 210 rpm. Both mutant and control growth cultures were removed from the growth chamber daily to record A_{730} readings for 9 consecutive days. Figure 7 shows that the T3A mutant cells grow photoautotrophically to similar maximum rates as the control cells. Unlike the control cells and the T3A cells, the T3D mutant cells did not exhibit photoautotrophic growth. These results indicate that the T3D mutation produces a defect within the PSII complex of these mutant cells.

Photoheterotrophic Growth of Control and Mutant Strains T3A and T3D. It has been well documented in the literature that *Synechocystis* 6803 may be grown in glucose containing media in the absence of a functional PSII complex allowing for the isolation of mutants with impaired PSII activity (Dzelzkalns and Bogorad 1988). Figure 8 documents photoheterotrophic growth of the control cells and the T3A and T3D cells in BG-11 media supplemented with 5 mM glucose. This data represents the average of three independent growth experiments. Under these conditions all of the cell lines grew at nearly identical rates. The significance is that growing the T3D cells photoheterotrophically in the presence of 5 mM glucose restores the T3D cells growth rate to comparable control levels. This data, coupled with the data on photoautotrophic growth, convincingly demonstrates that the lesion resulting from the introduction of the mutation resides within PSII.

Photoautotrophic Growth of the T3D "Revertant" Strain. In the experiment depicted in Figure 10, growth rates of the control cells, T3D cells, and T3D cells which have been transformed with non-mutagenized pTZ18U-*psb*C plasmid (T3D "revertant") were examined in the absence of glucose. DNA sequencing confirmed that this transformation converted the T3D mutation back to the original N-terminal threonine residue found in the control cells. This data shows that the T3D mutant cells failed to grow photoautotrophically. But, transformation of the T3D cells with the non-mutagenized pTZ18U-*psb*C plasmid resulted in restoration of photoautotrophic growth rates similar to those of the control strain. This data, along with the sequencing data, confirms that there is only a single mutation in the T3D cells which is responsible for the mutagenic phenotype.

Photoheterotrophic Growth of the T3D "Revertant" Strain. Figure 9 shows a comparison of photoheterotrophic growth rates in 5 mM glucose of control cells, T3D mutant cells, and T3D cells which have been transformed with the non-mutagenized pTZ18U-*psb*C plasmid (T3D "revertant"). This data represents the average from three independent growth experiments. When grown in the presence of glucose, there are no significant differences in the growth rate between the three cell types, as would be expected.

Oxygen Evolution

Oxygen Evolution in Control and Mutant Strains T3A and T3D. Figure 11 illustrates steady state oxygen evolution rates (water to DCBQ) of the control cells, the T3D cells grown in the presence of 5 mM glucose, and the T3A cells grown in both the presence and absence of glucose. Note that the T3D cells do not grow in the absence of glucose, and therefore an oxygen evolution rate could not be determined under these conditions. These rates are the average of three independent experiments. The error bars are plus and minus 1.0 standard deviation. The oxygen evolution rates for the control cells in the presence and absence of glucose were 797 and 683 μmoles O_2/mg chl/ hour, respectively. The oxygen evolution rate of the T3A cells was 619 μmoles O_2/mg chl/ hour in the presence of glucose and 525 μmoles O_2/mg chl/ hour in the absence of glucose. Expressed as a percentage of the control, the T3A cells evolved oxygen at approximately 77% of the control cells in both the presence and absence of glucose. A completely different result was obtained for the T3D mutant cells. Grown in the presence glucose, the T3D cells lacked any ability to evolve any oxygen. These results clearly show that the T3D cells are severely damaged and contain a lesion imparing PSII oxygen evolving activity.

Oxygen Evolution in the T3D "Revertant" Strain. Table 3 shows oxygen evolution rates in the presence and absence of 5 mM glucose of control cells, T3A cells, T3D cells, and T3D cells which have been transformed with non-mutant pTZ18U-*psb*C plasmid (T3D "revertant"). The oxygen evolution rates are expressed as a percentage of the control oxygen evolution rate (100%) and are the averages of three independent experiments. The T3A cells evolved oxygen to a similar rate with and without glucose; to 78% of the control rate in the presence of glucose and 77% of the control rate in the absence of glucose. This rate was not significantly different from that of the control. The T3D cells, which only grew photoheterotrophically, were severely damaged and lacked the ability to evolve oxygen. However, when the T3D cells were transformed with the non-mutagenized pTZ18U-*psb*C plasmid, oxygen evolution rates were restored to 81% of the control in the presence of glucose and 91% of the control in the absence of glucose. These results clearly demonstrate that the T3D cells were impaired in the ability to evolve oxygen, but were restored close to control levels upon restoration of the threonine residue.

Figure 7. Photoautotrophic growth of control cells, T3A mutant cells, and T3D mutant cells. These are averages of three independent growth experiments. Error bars are ± one standard deviation.

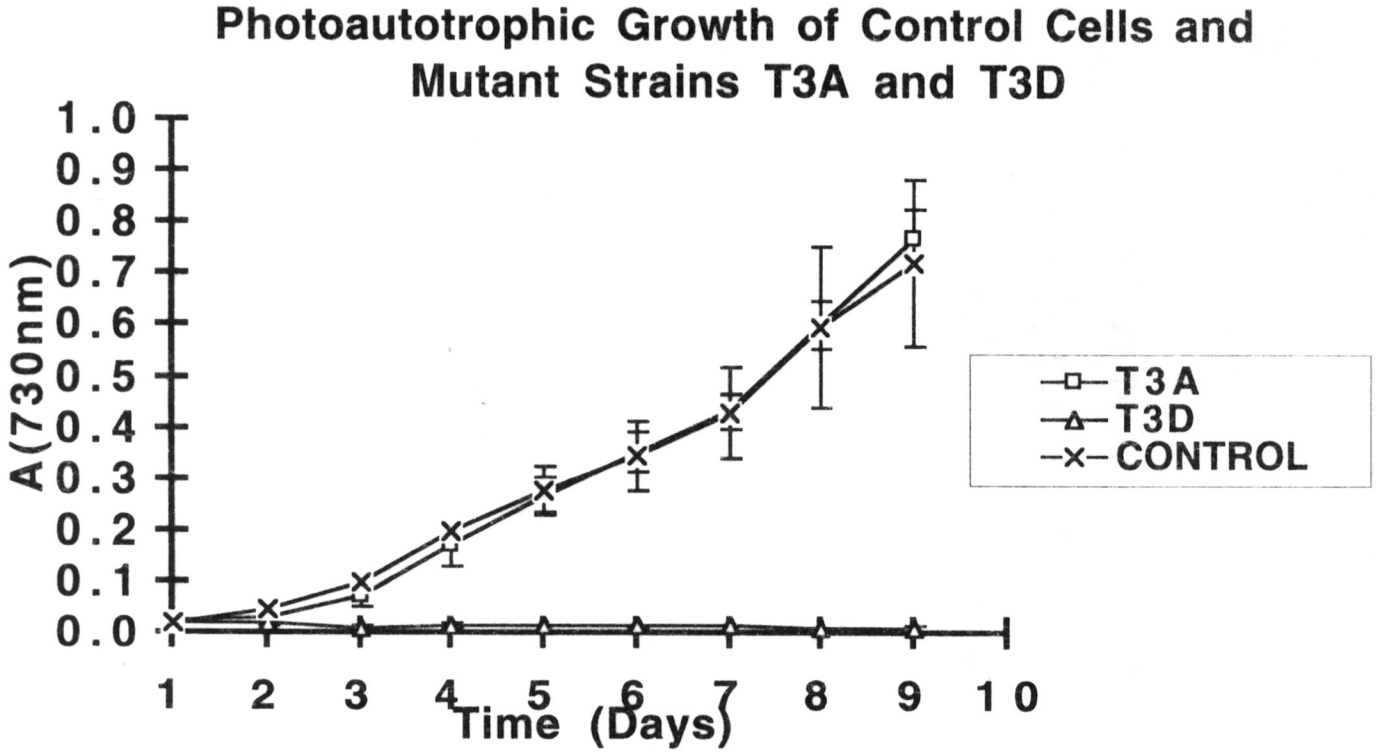

Photoautotrophic Growth of Control Cells and Mutant Strains T3A and T3D
A(730nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
4
5
6
7
8
9
10
Time (Days)
T3A
T3D
CONTROL

Figure 8. Photoheterotrophic growth in 5mM glucose of control cells, T3A mutant cells and T3D mutant cells. These data represent the average from three independent growth experiments. Error bars are ± one standard deviation.

Photoheterotrophic Growth of Control Cells and Mutant Strains T3A and T3D

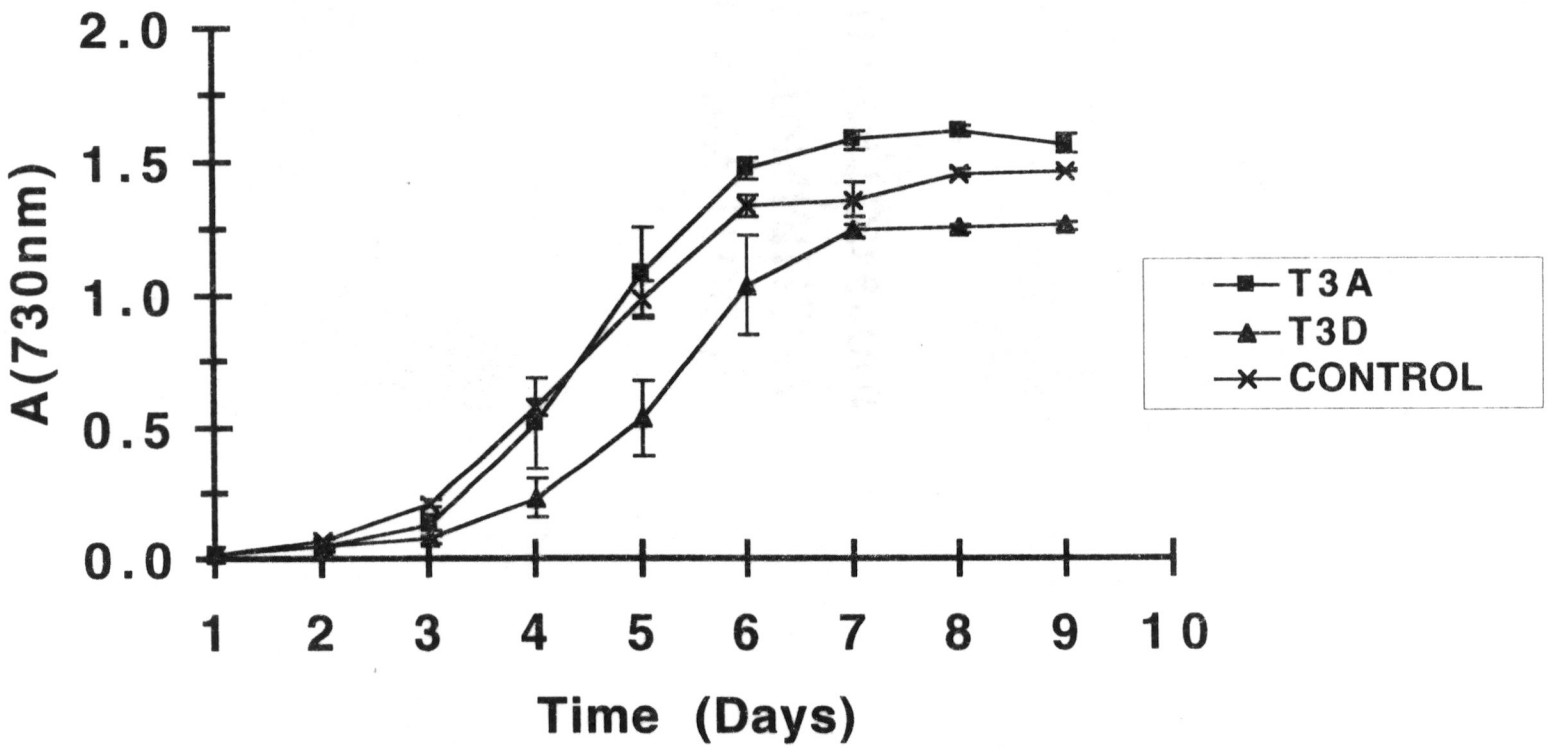


Figure 9. Photoheterotrophic growth in 5mM glucose of control cells, T3D mutant cells, and T3D cells transformed with non-mutant pTZ18U-*psb*C plasmid (T3D "revertant"). These data represent the average from three independent growth experiments. Error bars are ± one standard deviation.

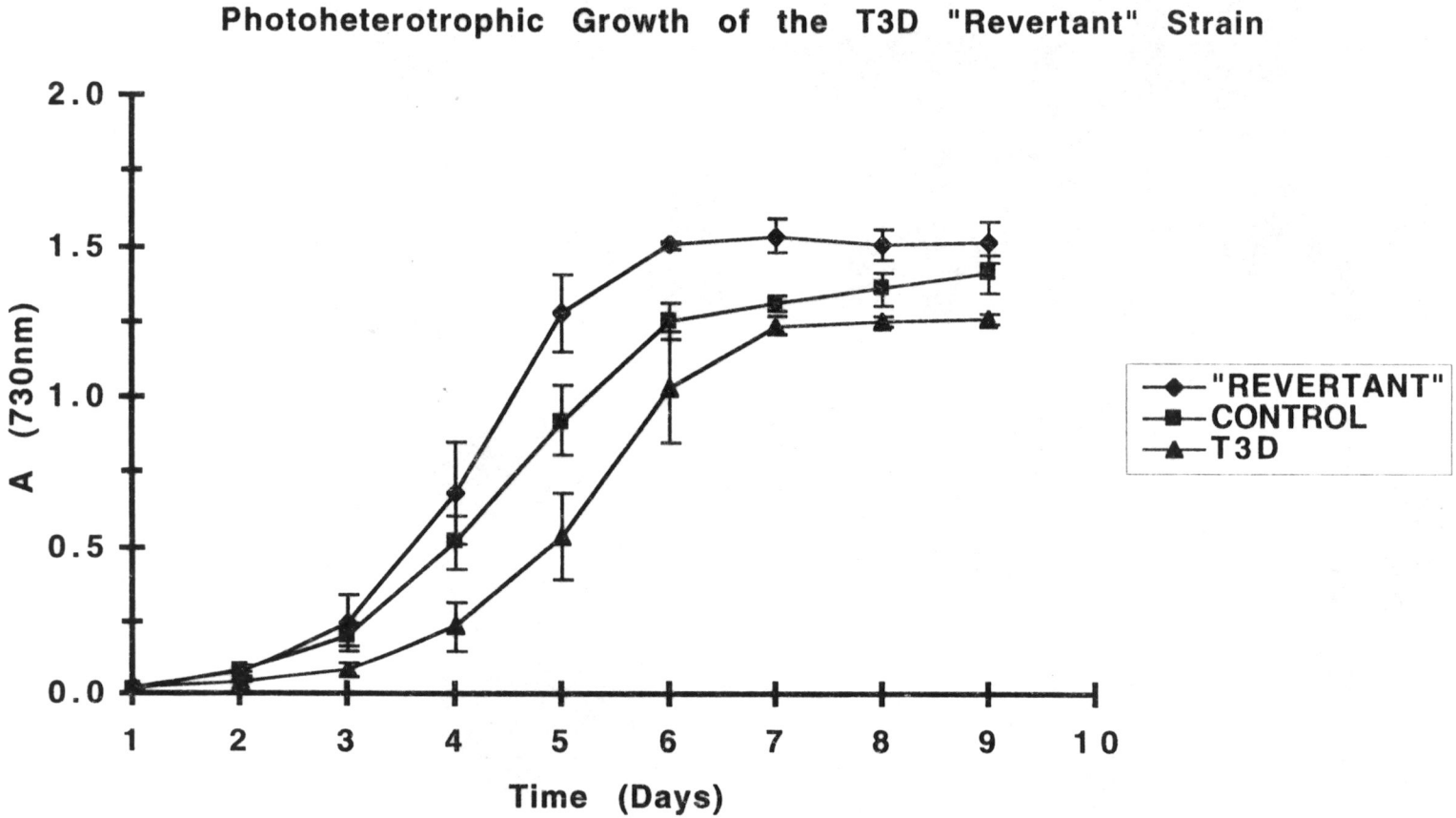

Photoheterotrophic Growth of the T3D "Revertant" Strain
2.0
1.5
1.0
0.5
0.0
A (730nm)
1
2
3
4
5
6
7
8
9
10
Time (Days)
"REVERTANT"
CONTROL
T3D

Figure 10. Photoautotrophic growth of control cells, T3D mutant cells, and T3D cells transformed with non-mutant pTZ18U-*psb*C plasmid. (T3D "revertant"). These are averages of three independent growth experiments. Error bars are ± one standard deviation.

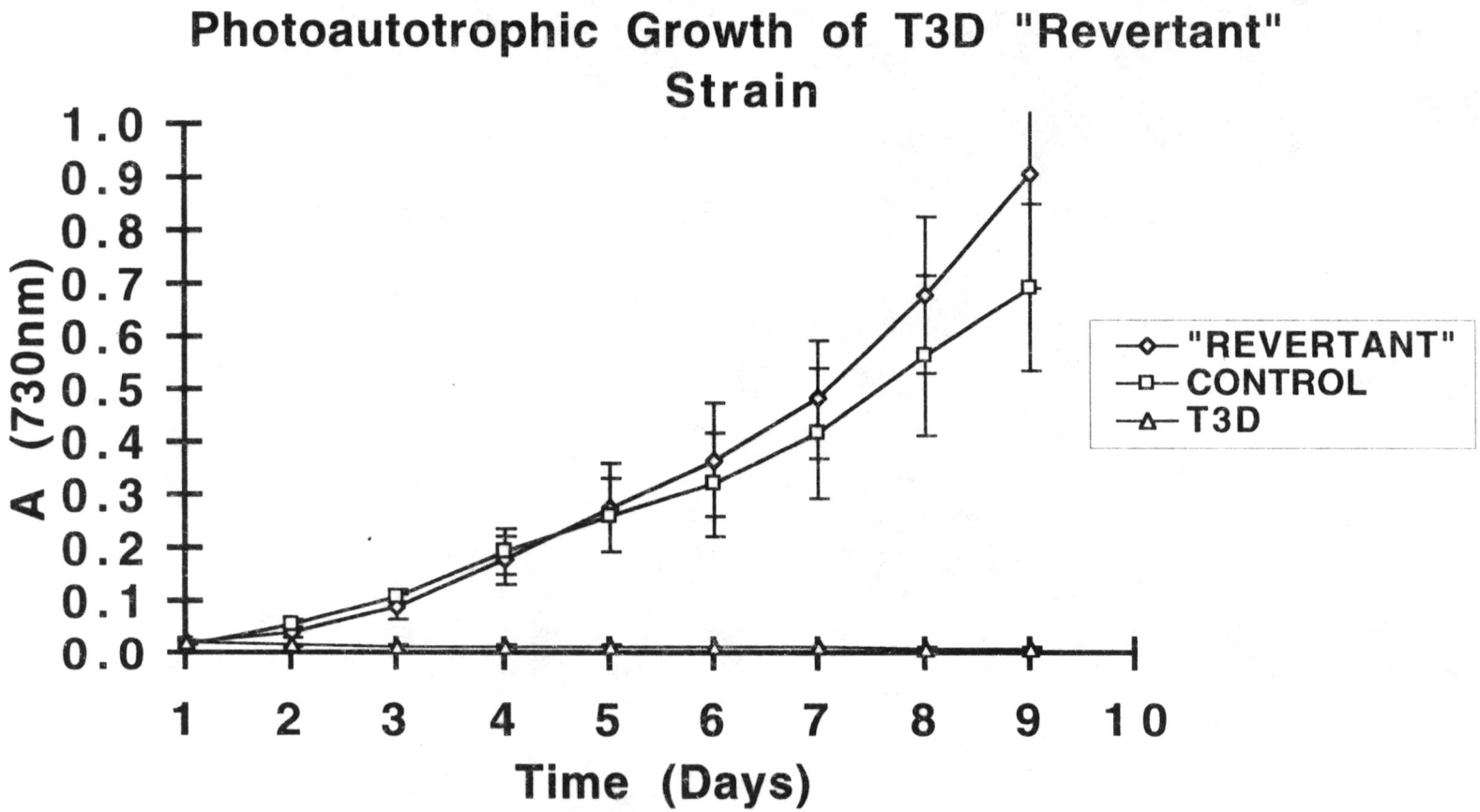
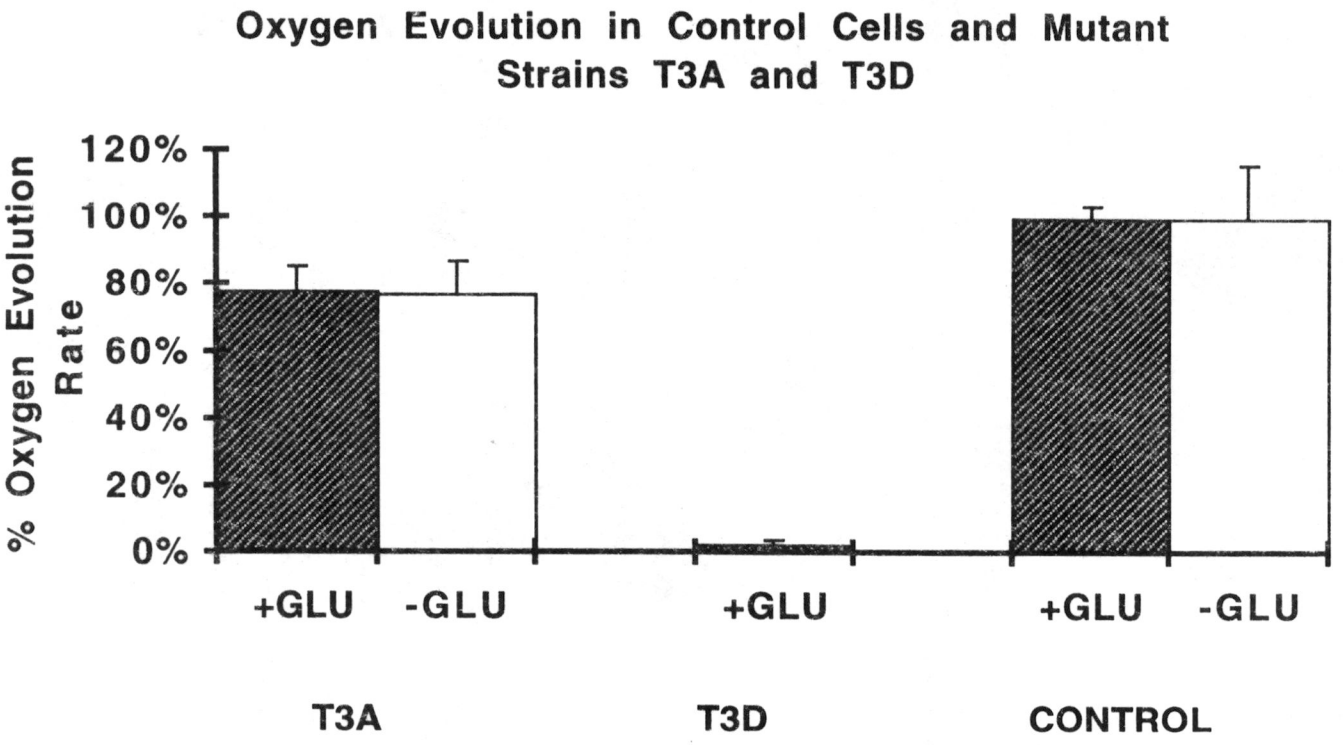

Photoautotrophic Growth of T3D "Revertant" Strain
A (730nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
4
5
6
7
8
9
10
Time (Days)
"REVERTANT"
CONTROL
T3D

Figure 11. Oxygen evolution (from water to DCBQ) of the control strain, T3D cells grown in the presence of 5mM glucose, and T3A cells grown in the presence or absence of glucose. These data are the average of three independent experiments. The error bars represent ± one standard deviation.


Oxygen Evolution in Control Cells and Mutant Strains T3A and T3D
% Oxygen Evolution Rate
120%
100%
80%
60%
40%
20%
0%
+GLU
-GLU
+GLU
+GLU
-GLU
T3A
T3D
CONTROL

Oxygen Evolution

Strain	μmoles/O_2/mg chl/hr. (% control)	
	+ Glu	-Glu
CONTROL	100%	100%
T3A	78% ± 8	77% ± 10
T3D	2% ± 2	----
"REVERTANT"	81% ± 14	91% ± 10

Table 3. Oxygen evolution rates (from water to DCBQ) of T3A cells, T3D cells, and T3D cells transformed with non-mutant pTZ18u-*psb*C plasmid (T3D "revertant"). These rates are expressed as a percentage of the control.

DISCUSSION

Site-directed mutagenesis is a useful method for probing the structural and functional significance of specific amino acid residues within proteins. Thus, site-directed mutagenesis was utilized as a tool in this project to determine the functional significance of the N-terminal threonine residue of the *Synechocystis* 6803 CP43 protein of PSII. Sequence information has already revealed that the N-terminal threonine residue is conserved between the CP43 protein of higher plants, green algae, and cyanobacterial species (Arntzen and Pakrasi 1986). In addition, it has been determined that numerous PSII proteins are phosphorylated in higher plants at the N-terminal threonine residue including LHCII, CP43, D1, D2, and *psb*H (Harrison and Allen 1991). The significance of this phosphorylation remains a subject of speculation in higher plants, but several hypothesis have been put forth (discussed below). Although the N-terminal threonine residue is conserved within the CP43 protein of cyanobacteria, phosphorylation of this residue has not been unequivocally demonstrated. The fact that it is conserved in all species examined to date suggests that this residue plays a functional role.

This research involved alteration of the N-terminal threonine residue of the *Synechocystis* CP43 protein as a direct test of its significance to PSII structure and function. Overall, the results from this research support the hypothesis that, as in higher plants, the N-terminal threonine residue of the CP43 protein of *Synechocystis* 6803 is required for a stable, functional PSII complex. This conclusion stems from the altered phenotype observed with the T3D mutant cells. The T3D mutant cells are devoid of oxygen evolving activity and are unable to grow photoautotrophically. Our working hypothesis is that the persistence of a negative charge at this site by replacement of threonine with aspartate results in a defect which renders the PSII complex inactive

(discussed further below). However, replacing the N-terminal threonine residue with an uncharged alanine residue has little effect on PSII function.

Growth Characteristics of the T3D and T3A Mutant Strains

A major support for the hypothesis that the N-terminal threonine residue of the *Synechocystis* CP43 protein is critical to PSII function is the failure of the T3D mutant cells to grow photoautotrophically (Figure 7). Unlike the T3D mutant cells, the T3A mutant cells grow photoautotrophically to approximately the same level as the control cells. Both the control cells and the T3A mutant cells grow to a maximum A_{730} of approximately 0.75 after nine days. By contrast, the T3D mutant cells exhibit no photoautotrophic growth during the same growth period. An intact PSII complex is absolutely required for photoautotrophic growth. Failure to grow photoautotrophically is an indication of a lesion within PSII.

As mentioned previously, *Synechocystis* 6803 may be grown photoheterotrophically in the absence of a functional PSII complex in media containing glucose. This allows for the propagation of mutants with impaired PSII activity (Dzelzkalns and Bogorad 1988). The ability of *Synechocystis* to grow photoheterotrophically in the absence of a functional PSII complex is well known (Williams, 1988). Here the only requirement for growth is an intact PSI.

Results from the photoheterotrophic and photoautotrophic growth data together demonstrate that the lesion resulting from the introduction of the T3A and T3D mutations resides within PSII. Under photoheterotrophic conditions, when grown in BG-ll media supplemented with 5mM glucose, the T3A and T3D mutant cells grow at a similar rate as the control cells (Figure 8). Even though T3D cells grow at a similar rate compared to the control, they do exhibit a lag of approximately one day. Also, the maximal A_{730} attained by

the T3D cells is slightly lower than that for the control. The reason for these latter observations is unclear at this time.

Growth Characteristics of the "Revertant" Strain

To further verify that the observed phenotype of the T3D mutant resulted from the intended mutation, the T3D cells genetically complemented back to wild type by transformation of the T3D mutant cells with non-mutagenized pTZ18U-*psb*C plasmid. This resulted in the restoration of threonine at position 3. This data confirms that only a single mutation is present within the N-terminus of the CP43 protein. Furthermore, this single mutation is responsible for the mutagenic phenotype of the T3D mutant cells. The photoheterotrophic growth rates of the T3D mutant cells and T3D "revertant" cells are also identical, as would be expected, since *Synechocystis* 6803 mutants may be maintained in BG-11 media containing glucose (Dzelzkalns and Bogorad 1988).

Oxygen Evolution

The importance of the N-terminal threonine residue of the *Synechocystis* 6803 CP43 protein on PSII function is also evident in the drastic difference in the steady-state oxygen evolution rates between the T3D mutant cells and the control cells (Figure 11). The T3D mutant cells, when grown in BG-11 media supplemented with 5mM glucose, are unable to evolve oxygen. An oxygen evolution rate for T3D mutant cells grown in the absence of glucose cannot be determined, since the T3D mutant cells do not exhibit photoautotrophic growth. A dramatically different pattern is observed in the T3A mutant cells. Unlike the T3D cells, the T3A cells, when grown in both the presence and absence of glucose, evolve oxygen at a fairly similar rate as the control cells (78% of the control rate in the presence of glucose and 77% in the absence of glucose). These results clearly

demonstrate that the PSII complexes in the T3D mutant strain are defective and lack the ability to evolve oxygen.

Again, these data are strengthened by the restoration of oxygen evolving activity observed with the T3D "revertant" cells (Table 3). These results show that upon restoration of the N-terminal threonine residue, oxygen evolution rates of the T3D mutant cells are restored to 81% of the control cells in the presence of glucose, and to 91% of the control cells in the absence of glucose. The oxygen evolution rates of the T3D "revertant" cells are also approximately the same as the T3A mutant cells.

The results from alteration of the N-terminal threonine residue of the *Synechocystis* 6803 CP43 protein strengthen the hypothesis that CP43 is critical to normal PSII structure/function. The CP43 protein has a major role in PSII activity. CP43 serves primarily as a chlorophyll-a light harvesting antennae in PSII (Bassi et al. 1987) and is one of the integral membrane proteins necessary for the stability of the PSII complex (Rochaix et al. 1989). Site-directed mutants in *Chlamydomonas reinhardtii*, which were unable to accumulate CP43, had reduced levels of other PSII proteins including CP47, D1, and D2 (Rochaix et al. 1989). These mutants were deficient in PSII activity. *Synechocystis* mutants lacking the *psb*C gene accumulate only 10% of the normal complement of PSII core complexes and cannot grow photoautotrophically or evolve oxygen (Rogner et al. 1991; Carpenter et al. 1990).

A number of hypotheses can be presented to explain the results herein. Our working hypothesis is that the altered phenotype of the T3D cells is a result of the presence of the negative charge contributed by the introduced aspartate residue. We envision that phosphorylation of the native threonine at this position may be a reversible molecular "switch." In the non-phosphorylated state normal PSII activity is observed. Phosphorylation then renders, by an unknown mechanism, the PSII complex inactive.

Thus far, little information has accumulated regarding the function of phosphorylation of the CP43 protein in higher plants. The majority of phosphorylation research has focused upon the D1 protein of higher plants. Phospho- D1 appears to regulate the repair cycle of photodamaged PSII by regulating the turnover of other PSII proteins during photoinhibition (Rintamaki et al. 1994a). Similar evidence exists for the phosphorylation of the D2 protein as well, although the turnover rate of phospho-D2 is much slower (Koivuniemi et al. 1995). The phosphorylation of both of these proteins occurs at the N-terminal threonine residue (Aro et al. 1992). Turnover of CP43 and D2 upon illumination with strong photoinhibitory light (5000 μmoles photons/m^{-2}/s^{-1}) has also been demonstrated by Yamamoto and Akasaka (1995) in tris-washed spinach thylakoid membranes. Three degradation products of CP43 were formed, as well as extensive crosslinks between the CP43, CP47 and D1 proteins. The degradation of CP43 during photoinhibition caused the entire PSII complex to become unstable and disorganized, leading to accelerated disintegration of the PSII complex. Whether or not CP43 was phosphorylated under these conditions was never shown. It is possible that it is actually phospho-CP43 that is involved in turnover during photoinhibition as is the case with D1 and D2. Even under non-photoinhibitory conditions, phosphorylation may be a molecular "switch" that targets CP43 for normal turnover. If so, then the presence of a permanent negative charge would signal for turnover of CP43 and the entire PSII complex. This would then give CP43 phosphorylation a similar role to phospho-D1 and phospho-D2.

Another hypothesis is that the negative charge of phopho-CP43 functions in repelling phospho-LHCII during a state 2 transition, in which LHCII is dissociated from PSII resulting in a shift of exicitation energy away from PSII (Ikeuchi et al. 1987; Michel et al. 1988). The kinase responsible for phosphorylating CP43 is redox controlled at the level of the plastoquinone pool. State transitions also occur in cyanobacteria and appear to be

regulated by the redox state of plastoquinone as well (Mullineaux and Allen 1986; Bruce et al. 1989), though phosphorylation of CP43 in response to plastoquinone reduction has not been conclusively demonstrated.

An additional possibility is that any covalent modification of the CP43 protein (such as the introduction of a negative charge) could result in a conformational change within the PSII complex which may affect the binding of other essential PSII components, therefore decreasing PSII activity. At the moment we cannot distinguish between this possibility and the possibility that we have identified a true functional domain. This is the obvious problem in all mutagenesis studies. The definitive answer awaits the solving of the x-ray crystal structure of PSII.

Conclusion

The results from this study strengthen the hypothesis that the conserved N-terminal threonine residue in the CP43 protein of *Synechocystis* 6803 is required for normal PSII activity. Alteration of the N-terminal threonine residue of CP43 to a negatively charged aspartate residue results in the loss of photoautotrophic growth and oxygen evolution. We speculate that covalent modification of the N-terminal threonine residue may be the underlying cause of the observed phenotype. This work does not attempt to prove whether the N-terminal threonine residue is or is not phosphorylated. Nor does it reveal the precise mechanism for control of protein phosphorylation by kinases and phosphatases.

Future Directions

The results from this research provide the foundation for further characterization of the potential effects of covalent modification of the N-terminal threonine residue on the structure and function of PSII in *Synechocystis* 6803. These next steps include the following. First, it would be of interest to replace the N-terminal threonine residue of the

CP43 protein with a serine residue. This would determine whether another amino acid residue with a phosphorylatable R-group similar to threonine would result in a wild-type phenotype. If our hypothesis is correct, then introduction of glutamate at this site would also produce the altered phenotype observed with the aspartate mutant. Secondly, it is of interest to assess whether or not PSII complexes are actually assembled in the T3D mutant, as opposed to complexes being present which are inactive. Immunoblots are necessary to determine the effects of T3A and T3D mutations on the assembly of a functional PSII complex. Polyclonal antibodies against the major PSII proteins are readily available to determine the presence or absence of PSII proteins in these mutants. Fluorescence assays should also be performed which would provide an indication of the number of functional PSII centers in the T3A and T3D cells as compared to the control cells. It can be hypothesized that the T3A mutant cells would contain approximately the same number of centers as the control cells, whereas the T3D mutant cells would contain either very few centers or a fairly normal complement of centers which would be inactive (both indicative of the decreased PSII activity). The T3D "revertant" cells, which restore photoautotrophic growth and oxygen evolution rates comparable to the control cells, should contain approximately the same number of centers as the control cells.

In addition to the above mentioned experiments, photoinhibition studies should be performed. In these assays, the T3A and T3D mutants would be exposed to intense light conditions (4,000 μmoles photons/m^{-2}/s^{-1}). These studies would probe the possible role of the N-terminal threonine residue of the *Synechocystis* 6803 CP43 protein in increased susceptibility to or protection from photoinhibition.

Additionally, in-vitro phosphorylation assays, in which the T3A and T3D mutants would be grown in the presence of ^{32}P-orthophosphate, would demonstrate whether or not the N-terminal residue of the CP43 protein is actually phosphorylated.

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