Photosystem II Function and Integrity in Spite of Drastic Protein Changes in a Conserved Region of the D2 Protein†

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ABSTRACT: D1 and D2 are structurally related proteins forming the core of the photosystem II reaction center. The two proteins have several loop regions including an extended stroma-exposed loop between transmembrane helix *D* and parallel helix *de*. This loop (the *D–de* loop) is phylogenetically conserved in both proteins. The role of the *D–de* loop in photosystem II was studied in *Synechocystis* sp. PCC 6803 by constructing a chimeric D2 protein in which the stroma-exposed loop of D1 replaced that of D2. In one of the transgenic lines, a single-base deletion shifted the reading frame of the chimeric gene leading to loss of D2 accumulation and photosystem II assembly. Selection for spontaneous reversion to photoautotrophy yielded several suppressor mutants, five of which were analyzed. In all, further frameshifts in the inserted loop piece restored the original reading frame allowing readthrough to the normal carboxy terminus. However, the sequences in the restored *D–de* loop varied widely among the mutants. Changes ranged from a deletion of one amino acid residue to an insertion of 31, while the net charge of the *D–de* loop increased by up to 12 units. Mutant electron transfer rates and photoautotrophic growth were only mildly affected as compared to wild type. Nevertheless, in all mutants, the hydropathy profile of the stroma-exposed *D–de* loop region maintained its hydrophilic character including turns in similar locations. We conclude that the stroma-exposed, *D–de* loop of the D2 protein can accommodate drastic composition and size changes without extensive functional consequences in photosystem II. Hydrophilicity appears to be the major structural information encoded in this region.

The current model for the photosystem II (PSII) reaction center supposes a heterodimer arrangement of the D1 and D2 proteins, with accompanying chromophores and cofactors symmetrically oriented in the photosynthetic membrane [reviewed by Mattoo et al. (1989) and Vermaas and Ikeuchi (1991)]. The model (Trebst, 1986) is based on the high-resolution crystal structure of the functionally-homologous reaction center proteins L and M in anoxygenic purple bacteria (Michel & Deisenhofer, 1988). The sequences of D1 and D2 are extended at several locations as compared to the bacterial L and M subunits which may correspond to unique features of PSII. One such location faces the stroma and connects between transmembrane helix *D* and parallel helix *de* of both the D1 and D2 proteins. This region is termed the *D–de* loop.

The *D–de* loop of the D1 protein (residues 225–250) is in the vicinity of the secondary quinone (Q_B) and PSII-herbicide binding niche (Dostatni et al., 1988), while in D2, the *D–de* loop (residues 224–248) is near the binding pocket of the primary quinone (Q_A). Major D1 and D2 trypsinization sites are present in the *D–de* loop (Marder et al., 1984; Geiger et al., 1987). Application of PSII herbicides known to displace Q_B from its binding niche on D1 retards trypsinization of both proteins and has been interpreted to indicate a contact site between the two in this region (Trebst, 1991). The *D–de* loop of D1 was proposed as the site of primary cleavage of D1 during light-dependent degradation of the protein (Greenberg et al., 1987).

Loop regions in proteins often are involved in binding and recognition at surface areas as exemplified by those in DNA-binding proteins and immunoglobulins (Pabo & Sauer, 1984; Mariuzza et al., 1987). Exposed loop regions are generally flexible and mobile and can accommodate sequence modifications although they tend to adopt particular conformations (Thornton et al., 1988; Chothia et al., 1989).

The *D–de* loop region of the D1 and D2 proteins are twice as long in PSII as in bacterial reaction centers and are presumed to be exposed to the stromal, rather than lumenal side of the photosynthetic membrane (Michel & Deisenhofer, 1988). The *D–de* loops of D1 and D2 are each highly conserved phylogenetically, 18 of 25 residues being identical among 27 published sequences of D1 and the same number among 15 sequences of D2 (Svensson et al., 1991). Yet, there is less than 30% sequence homology between the loops of the two proteins suggesting particular roles for each.

We used *Synechocystis* sp. PCC 6803 to engineer an exchange of an 18 amino acid stretch of the *D–de* loop of D1 for the comparable loop region of D2. This appears to lead to a relatively normal photoautotrophic phenotype. However, among the resulting transformants, one was found that lacked PSII activity. This mutant had a one-base deletion in *psb*DI, leading to an altered reading frame. From this mutant, photoautotrophic pseudorevertants were isolated. Analysis of these strains revealed additional shifts in that gene region (intragenic suppression) which result in dramatic changes in composition, length, and net charge of the *D–de* loop in the D2 protein. This indicates that extensive sequence alterations

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Table I: List of Oligonucleotides

	oligonucleotide sequence[a]	corresponding gene position[b]	restriction site modification[c]
I	CACCCTGTTTCGCGAAGATGGG	*psb*DI 660-678	*Nru*I
II	CGAAACCACCGAAGTTGAATCCCAGAACTACGG TTACAAATTCGGCCAAGAAGAAGAAACCTACTC	*psb*AII 677-738	*Nru*I;*Hae*III;*Nco*I
III	CATGGAGTAGGTTTCTTCTTCTTGGCCGAATTTGT AACCGTAGTTCTGGGATTCAACTTCGGTGGTTTCG	*psb*AII 677-738	*Nco*I;*Hae*III;*Nru*I
IV	GCAGTCGGATCCGCCCCAG	*psb*DI 10-28	*Bam*HI
V	GAGCGTTAACACGGGGGAG	*psb*DI 1054-1036	*Hin*cII
VI	GCGGGAATTCTCCGGTTCA	*psb*DI 526-544	*Eco*RI
VII	GGCCCGAAGCTTCAAGGC	*psb*DI 885-868	*Hin*dIII
VIII	GGTTCCACAACTGGACTT	*psb*DI 560-577	
IX	CGCTTGTTGGAGAAAGCA	*psb*DI 777-794	
X	CGTAGCTCCTGGGAGA	*psb*DI 895-911	
XI	CCGTATTCTTGATCTACCC	*psb*AII 467-485	
XII	GGATTAATTCTCTAGACTC	*psb*AII 1245-1227	

[a] Oligonucleotides are listed in a 5′ to 3′ direction. [b] Numbering for *psb*DI is according to Williams and Chisholm (1987) and for *psb*AII is according to Ravnikar et al. (1989). [c] Restriction site modifications are underlined and listed from left to right. (Not all modifications were utilized in this study).

in a highly conserved protein region only mildly affect basic PSII function. The hydrophilic character of the *D–de* loop was maintained in all of the modified D2 proteins.

MATERIALS AND METHODS

Strains and Growth Conditions. Synechocystis sp. PCC 6803 cells were grown in BG-11 medium (Williams, 1988). The wild type strain for these studies lacks *psb*DII, which was replaced by a spectinomycin-resistance cartridge, and contains a kanamycin-resistance cartridge downstream of *psb*DI/C (Vermaas et al., 1990a). *psb*DI/C carrying site-directed mutations was introduced along with the kanamycin-resistance cartridge into the above wild type to obtain the mutant line D2-1*. Mutant cultures were maintained on plates in the presence of 20 μg/mL spectinomycin and 10 μg/mL kanamycin. Five millimolar glucose and 20 μM atrazine were added to maintain PSII-independent photoheterotrophic growth. Liquid cultures were grown in 250-mL flasks containing 40 mL of medium on a gyratory shaker (100 rpm) at 30 °C and 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ fluorescent light. Cell density was measured at 730 nm by diluting cultures to an OD of <0.5, where turbidity is roughly proportional to cell number.

Transformation and Nucleic Acid Manipulations. Transformation of *Synechocystis* sp. PCC 6803 (Williams, 1988) and site-directed mutagenesis (Kunkel et al., 1988; Vandeyar et al., 1988) were performed as described. The oligonucleotides used in this study are listed in Table I. D2 mutations were initially prepared in a 533-bp *Xba*I–*Nco*I fragment of the *psb*DI gene cloned in pUC120. A codon for arginine, creating a unique *Nru*I site, was inserted between nucleotides 669 and 670 of *psb*DI [numbering according to Williams and Chisholm (1987)] using oligonucleotide I (Table I). The presence of the desired mutation was verified by sequencing (Sequenase, U.S. Biochemical) the single-stranded DNA induced from this plasmid (Vieira & Messing, 1987). Then, a double-stranded DNA fragment corresponding to *psb*AII sequence 677–738 [numbering according to Ravnikar et al. (1989)] but having additional *Nru*I and *Nco*I sites at the ends and a *Hae*III site in between, was synthesized using oligonucleotides II and III as templates. This DNA fragment was inserted between the unique *Nru*I and *Nco*I sites (position 734) in the *psb*DI gene and subcloned to replace the wild type sequence in a *psb*DI/C operon construct (Vermaas et al., 1990a) in pUC18. This plasmid contained an additional kanamycin-resistance cartridge just downstream of *psb*C and was used to transform a *Synechocystis* mutant carrying a deletion of its *psb*DII and *psb*DI/C genes as described (Vermaas et al., 1988, 1990a).

Kanamycin-resistant colonies were isolated, and the presence of the introduced mutation (following double recombination between homologous sequences up- and downstream of *psb*DI/C in the plasmid and the genome) was verified by the *Hae*III restriction pattern of a PCR-generated DNA amplified from the genomic *psb*DI gene (using oligonucleotides IV and V) and by direct sequencing of that PCR product. PCR amplification (30 cycles of 20 s at 93 °C, 40 s at 58 °C, and 40 s at 73 °C) was performed (Ericomp) using *Taq* DNA polymerase as recommended (U.S. Biochemical). Asymmetric PCR (McCabe, 1990) for sequencing was performed (40 cycles at the above conditions) starting with 5 ng of the above PCR-generated double-stranded DNA and using either oligonucleotides VI or VII. Single-stranded DNA sequencing of the asymmetrically-amplified PCR products was performed using *Taq* DNA polymerase (Taquence, U.S. Biochemical) as described (Brow, 1990). Both DNA strands were sequenced using either oligonucleotides VI, VIII, IX, or X. PCR amplification of the *psb*AII gene region was performed using oligonucleotides XI and XII.

Oxygen Evolution Assays. The steady-state rate of oxygen evolution was determined in intact cells at a chlorophyll concentration of 5 μg/mL using a Clark-type electrode. Measurements were performed at 25 °C in 25 mM Hepes/NaOH, pH 7.2, in the presence of electron acceptors 2,5-dimethyl-*p*-benzoquinone (0.1 mM) and $K_3Fe(CN)_6$ (1 mM). The light from a halogen projector was filtered through an OG 570 (Schott) filter and was saturating for maximal rates of electron transfer.

PSII Fluorescence Induction Measurements. PSII fluorescence induction measurements were performed using whole cells at a final concentration of 2 μg of chlorophyll/mL. The excitation wavelength was 620 nm and the intensity was 70 $\mu E \cdot m^{-2} \cdot s^{-1}$. The emitted fluorescence was detected through a RG 665 glass filter (Schott) transmitting above 665 nm, amplified, and recorded by a storage oscilloscope. The initial basal fluorescence (F_0) was determined by extrapolating the slope of the initial fluorescence kinetics following the full shutter opening back to a time point equal to the half-time of the shutter opening (2.5 ms). The steady-state fluorescence (F_s) was recorded after 2 s in the light, and the maximal fluorescence (F_m) was determined as F_s but in the presence

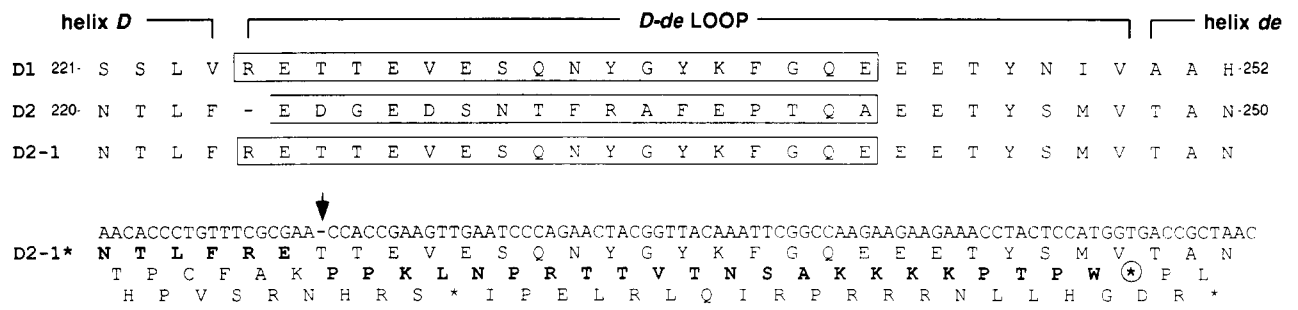

FIGURE 1: Origin of the D2-1* mutant. The protein sequence $D1_{225-242}$ replaced $D2_{224-240}$ to form the chimeric D2-1 protein. The manipulated residues are boxed. As a result of a nucleotide deletion (arrow) in one of the clones, mutant line D2-1* was formed. Bold letters indicate the shifted reading frame resulting from the deletion, and the circled asterisk represents the stop codon. The position of the *D–de* loop and the edges of helix *D* and *de* are indicated at the top.

of 0.1 mM DCMU (Schuster et al., 1988; Ohad & Hirschberg, 1992). The steady-state capacity of PSII for electron transfer is calculated as $f = [F_m - F_s]/[F_m - F_0]$ (Braun & Malkin, 1990).

LDS–PAGE and Immunoreactions. Total membranes were isolated after cell disruption using a Mini Bead Beater (Biospec products, Bartlesville, OK). Cells (up to 5 μg of chlorophyll) were suspended in a microfuge tube containing 0.2 mL of STN buffer (0.4 M sucrose, 10 mM Tris, pH 8.0, 10 mM NaCl) and 0.2 mL of 0.1-mm beads (suspended in STN) and vibrated for 30 s at maximal speed. Total membranes were collected from the pellet after centrifugation (12 000 rpm, 15 min) and solubilized in STN followed by LDS-containing buffer as described (Callahan et al., 1989). Membrane proteins (equivalent to 2.5 μg of chlorophyll) were fractionated by LDS–PAGE (10–20% gradient gel) containing 5 M urea at 4 °C and blotted using a semidry blotter (E&K Scientific Products). Immunoreaction was performed in PBS (100 mM NaCl, 100 mM phosphate buffer, pH 7.5) containing 1% Tween-20 with primary antisera raised against spinach D1 and D2 polypeptides (gift of N. Nelson) and against residues 237–245 of D2 (gift of R. Sayre). The signal was amplified and detected using an enhanced chemoluminescence (ECL) system (Amersham).

RESULTS

Physical Characterization. Synechocystis sp. PCC 6803 was engineered to have codons 225–242 of the *psb*AII sequence replace the analogous codons (224–240) of the *psb*DI sequence. This represents most of the *D–de* loop region of the proteins (Figure 1). Among several colonies resulting from the mutagenesis experiment, one was found to be photosynthetically incompetent. This mutant, D2-1*, was isolated and analyzed by direct sequencing of the PCR product amplified from its genomic *psb*DI gene. A deletion of an adenosine nucleotide in the inserted piece was revealed which could cause a reading frame shift and termination after residue 247 (Figure 1). Photosynthetically incompetent mutants having truncated *psb*D transcription units and lacking PSII reaction centers also have been described in *Chlamydomonas* (Erickson et al., 1986; de Vitry et al., 1989).

Spontaneous mutations in D2-1* leading to restoration of photoautotrophy were selected for on BG-11 plates without glucose. This approach yielded several photosynthetically competent colonies. Transformation of D2-1* with PCR-DNA fragments amplified from *psb*DI of the photoautotrophic revertants was then carried out to determine if this region of the *psb*DI is the genetic location responsible for the reversion.

Table II: Transformation of D2-1* with PCR-Amplified DNA Fragments of *psb*DI[a]

transforming DNA[b]	colonies/plate[c] expt 1	expt 2
WT *psb*DI-plasmid		80
WT *psb*DI-PCR	120	
D2S-A *psb*DI-PCR	300	20
D2S-C *psb*DI-PCR	300	15
D2S-E *psb*DI-PCR	150	13
none	47	0
D2-1* *psb*DI-PCR	50	
D2S-A *psb*AII-PCR	55	

[a] D2-1* cells were grown in the presence of glucose and then separately transformed with approximately 0.3 μg of a PCR-amplified DNA fragment from the genomic *psb*DI gene of each of the suppressor mutant lines. [b] The transforming DNA fragment (see Materials and Methods) contained, in each case, 99% of the entire *psb*DI gene and was amplified using oligonucleotides IV and V (Table I). [c] Following transformation, cells were grown on BG-11 plates without glucose, and the number of colonies capable of photoautotrophic growth was determined. Transformation without DNA (none), or with PCR-DNA amplified from the *psb*AII gene (D2S-A *psb*AII-PCR), served as negative controls. Transformation with a plasmid containing a 533-bp cloned fragment of the *psb*DI gene (WT *psb*DI-plasmid; see Materials and Methods) served as the positive control.

A marked increase in the frequency of photosynthetically competent colonies was indeed observed when such fragments were used (Table II). This indicates that suppression of photoautotrophic incompetence in the mutants generated from D2-1* is due to an intragenic change in the *psb*DI gene. The variability in the level of D2-1* transformation efficiency and reversion to photoautotrophic phenotype between experiments (Table II) may reflect differences in physiological competence of the cells at the time of transformation.

Five intragenic suppressor mutants isolated from D2-1* were characterized. Sequence analysis revealed that in all five a reversion to the original reading frame had occurred, resulting in a full-sized D2 protein. However, the location of the frameshift reversion varied among the mutants, leading to widely differing and novel protein sequences in the exposed *D–de* loop region (Figure 2): (a) Suppressor mutant D2S-C has a deletion of two nucleotides (CC) just following the original D2-1* deletion (Figure 2). This caused a shift back to the original reading frame with the deletion of a single amino acid. (b) Suppressor mutant D2S-A has an insertion of a tetranucleotide (CAAA) 54 bp after the original change in D2-1* (Figure 2). This restored the original reading frame, leading to a new D2 protein sequence with two amino acids of the inserted D1 piece (R and E) located before the frameshift position in D2-1*, followed by 19 residues (6 of which are

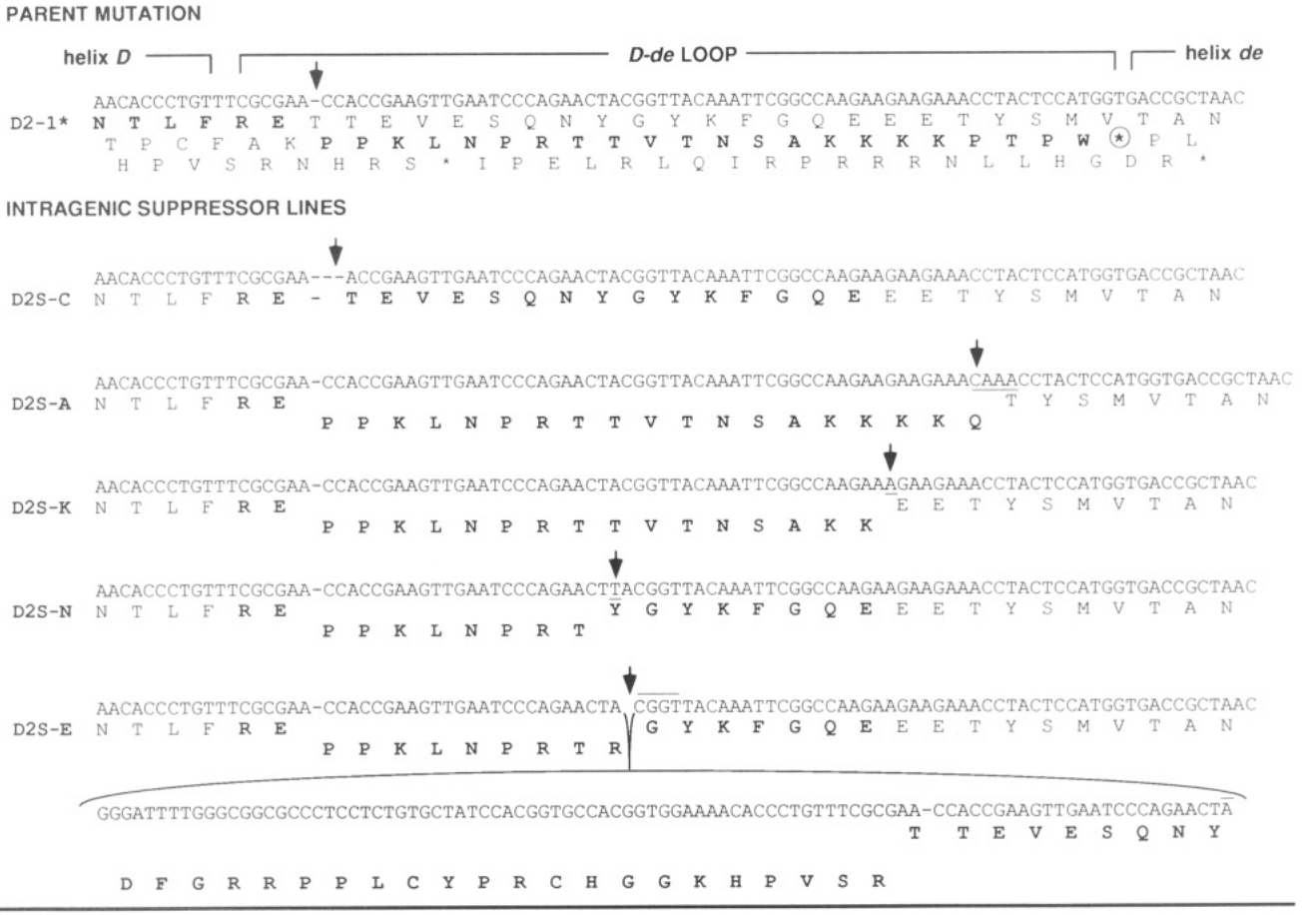

FIGURE 2: Nucleotide and protein sequences of suppressor mutants. All photoautotrophic suppressor mutants (intragenic suppressor lines) originated from line D2-1* (parent mutation, described in Figure 1). Positions in suppressor lines which shifted the reading frame back leading to a full-sized D2 protein are indicated by arrows. The short insertions in D2S-A, D2S-K, and D2S-N are underlined. Bold letters indicate the reading frame within the transgenic piece. A repeated head to head motif (ACGGT) found at the position where the duplicated piece was inserted in D2S-E (and where the piece initiated its duplication at position 607, not shown) is overlined.

charged) originating from neither D1 nor D2. (c,d) In two other suppressor mutants, D2S-K and D2S-N, the reading frame was shifted back to the original one by insertion of a single nucleotide 47 and 24 bp, respectively, after the original D2-1* change. These insertions produced unique protein sequences in the *D–de* loop region (Figure 2). (e) The most pronounced change in the D2 protein occurred in suppressor mutant D2S-E, where a 94-bp DNA fragment from an upstream region of the *psb*DI gene was duplicated and inserted 25 bp after the original frameshift in D2-1* (Figure 2). The resulting increase in the size of the mutated *psb*DI gene is visible in Figure 3. The duplication/insertion event caused the reading frame to shift back to the original one but the D2 protein was extended by 31 residues in the *D–de* loop region. A head-to-head repeated motif of five nucleotides (TGGCA) exists at the position where the duplicated piece was excised (*psb*DI nucleotides 602–606) and the location where it was inserted (Figure 2, D2S-E, overlined nucleotides). This motif appears several times at additional locations in this part of the gene and participates in putative stem–loop structures (not shown). Interestingly, two codons of arginine (AGA, AGG) which are not present in any of the known gene sequences of *Synechocystis* sp. PCC 6803 appear in the mutant *D–de* loops of the functionally-expressed D2 proteins.

Hydropathy plots of the decoded wild type and mutant D2 sequences were compared for the region between helices *D* and *E*. Although the polypepide sequences differ widely among the *D–de* loop region of wild type, D2S-C, D2S-A, and

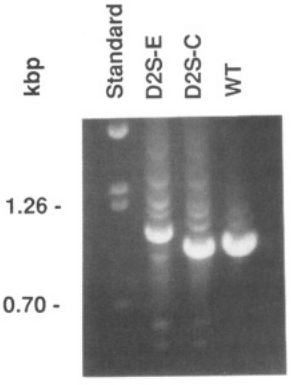

FIGURE 3: DNA fragments amplified by PCR from the genomic *psb*DI gene. An approximately 1-kbp fragment was amplified from wild type and photoautotrophic suppressor mutants D2S-E and D2S-C using oligonucleotides IV and V (see Table I). DNA (0.3 μg) was electrophoresed on a 1% agarose gel and stained with ethidium bromide. Size standards are of λ phage DNA digested with *Bst*EII.

D2S-E, all maintain a hydrophilic character (Figure 4). Likewise, the *D–de* loop region of D2S-K, D2S-N, and the D1 protein are hydrophilic (not shown). Furthermore, loop turns (Chou & Fasman, 1978), containing asparagine and glycine residues in the wild type D1 and D2 proteins, were retained in the *D–de* loop region of all the mutants at similar locations which now contained proline residues (not shown).

The presence of the D1 and D2 proteins in thylakoids of the suppressor mutants was revealed by immunoblotting

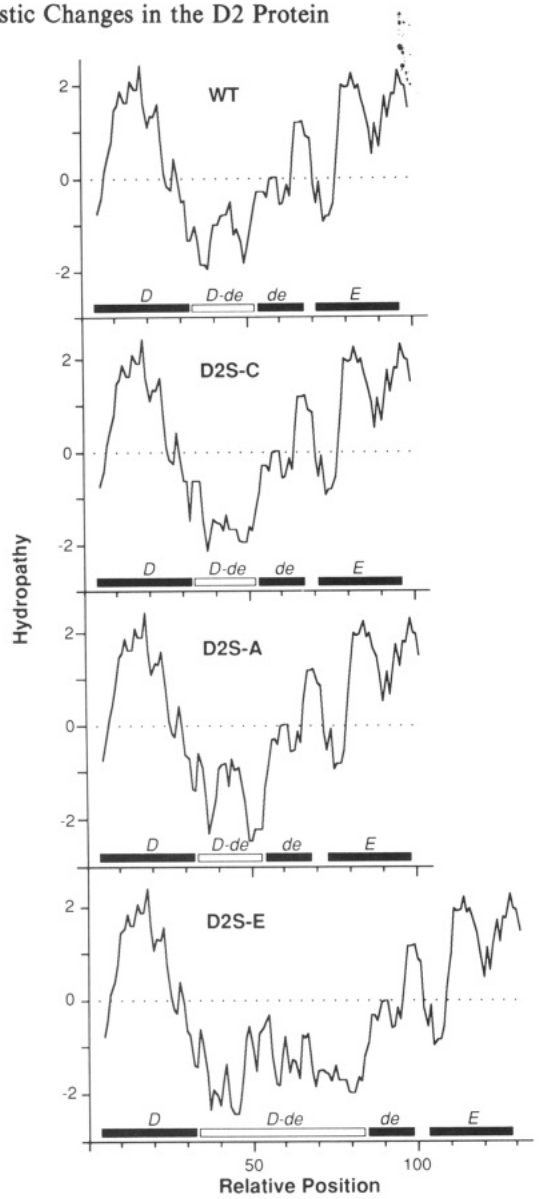

FIGURE 4: Hydropathy plots of helices *D* and *E* of the D2 protein. Analyses for wild type (WT) and suppressor mutants D2S-C, D2S-A, and D2S-E are shown. Sequences were analyzed according to Kyte and Doolittle (1982) using a window size of 9 amino acid residues. The position and lengths of helices *D*, *de*, and *E* are indicated by black bars, and those of the *D–de* loop are indicated by a white bar. The plots are aligned at the N-termini of the *D* helix (amino acid residue 191). Relative positions of elements are shown.

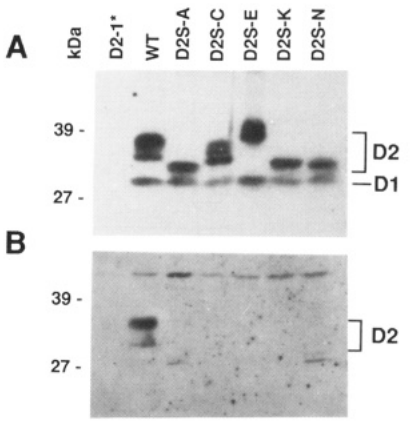

FIGURE 5: Immunoblots of D1 and D2 proteins. Membrane proteins of wild type (WT), D2-1*, and suppressor mutants (D2S-A, -C, -E, -K, -N) were isolated, electrophoresed at 4 °C on 10–20% gradient polyacrylamide gels containing LDS and 5 M urea, blotted as described, and detected using chemoluminescence. (A) Immunoreaction with polyclonal antibodies raised against both D1 and D2 proteins. (B) Immunoreaction with epitopic antibodies raised against amino acid residues 237–245 of the D2 protein.

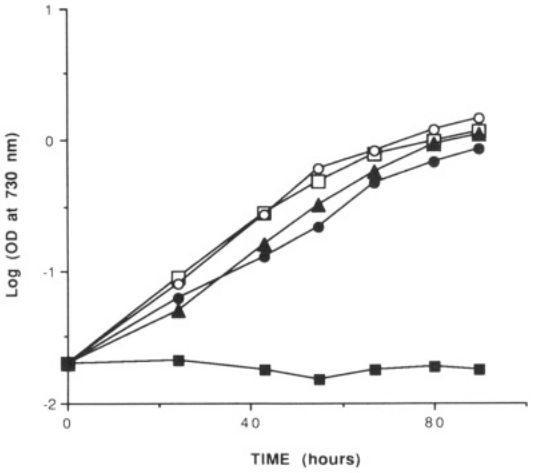

FIGURE 6: Growth rates of wild type (□), D2-1* (■), and suppressor mutants D2S-A (▲), D2S-C (○), and D2S-E (●) in the absence of glucose. Cell turbidity at 730 nm was measured as a function of time.

(Figure 5A). The results indicate small differences (less than 2-fold) in the apparent steady-state levels of the proteins in the suppressor mutants versus wild type. The considerable shift in electrophoretic mobility of D2 in various mutants, reflects changes in net charge, size, and/or conformation. The increased mobility of the D2 protein in mutants D2S-A, D2S-K, and D2S-N correlates with an increased net charge in the *D–de* loop of +12, +8, and +6, respectively, versus wild type. The charge effect of +9 in mutant D2S-E is apparently masked by the substantial increase in size of the mutant protein in this case.

D2 antisera raised against wild type amino acid residues 237–245 located in the *D–de* loop region failed to recognize the altered D2 protein in the membranes of all the suppressor mutants (Figure 5B). This confirms that the protein sequence in this region has indeed been modified.

Functional Characterization. The photosynthetic capabilities of the suppressor mutant lines were assessed by determining photoautotrophic growth rates at standard conditions of nutrition, aeration, temperature, and light (Figure 6). The D2-1* frameshift mutant was unable to grow under these conditions; however, the growth rate of suppressor mutant D2S-C was 100%, and that of mutants D2S-A and D2S-E was about 80% that of wild type. Thus, severe alterations in the *D–de* loop of the D2 protein do not appreciably interfere with photoautotrophic growth under permissive regimes.

The capacity of PSII electron transfer under steady-state conditions was evaluated using chlorophyll *a* fluorescence kinetics measurements (Braun & Malkin, 1990; Ohad & Hirschberg, 1992). The results indicate that the suppressor mutants are only mildly affected in overall PSII electron transfer potential, possessing 78–89% of the wild type capacity (Table III). Photosynthetic oxygen evolution rates under saturating light conditions were somewhat variable in the suppressor mutants, depending on the experiment.

DISCUSSION

Genetic and Structural Consequences. The capability of *Synechocystis* sp. PCC 6803 for spontaneous gene modification, coupled with strong selection pressure for a photoautotrophic phenotype, allowed isolation of several intragenic

Table III: Relative PSII Activities of D2 Mutants

cell line[a]	electron transfer capacity of PSII[b] (% of WT)	oxygen evolution[c] (% of WT)
WT	100	100
D2-1*	0	0
D2S-C	86 ± 8	56 ± 22
D2S-A	89 ± 4	57 ± 31
D2S-K	87 ± 4	71 ± 54
D2S-N	78 ± 6	56 ● 27
D2S-E	86 ± 8	66 ± 54

[a] See Figures 1 and 2 for descriptions of mutant lines. [b] Chlorophyll *a* fluorescence kinetics parameters (see Materials and Methods) were used to calculate the steady-state electron transfer capacity of PSII. Average values ± SD are for 5 measurements and are normalized to wild type. The absolute ratio $[F_m - F_s]/[F_m - F_0]$ of wild type is 0.86 ± 0.05. [c] Oxygen evolution rates were measured using whole cells in the presence of 2,5-dimethyl-*p*-benzoquinone and $K_3Fe(CN)_6$. Values for 5 measurements are normalized to wild type activity (183 ± 11 μmol of O_2·(mg of Chl)$^{-1}$·h^{-1}).

suppressor mutants from the obligate photoheterotrophic mutant D2-1*. The frame shift in the *psb*DI gene of D2-1*, caused by a single nucleotide deletion, was annulled by an additional deletion (2 bp) or by insertions (1, 4, or 94 bp). The location of the intragenic suppressor mutation varied from immediately following the original deletion to 54 nucleotides beyond that point. Although feasible, no intragenic suppressor mutant was found in which a frame shift occurred upstream to the original change in D2-1*. This may indicate that transmembrane helix *D* (the upstream region) is under greater constraints than the *D–de* loop to functionally accept dramatic changes.

The loop region between helices *D* and *de* of the D2 protein can accommodate viable alterations of composition, length, and charge. Insertion of 31 residues in mutant D2S-E suggests that this region has considerable "elbow room" for expansion. Functionally allowable modification of up to 21 residues in the *D–de* loop of the D2 protein correlates with a similar capacity of the loop in the D1 protein to accommodate drastic modifications (H. Kless, L. McIntosh, & M. Edelmen, unpublished). This marked plasticity raises a question as to the specificity of the proposed contact site in the vicinity of positively charged residues $D1_{238}$ and $D2_{234}$, respectively (Trebst, 1991). Furthermore, while it has been shown in purple bacteria that the *D–de* loop of subunit M participates in hydrophobic interactions with subunit H to stabilize the reaction center (Deisenhofer et al., 1985; Feher et al., 1989), the plasticity of the *D–de* loop of the D2 protein (the M homologue) argues against similar interactions playing an important part in stabilizing the stromal face of the PSII reaction center. Similarly, the plasticity in net charge of the *D–de* loop from −6 (in wild type) up to +6 (in mutant D2S-A) suggests that the electric dipole is not an important factor in determining orientation of the PSII reaction center proteins in the membrane, in contrast to its suggested role in the bacterial reaction center (Michel & Deisenhofer, 1989).

Exposed regions of proteins may tolerate alterations without significant consequences to the hydrophobic core (Bowie et al., 1990). Furthermore, exposed loop regions vary more between homologous proteins than do buried regions, and may contain deletions and insertions (Fetrow et al., 1989; Pompliano et al., 1990). Yet, the hydrophilic character of the *D–de* loop region was maintained in all the D2 suppressor mutants, and turns are predicted in the mutant sequences at similar locations as in wild type. These turns are α–α loop templates which contain either proline or glycine/asparagine residues (Milner-White & Poet, 1987; Thornton et al., 1988). Hydrophilicity may thus be an important component of the information stored in this region, modulating proper protein folding and insertion of hydrophobic helices into the membrane (Michel & Deisenhofer, 1989) without strict requirements for length and/or sequence composition.

Functional Considerations. Existence of the D2 suppressor mutants indicates that structural requirements in the *D–de* loop can be relaxed. Nonetheless, in nature, both sequence and length of the *D–de* loop of the D2 protein are phylogenetically conserved (Svensson et al., 1991), suggesting some evolutionarily important role for this region. Since all of the D2 suppressor mutants show some reduction in their PSII performance, it is possible that the *D–de* loop may have a regulatory function which is not rate limiting under optimal growth conditions but is revealed mainly under marginal conditions (stress) and may serve to "fine-tune" PSII interactions for optimal activity. Such functions might very well be features which optimize photoautotrophic fitness and thus have been conserved.

In conclusion, we note that the genetic flexibility of the *D–de* loop, coupled with strong selection pressure for autotrophy, should provide a useful engineering tool for further analysis of the PSII reaction center.

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