Functional Analysis of Combinatorial Mutants Altered in a Conserved Region in Loop E of the CP47 Protein in *Synechocystis* sp. PCC 6803†

Martin Tichy and Wim Vermaas*

Department of Plant Biology and Center for the Study of Early Events in Photosynthesis, Arizona State University, Box 871601, Tempe, Arizona 85287-1601

Received September 25, 1997; Revised Manuscript Received November 19, 1997

ABSTRACT: Regions in the large lumenally exposed region (loop E) of CP47 affect properties of the water-splitting system in photosystem II (PS II). To investigate the role of these regions, we developed a method for functional complementation of obligate photoheterotrophic mutants carrying a deletion in one such region. Using an obligate photoheterotrophic mutant that carries a short deletion (Δ(D440-P447)) in loop E of CP47, completely degenerate sequences of eight codons in length were introduced at the site of the deletion. Transformants that were complemented to photoautotrophic growth were selected, and 20 such mutants were studied. Sequence analysis revealed that, as expected, in each of them CP47 had been restored to its wild-type length. However, none of the amino acid residues in the deleted region were found to be critical for function. A negatively charged residue at position 440 and a positively charged one at position 444 were favored but not required. Photoautotrophic growth of mutants obtained varied from almost normal to significantly impaired. The mutants contained 20−100% of the amount of PS II present in the wild type, with PS II amounts correlating with the initial rates of oxygen evolution. The mutants had a high rate of photoinactivation, and many mutants showed an up to 1000-fold increase in chloride requirement for photoautotrophic growth. These phenotypic effects were a direct consequence of the CP47 mutations and were not caused by altered binding of one of the extrinsic proteins. No particular amino acid residues in positions 440−447 of CP47 were found to be indispensable for photoautotrophic growth, and many amino acid combinations in this region support PS II function. However, the mutagenized region is shown to interact with the oxygen-evolving site of PS II and appears to have a direct role in chloride binding.

PS II[1] is a multimeric pigment−protein complex located in the thylakoid membrane of all oxygenic photosynthetic organisms. Light energy absorbed by PS II causes the separation of charge between the primary electron donor (P680) and the primary electron acceptor in the reaction center and eventually induces oxidation of water. Manganese, calcium, and chloride are associated with the water-oxidizing complex of PS II. However, the binding sites of these cofactors have not yet been elucidated (reviewed in refs *1* and *2*).

Two homologous chlorophyll *a* binding PS II proteins, CP47 and CP43, serve primarily as an antenna for light harvesting and transfer energy to the reaction center. Additionally, both CP47 and CP43 are involved in functions other than light harvesting. In the absence of CP47, stable PS II assembly does not occur, and without the CP43 gene, some PS II is assembled but no oxygen evolution is observed (*3*, *4*). The predicted topology of both proteins includes six membrane-spanning regions and three lumenally exposed loops, of which the terminal one (loop E) is very large. Loop E, connecting helices V and VI, consists of approximately 200 amino acid residues in CP47 (G254−G449) and is a little shorter in CP43 (*5*, *6*). Because of its lumenal orientation and unusual length, loop E has been targeted in searches for roles of CP47 in PS II stability and water oxidation.

Several lines of evidence indicate a direct or indirect interaction between CP47 and the extrinsic 33-kDa Mn-stabilizing protein (OEE-1). In spinach, extraction of OEE-1 increases the accessibility of proteases and labeling reagents to CP47 (*7*, *8*). Cross-linking experiments corroborate a proximity between OEE-1 and CP47 (*8*, *9*): treatment with a zero-length cross-linker led to covalent immobilization of all OEE-1 to CP47 and to stabilization of the oxygen-evolving activity of PS II membranes (*10*). Loop E of CP47 was found to be cross-linked with the N-terminal region of OEE-1 (*11*). Another domain of OEE-1 interacts with loop A of CP47 (*12*). In other experiments the direct association between CP47 and the manganese cluster that is critical for water oxidation has been studied. The epitope P360−S391 in loop E of CP47 that is recognized by a monoclonal antibody is exposed only after depletion of the strongly bound pool of manganese associated with the oxygen-evolving system (*13*). However, this does not necessarily imply that

†This work was funded by a grant from the U.S. Department of Energy (Grant DE-FG03-95ER20180).

* Corresponding author.

[1] Abbreviations: chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMBQ, dimethyl-*p*-benzoquinone; HA, hydroxylamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; OD_{730}, optical density at 730 nm; OEC, oxygen-evolving complex; PS II, photosystem II; *Synechocystis* 6803, *Synechocystis* sp. PCC 6803; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

S0006-2960(97)02381-7 CCC: $15.00
Published on Web 01/22/1998

CP47 provides ligands to the manganese cluster, since manganese removal may be accompanied by a conformational change.

The studies mentioned above were carried out on PS II complexes from higher plants. However, these studies are likely to be extrapolatable to cyanobacterial systems, as the cyanobacterial and plant OEE-1 proteins are functionally interchangeable (*14*) despite substantial differences in their primary structure. In cyanobacteria either OEE-1 or one of the other two proteins on the donor side of PS II (cyt c_{550} and a 12-kDa protein) can be deleted without abolishing the ability of the resulting mutants to grow photoautotrophically (*15*−*17*). Interestingly, upon simultaneous deletion of OEE-1 and cyt c_{550} the cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803) becomes an obligate photoheterotroph (*18*). As the cyanobacterial oxygen-evolving complex can be modified significantly without necessarily abolishing function, cyanobacteria represent a good experimental system to study the role of specific proteins in donor side reactions.

In a quest to determine the role of the CP47 protein in water oxidation, a number of *Synechocystis* 6803 strains carrying short deletions in loop E of the CP47 protein were constructed (*19*, *20*). The mutants obtained covered a wide range of phenotypes, from obligate photoheterotrophic to photoautotrophic with nearly wild type phenotype, depending on the position of the deletion. Several photoautotrophic mutants with interesting phenotypes were studied in more detail (*21*, *22*). Indeed, in some mutants the properties of the water-splitting system and the binding of OEE-1 were influenced significantly. In particular, the two deletion mutants Δ(A373-D380) and Δ(R384−V392) were found to have phenotypes similar to the strain lacking OEE-1: they exhibited fast photoinhibition, a decreased rate of steady-state oxygen evolution, and dark inactivation of the oxygen-evolving complex (OEC). However, these mutants did not exhibit an increased Ca^{2+} demand for photoautotrophic growth (*20*) in contrast to what was found for the OEE-1 deletion mutant (*15*). The deletions present in the two CP47 mutants both overlap with domains identified to bind OEE-1 in plants.

The cyanobacterial system also has been used to modify many of the charged residues in loop E in order to evaluate their involvement in donor side reactions (*23*−*26*). Two interesting mutants were found. The mutant R384G/R385G has a phenotype similar to the mutant lacking OEE-1 (*23*), and interestingly, PS II particles prepared from this mutant entirely lack OEE-1 (*25*). The two mutated residues in this strain are in the domain that was deleted in the Δ(R384−V392) mutant mentioned above. The second mutant, R448G, has an increased chloride requirement and is an obligate photoheterotroph under chloride-limiting conditions (*24*). In loop C, alteration of a single amino acid residue (W167S) resulted in 75% loss in oxygen evolution capacity and the mutant accumulated very few PS II reaction centers (*27*).

Chloride is important for water oxidation by PS II, but its stoichiometry, precise role, and location is unknown. In dark-adapted Cl^- depleted samples (with the water-splitting system primarily in the S_1 state), only two electrons are rapidly transferred to $P680^+$, which was explained as formation of the $Y_Z^{ox}S_2$ state (reviewed in ref *28*). No S_2 EPR multiline signal can be detected in chloride-depleted PS II, unless chloride is added after illumination (*29*). It was concluded that the $S_2 \rightarrow S_3$ transition was blocked in the absence of Cl^-. Chloride apparently binds to two types of sites in PS II that differ from each other in the kinetics of exchange, with the slowly exchanging chloride influencing the oxygen evolution activity and S_2 state EPR signals (*30*). In current models of the OEC both Ca^{2+} and Cl^- are integral components of the active site. Chloride is thought to serve as a terminal ligand of the manganese cluster (*31*, *32*), operating in a ligand exchange with H_2O to prevent premature oxidation of water and formation of H_2O_2 (*33*).

In this paper we report the results of combinatorial mutagenesis to probe the function of one conserved region (D440−P447) in loop E of the CP47 protein. Deletion of this region led to an obligate photoheterotrophic phenotype and to a disappearance of PS II from the thylakoid membrane (*20*). In our combinatorial approach, random eight-codon sequences were introduced at the site of the deletion and transformants that had regained photoautotrophic capacity were selected. To identify protein sequences that can be accommodated, mutants were sequenced and their phenotypes were characterized. The results show that the combinatorial mutagenesis method is efficient to study the sequence requirements in a protein domain and to generate interesting mutants with altered PS II properties.

MATERIALS AND METHODS

Mutant Construction. The deletion mutant lacking residues D440−P447 of CP47 (Δ12) that was used in this study has been constructed by Haag et al. (*20*). This mutant is an obligate photoheterotroph. The combinatorial mutagenesis protocol used was based on methods developed for in vitro mutagenesis using a single-stranded bacteriophage template (*34*, *35*). The bacteriophage M13mp19 containing the *psbB* gene (coding for CP47) with the Δ(D440-P447) deletion was used as a template. We used the uracil-containing template isolated from *Escherichia coli* strain CJ236 to suppress the amplification of the template phage strand after mutagenesis (*36*). As mutagenic primer a 67-nucleotide-long oligonucleotide was used whose 5′ end contained 21 nucleotides corresponding to the *psbB* sequence immediately upstream; at its 3′ end it contained 22 nucleotides downstream of the deletion; in the middle were 24 nucleotides that were completely degenerate. After hybridization, the highly processive T7 DNA polymerase was used for synthesis of the second strand, and T4 ligase was used for ligation. Heteroduplex phage DNA was then electroporated into *E. coli* strain MV1190 and amplified for 2 h in liquid culture. Single- and double-stranded M13 DNA was isolated and used for transformation of the photoheterotrophic Δ(D440-P447) strain. For transformation, 200 μL of concentrated cells ($OD_{730} = 5$) in BG-11 medium containing 5 mM glucose was incubated with phage DNA for 1 h, and then plated directly on BG-11 medium without glucose. Photoautotrophic transformants were selected, the part of their *psbB* corresponding to the loop E was amplified by PCR, and this PCR product was sequenced on an ABI-377 DNA sequencer using a sequencing primer 50 nucleotides upstream of the mutagenized region.

PS I-less strains were obtained by transformation of the PS I-less/*psbB*$^-$ strain [in which a large part of *psbB* had

been replaced by a spectinomycin-resistance cassette (*37*)] with the genomic DNA of the combinatorial photoautotrophic mutants. Transformants were selected for kanamycin resistance; a kanamycin-resistance cassette was present downstream of the original Δ(D440-P447) mutant (*20*).

Growth Conditions. Wild-type and mutants of *Synechocystis* 6803 were grown in liquid BG-11 medium (*38*) at 30 °C at 40 μE/(m^2•s) [5 μE/(m^2•s) for PS I-less strains] on a rotary shaker. During segregation, the PS I-containing mutants were grown photoautotrophically; after segregation, all media were supplemented with 5 mM glucose (15 mM for PS I-less strains). Solid media were supplemented with 1.5% (w/v) agar, 0.3% (w/v) sodium thiosulfate, and 10 mM TES−NaOH buffer, pH 8.2. For all mutants on solid media, 20 μM atrazine was added to suppress selection for spontaneous secondary pseudoreversions that might lead to increased PS II activity. For nutrient (Ca, Cl) deprivation experiments, cells were subcultured 2−4 times by diluting about 40-fold to an optical density at 730 nm (OD_{730}) of 0.02 in liquid BG-11 depletion medium with 5 mM glucose. In this depletion medium $CaCl_2$ was omitted, $MnCl_2$ was replaced by $MnSO_4$, and $NaNO_3$ (a major component of BG-11 that contains significant contaminating Cl^- concentrations) was substituted by KNO_3. Chemicals used for the media were analytical grade and may have contained traces of Ca^{2+} and Cl^-. The cells were considered to be calcium- and chloride-depleted when their oxygen evolution did not decrease between two subcultures. After depletion, the rate of photoautotrophic growth was measured in the depletion medium without glucose but, if indicated, after addition of one of the depleted ions.

Oxygen Evolution. Oxygen evolution was measured directly using samples taken from liquid cultures (OD_{730} of the cultures was 0.5−0.8). Measurements were performed using a Clark-type electrode in 25 mM HEPES/NaOH pH 7.0, in the presence of 1 mM potassium ferricyanide and 0.1 mM dimethyl-*p*-benzoquinone (DMBQ). The temperature was 25 °C and the light intensity 4000 μE/(m^2•s). The light was passed through an orange filter, cutting off light with wavelengths < 570 nm. The chlorophyll (chl) *a* concentration was about 2 μg/mL (0.4 μg/mL for PS I-less cells), as determined by absorption at 663 nm after chl extraction from cells in 100% methanol.

Herbicide-Binding Assay. The concentration of assembled PS II centers in intact cells of *Synechocystis* 6803 strains was determined by measuring the binding of ^{14}C-labeled diuron (*39*). The extrapolated amount of bound, atrazine-displaceable diuron at infinite concentration of free diuron is equal to the amount of PS II. The concentration of chl in samples used for these experiments was 40−80 μg/mL.

Fluorescence Measurements. Chl *a* fluorescence induction in the presence of hydroxylamine was detected with a Walz pulse-amplitude-modulation (PAM) fluorometer and recorded using the program FIP (Q_A Data, Turku, Finland). Continuous illumination came from a PAM 102 L LED lamp. Fluorescence induction was measured directly in samples of liquid cultures of PS I-less strains (OD_{730} = 0.5−0.7, containing about 0.4 μg/mL chl). After a 1-min dark adaptation of cells in 25 mM HEPES/NaOH, pH 7.0, DCMU was added to a final concentration of 50 μM, followed by 20 mM hydroxylamine (HA). The weak monitoring light was turned on 30 s later at 1.6 kHz, followed after 10 s by actinic illumination.

RESULTS

Mutagenesis and Mutant Selection. The goal of combinatorial mutagenesis was to identify structurally and functionally important amino acid residues in the region that was deleted in the Δ(D440-P447) deletion mutant. Eight codons (24 nucleotides) that spanned residues D440-P447 of CP47 of *Synechocystis* 6803 were introduced by random nucleotide sequences as was described in Materials and Methods. Central features of the procedure are (1) the utilization of single-stranded bacteriophage template M13 carrying the *psbB* gene with the Δ(D440-P447) deletion and (2) the application of oligonucleotide-directed mutagenesis using a degenerate primer with random 24-nucleotide sequences at the site of the deletion. The M13 DNA mixture that resulted from this mutagenesis was used for *E. coli* transformation to achieve DNA amplification. The resulting DNA was mixed with the *Synechocystis* 6803 Δ(D440-P447) deletion mutant for transformation. Screening for a desired phenotype occurred at the level of selection of photoautotrophic transformants of the obligate photoheterotrophic Δ(D440-P447) mutant. Therefore, there was no need to isolate individual M13 clones or to screen them for whether they have incorporated the 24 nucleotides. The percentage of clones carrying an inserted sequence at the 440−447 position is not as important as in most site-directed mutagenesis protocols. However, the total yield of different mutants is important for this combinatorial mutagenesis approach, because only a small fraction of possible sequences is likely to support photoautotrophic growth in *Synechocystis* 6803.

The Δ(D440-P447) region of four resulting M13 phages was sequenced. Two phages were shown to have a sequence identical to that of the original Δ(D440-P447) mutant, and the other two carried different mutations. Two steps were found to be critical to obtain a high number of phages with random inserts: optimal primer phosphorylation and mutant strand ligation. Ligation for 4 h at 20 °C in a separate step after polymerization using a high concentration of T4 DNA ligase (final concentration 5 units in 20 μL) was particularly important for a good mutant yield. The specific cleavage of the original phage strain and its degradation by exonuclease recommended in ref *35* to increase the proportion of transformants carrying a mutation were omitted. In particular, the recommended treatment with exonuclease (*35*) decreased the mutant yield after electroporation into *E. coli* by one order of magnitude and was therefore not performed. The transformation efficiency of *Synechocystis* 6803 using single-stranded phage DNA was one order of magnitude lower (on a per molecule basis) than when double-stranded M13 DNA was used (data not shown).

Two mutagenesis experiments and transformations yielded photoautotrophic mutants with protein sequences as presented in Figure 1. A total of 50 photoautotrophic *Synechocystis* 6803 colonies were obtained, 30 of which were sequenced yielding 20 different nucleotide sequences. The apparent redundancy (six sequences obtained twice, two obtained three times) is a result of the phage amplification in *E. coli*. This 2-h amplification is necessary to segregate phage heteroduplexes and to obtain a sufficient amount of phage DNA for

Table 1: Growth Characteristics of the Photoautotrophic Combinatorial Mutants

strain	doubling time (h)	oxygen evolution [μmol of O_2/(mg of chl·h)]	photoinactivation $t_{1/2}$ (min)	growth rate after depletion[a] (% normal growth rate)			herbicide binding (chl/PS II)
				Ca, Cl depleted	Ca depleted	Cl depleted	
wt	12	320	>5	40	60	75	610
d12.1	12	350	5	50	50	80	550
d12.5	18	290	3	10	50	20	750
d12.7	14	250	3	10	40	10	680
d12.10	23	280	2	0	30	0	980
d12.12	17	260	3	10	35	15	830
d12.14	24	300	2	0	50	0	830
d12.16	32	260	1	0	50	0	nd[b]
d12.18	30	250	3	0	30	10	1430
d12.19	16	220	2	0	20	0	nd
d12.20	37	170	1−2	0	50	0	2780

[a] Photoautotrophic growth rates upon depletion of calcium, chloride, or both are given as percentage of the growth rate in normal BG-11. [b] Not determined. Data reported here are an average of 2−4 independent measurements. The data shown were reproducible within 15% of the average.

	438		449
Eukaryo		S	
Oeno		SN	
Cyano		S A	
Prochlo		T	
wt	**NS**	**DGVFRTSP**	**RG**
d12 del	NS	.....	RG
d12.1	NS	ERVGRGSA	RG
d12.2	NS	KGVERMAL	RG
d12.3	NS	VNLGRGGL	RG
d12.4	NS	GNTGRLGV	RG
d12.5	NS	LAGGRAAP	RG
d12.6	NS	AGGSRGGA	RG
d12.7	NS	NGEAREVT	RG
d12.8	NS	GGRWGANS	RG
d12.9	NS	GGRQGCGA	RG
d12.10	NS	GGRLRNAV	RG
d12.11	NS	HSVRGGSA	RG
d12.12	NS	ESGRGATV	RG
d12.13	NS	ERLWGEGT	RG
d12.14	NS	ERVMASGP	RG
d12.15	NS	EGLASDGV	RG
d12.16	NS	DGYQGSAI	RG
d12.17	NS	GDMSGGRL	RG
d12.18	NS	REEWLVGV	RG
d12.19	NS	WEGRGGSV	RG
d12.20	NS	VNNTASGV	RG

FIGURE 1: Alignment of the amino acid sequence from residue 438 to residue 449 of CP47 from the wild type (wt), the Δ(D440-P447) deletion mutant (d12 del), and 20 oligonucleotide-directed mutants (d12.1−20). Above the wild-type sequence are substitutions found in CP47 sequences from the 25 different species presently in GenBank. Eukaryo is the sequence found in most eukaryotes, Oeno is from *Oenothera hookeri*, Cyano is from *Cyanophora paradoxa*, and Prochlo is from *Prochlorothrix hollandica*. d12 indicates that all mutants originated from the Δ12 mutant constructed by Haag et al. (*20*).

cyanobacterial transformation. As our sole selection criterion was reconstitution of photoautotrophic growth, we could not determine exactly the percentage of random sequences that complemented the photoheterotrophic *Synechocystis* 6803 strain to photoautotrophy. However, an approximation suggests that about 0.15% of the random sequences restores photoautotrophic growth. This estimate is based upon the generation of 1.8×10^4 different *E. coli* transformants per mutagenesis experiment (1 μg of template M13 DNA), a 50% frequency of M13 phages into which a random sequence has been inserted, and the generation of about 15 different photoautotrophic mutants per experiment. The number of transforming phages per experiment is relatively low because a uracil-containing template was used. Control M13 template (without uracil) yielded 10^6-10^7 transformants/μg of DNA.

Photosynthetic Performance. All mutants were selected on the basis of their ability to grow photoautotrophically. However, differences in their photosynthetic capabilities were apparent. The growth characteristics of 10 photoautotrophic mutants are summarized in Table 1. These strains represent essentially all obtained phenotypes. Other strains were characterized only partially; however, their characteristics were within the presented spectrum. With the exception of d12.20, the mutants showed initial oxygen evolution rates at saturating light intensity that were within 30% of those obtained in the wild type. However, the photoautotrophic growth of some mutants (d12.10, d12.14, d12.16, d12.18, and d12.20) was impaired by a factor of 2−3. Part of the reason for this phenomenon may be that oxygen evolution is less stable in many of the mutants, particularly in those that grew more slowly photoautotrophically. The rate of photoinactivation as shown in Table 1 is measured as the time in which oxygen evolution upon illumination at high light intensity [4000 μE/(m^2·s)] drops to half of the original value.

Several mutants with changes on the donor side of PS II have been reported to exhibit increased requirements for calcium or chloride (*15*, *40*), which are important ions for oxygen evolution. We tested our mutants for the ability to grow under chloride- and/or calcium-limiting conditions. Cells were first depleted of both calcium and chloride in a depletion medium containing glucose and were then transferred to the same medium without glucose. In samples indicated to be only calcium-depleted or only chloride-depleted, 0.5 mM chloride or 0.25 mM calcium, respectively, was added to the medium at the time of transfer to the medium without glucose. After depletion, all mutants except d12.1 were more impaired in photoautotrophic growth than the wild type, and particularly chloride depletion led to drastically reduced growth rates with the degree of reduction depending on the particular mutant (Table 1). This impairment was easily reversed by addition of the missing ion(s) (data not shown).

Chloride dependence of photoautotrophic growth was studied in more detail in the wild type and two of the mutants (d12.12 and d12.16) by growing these strains in media with normal (0.5 mM) and decreased (50, 5, and 0.5 μM) concentrations of added Cl^-. As shown in Figure 2, growth

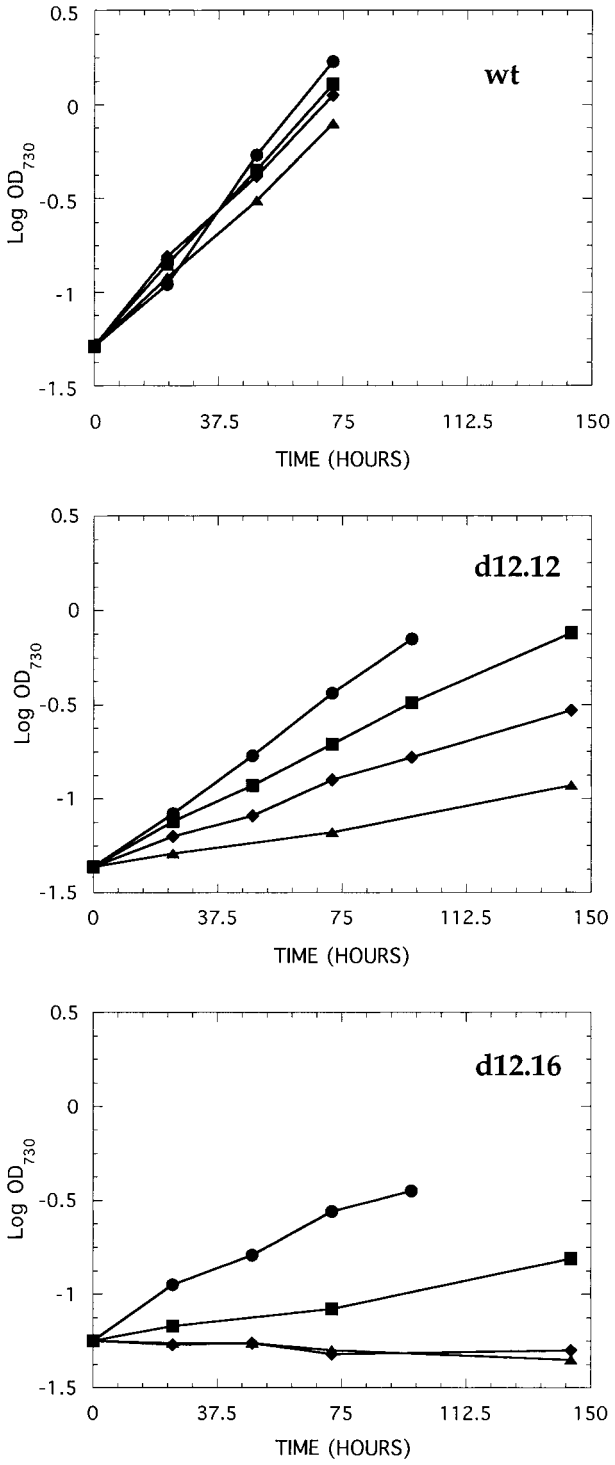

FIGURE 2: Growth rates of the wild type and two mutants, d12.12 and d12.16, at the following concentrations of added chloride to the growth medium: (●) 0.5, (■) 0.05, (◆) 0.005, and (▲) 0.0005 mM. The normal chloride concentration in BG-11 medium is 0.5 mM. Cells used for inoculation were deprived of both calcium and chloride for 2−4 subcultures in the depletion medium in the presence of glucose. All changes in growth rate were easily reversible by reintroducing chloride and calcium into the medium.

of the wild type was normal if only 5 μM added Cl^- was present and was only slightly impaired upon addition of 0.5 μM Cl^-. The growth rate of the d12.12 mutant decreased gradually with decreasing concentrations of chloride, whereas the d12.16 mutant did not grow autotrophically in media with a 100-fold or more decreased chloride concentration. This

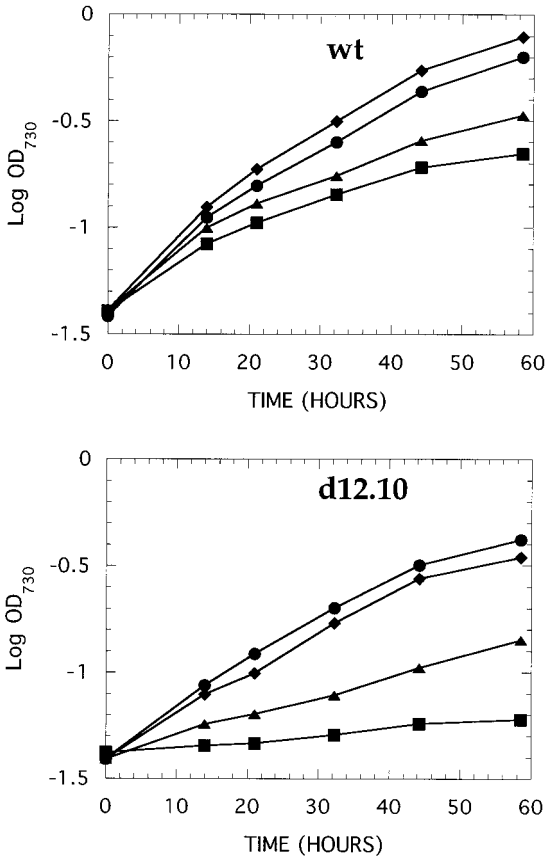

FIGURE 3: Photoautotrophic growth of the wild type and the d12.10 mutant in the presence or absence of Ca^{2+} and Cl^- in normal BG-11 medium and in BG-11 medium with Na^+ substituted by K^+: (●) $K^+ + CaCl_2$, (■) $K^+ - CaCl_2$, (◆) $Na^+ + CaCl_2$, and (▲) $Na^+ - CaCl_2$. Cultures were inoculated with cells grown on normal medium containing both Ca^{2+} and Cl^-. Notice the much slower depletion in the wild type. Several subcultures of the wild type were necessary to achieve complete depletion.

suggests that, for some strains, 0.5 mM chloride may not be sufficient for optimal photoautotrophic growth and that, therefore, the slow growth may be due in part to a limiting chloride concentration. To test this, two mutants with long photoautotrophic doubling times (d12.16 and d12.20) were put into a medium with 150 mM NaCl, which represents a 300-fold increase in chloride concentration over that present in BG-11 and which does not yet negatively affect growth of the wild type. The growth rate of these mutants increased by 30% and 20%, respectively. This suggests that the decreased growth rate is not due solely to a chloride limitation.

To increase consistency in the results of the depletion and repletion experiments a modified BG-11 medium was used, in which 98% of the sodium ions were replaced by potassium. As shown in Figure 3, in this medium a faster and more complete depletion was achieved, as measured by growth rate. The complete omission of sodium was lethal. This is in line with reports that the use of media without sodium is necessary for enhanced calcium limitation in *Synechococcus* 6301 (*41*). Also a difference in contaminating concentrations of Ca^{2+} and Cl^- cannot be excluded. The photomixotrophic doubling time of all strains in Table 1 depleted of both calcium and chloride was 22 (wt, d12.1) to 27 (d12.18, d12.20) h in the presence of 5 mM glucose.

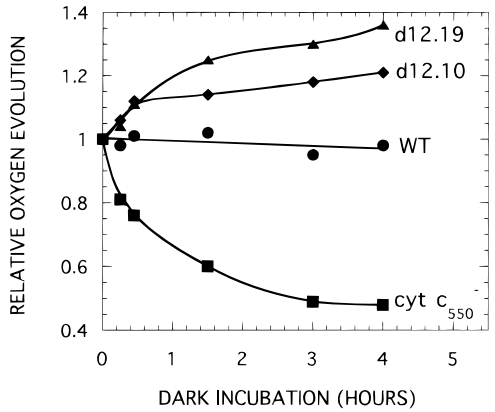

FIGURE 4: Dark (in)activation of oxygen evolution in the wild type (●), d12.10 (◆), d12.19 (▲), and the cyt c_{550} deletion (*psbV*$^-$) (■) strains. The initial oxygen evolution rate of cells grown under standard conditions was measured at time $t = 0$. Cells were incubated in darkness for the time indicated, and aliquots were taken to measure the initial oxygen evolution rate. The relative values of oxygen evolution, adjusted for cell growth, have been shown. The absolute values of oxygen evolution at time $t = 0$ for the wild-type, the cyt c_{550} deletion mutant, and the D440-P447 combinatorial mutants d12.10 and d12.19 were 320, 250, 270, and 220 μmol of O_2/(mg of chl·h), respectively.

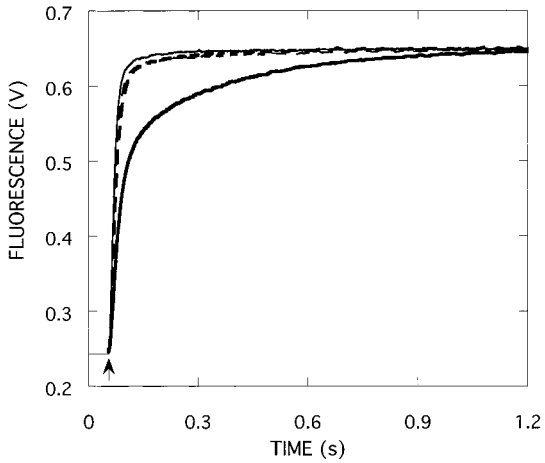

FIGURE 5: Fluorescence induction curves of the wild type (solid lines) and the d12.10 mutant (broken lines) in the presence of 50 μM DCMU and in the presence (thick lines) or absence (thin lines) of 20 mM hydroxylamine (HA). Actinic light was turned on at the time indicated by the arrow. Note that the lines with and without HA are essentially superimposed in the case of the d12.10 mutant.

To determine whether the effect of the CP47 mutations on water oxidation is direct or may be mediated through changed binding of one of the lumenal proteins, we measured the rate of dark inactivation of PS II electron transport in several mutants (Figure 4). Upon dark incubation, the oxygen-evolution capacity of the CP47 mutants that were tested was stable and even went up during a 4-h dark incubation. This is in contrast to the decrease in oxygen-evolving capacity that was observed in the cytochrome c_{550} deletion mutant. This indicates that the mutations in the D440−P447 region influence the chloride requirement. The actual increase of the oxygen evolution rate of the CP47 mutants upon dark incubation suggests that the mutants are partially photoinactivated under normal growth conditions [40 μE/(m^2·s)].

We also measured the fluorescence characteristics of the mutants shown in Table 1 after transfer of these mutations to a PS I-less background. The data shown in Figure 5 indicate that fluorescence induction in the presence of hydroxylamine (20 mM) and DCMU is much faster in the d12.10 mutant than in the control PS I-less strain. We found this feature to be characteristic for all mutants tested except for the d12.1 strain, although the extent of the difference was dependent on the strain and on the state of the cells used. The faster induction indicates that hydroxylamine is a better electron donor in the mutants than in a control strain, which may be caused by altered accessibility of hydroxylamine to its site of donation.

Functionality of the donor side of PS II can be characterized in vivo by measuring reoxidation kinetics of Q_A^- after a single saturating flash or after continuous illumination in the presence of DCMU, which blocks electron transfer between Q_A and Q_B. In the D440-P447 combinatorial mutants, the fluorescence decay after a single flash or after continuous illumination in the presence of DCMU was not significantly changed (data not shown). The half-times of charge recombination in the control strain after a single flash and continuous illumination were 300 and 360 ms, respectively. The rates of decay of variable fluorescence in both experiments were within 30% of that of the PS I-less control strain indicating rather normal properties of the OEC and of electron transfer at the donor side in the mutants. Measurements were conducted in a medium with normal chloride (0.5 mM) and calcium (0.25 mM) concentrations.

DISCUSSION

The obligate photoheterotrophic Δ(D440-P447) (Δ12) mutant of *Synechocystis* 6803 lacks assembled PS II, and therefore, PS II function of this mutant cannot be studied (*20*). However, the photoheterotrophic nature of this strain made it very useful as a background strain for functional complementation with an easy selection for photoautotrophy. A combinatorial mutagenesis approach was used to probe the importance of individual residues in the region that was deleted and to investigate the phenotype of the photoautotrophic mutants that were obtained.

Combinatorial mutagenesis has been used in many cases to determine the structural or functional role of individual amino acid residues (*42*−*45*) or to obtain mutants with altered phenotypes (*46*, *47*). The combinatorial approach, which allows the study of several residues at a time, is more complex than site-directed mutagenesis in that it involves multiple mutations simultaneously. Also, site-directed mutagenesis of a single residue requires some understanding of the structure of the interacting units (protein−protein, protein−cofactor) to decide which amino acid may be important. The combinatorial approach is complementary to site-directed mutagenesis because it is able to identify these important residues (*42*) and can be used for proteins of unknown structure (*48*). This approach usually requires selection for a "gain of function" phenotype that is easy to screen for. Acquisition of photoautotrophic growth capability is a good example of such positive selection. Our system employs (1) second-strand synthesis of single-stranded phage using a primer that is completely degenerate in the desired region and a template that lacks this region, followed by (2) limited amplification of the phage, (3) transformation of the photoheterotrophic cyanobacterial deletion strain, and (4)

selection for photosynthetic transformants on media that lack a fixed carbon source. This system proved to be convenient and efficient as it can produce about 10^5 different random sequences in a single experiment. As there are 20^8 (2.5×10^{10}) different combinations in which eight amino acid residues can be arranged, in our experiments we isolated only a small fraction of the possible amino acid combinations that will give rise to photoautotrophic growth. The transformation efficiency with the double-stranded phage DNA was about 10-fold higher than with single-stranded DNA. Single-stranded M13 DNA was reported not to be taken up by *Synechocystis* 6803 (*49*), yet two mutants originated from transformations using single-stranded M13 DNA.

It is obvious that although the deleted region of CP47 is very conserved (Figure 1), many sequences can satisfy the photoautotrophic growth requirement we selected for. The residue most frequently observed in the 8-residue sequence was glycine, whereas the positively charged arginine also occurred frequently. Although there are multiple codons for both residues (4 and 6), their frequency cannot be explained solely by this fact. No residue was strictly required at a particular position, and several nonconservative substitutions were found at each position. A negative charge at position 440, arginine at 444, and a hydrophobic residue at 447 were preferred but not required. These results are supported by site-directed mutants made by Putnam-Evans et al. (*25*) in the Δ(D440-P447) region. The D440N and R444G mutants have normal levels of oxygen evolution. Both mutations are represented in our collection (Figure 1) even though, of course, in a different context. The surprisingly high level of substitution tolerance is in accord with most other combinatorial studies on different proteins, usually also targeted to highly conserved regions (*43*, *44*, *50*, *51*).

The mutants that were obtained contained 20−100% of the amount of PS II present in the wild type, and these PS II amounts generally correlated with the initial rates of oxygen evolution. The increased rate of photoinactivation at high light intensity is a general feature of many mutations on the donor side of PS II that disturb the OEC, including the deletion mutants lacking some of the lumenal extrinsic proteins. Another feature shared by several of these mutants is an increased chloride requirement. Many of our mutants were limited in their photosynthetic capability by low chloride concentrations in the medium, and several were not able to grow photoautotrophically without added chloride. This result is similar to a report (*24*) in which the R448G mutant was shown to be an obligate photoheterotroph under chloride-limiting conditions. Interestingly, the R448 residue is adjacent to the Δ(D440-P447) deletion in close proximity to the proposed transmembrane helix VI (G449−G470). It is possible that the whole region close to this helix and not only a single residue such as R448 modulates chloride binding. It is also possible that the combinatorial mutations affected the ability of R448 or another residue to bind chloride. The residue(s) that directly interact with chloride have not yet been elucidated, and therefore, we currently cannot distinguish between these two possibilities. However, the importance of protein context was shown also in another study comparing the results of site-directed and combinatorial mutagenesis (*46*). In these experiments no single residue was found to determine a particular phenotype, which in this case was the absorption spectrum of a light-harvesting protein of photosynthetic purple bacteria.

An important question in our work and that of others is whether the increased chloride requirement was caused directly by the modification of CP47 or whether this effect was mediated by a changed interaction between CP47 and another protein, for example an extrinsic lumenal protein. There are three known extrinsic proteins associated with PS II in cyanobacteria. In addition to OEE-1, two other proteins, cytochrome c_{550} and a 12-kDa protein (encoded by *psbV* and *psbU*, respectively), are present in stoichiometric amounts with PS II (*52*). Photoautotrophy does not critically depend on the presence of either OEE-1 (*15*) or cytochrome c_{550} (*17*), but mutants lacking both are obligate photoheterotrophs (*18*). Mutants lacking either OEE-1 or cytochrome c_{550} were found to rapidly and reversibly lose oxygen evolution in darkness in a process called dark inactivation (*18*, *53*). Two photoautotrophic deletion mutants lacking a region of loop E of CP47 [Δ(R384-V392) and Δ(A373-D380)] that is implicated to be important for OEE-1 binding showed a similar phenotype (*22*). This is not the case in the Δ(D440-P447) mutants (Figure 4). In these mutants the oxygen evolution rate is increased after a dark incubation, indicating that these mutants are photoinhibited under normal growth conditions [light at 40 μE/(m^2•s)]. Therefore, the changes in the CP47 protein in the D440-P447 combinatorial mutants directly affect the chloride requirement, rather than that such effects are mediated via an extrinsic protein. The mutants appear to be deficient in chloride binding, and this impairment can be compensated to some extent by increasing the chloride concentration in the medium.

Chloride is an important cofactor for efficient water oxidation, but its exact location, precise role, and the number of chloride ions required for oxygen evolution are not known. There is little agreement about chloride interactions with the OEC in lower or higher S states (reviewed in ref *28*). It has been shown that, in PS II core complexes, chloride is important to prevent the formation of hydrogen peroxide, which has been suggested to be released upon the $S_2 \rightarrow S_0$ transition (*54*). This indicates that chloride is in close proximity to the OEC. Two chloride-binding sites with different binding kinetics or only one site with heterogeneous properties per PS II was proposed based on binding of labeled chloride (*30*, *55*). One binding site characterized by a high affinity for chloride and a slow exchange rate and with a stoichiometry of one chloride/PS II has been indicated to be responsible for high oxygen-evolving activity and normal EPR properties of the S_2 state (*30*). The ESEEM data on chloride/bromide exchange indicate that halides can closely approach the manganese (*56*). Interpretations of EXAFS results suggest that one chloride binds to manganese (*57*) with a Mn−Cl distance of approximately 2.3 Å. It is not clear if this chloride is identical to the slowly exchanging chloride. However, it is probable that the chloride, which is an integral component of the OEC, is identical to the one responsible for high oxygen evolution. This would indicate that the D440-P447 region of CP47, which is involved in chloride binding, is also close to the OEC.

We made two interesting observations during our chloride depletion experiments. In the first place, chloride depletion was more complete when calcium was depleted at the same time. An interrelationship between chloride and calcium is

assumed. A requirement for calcium binding is indicated in some models (reviewed in ref *28*). Second, the depletion was faster and more reproducible when a modified BG-11 medium was used in which most sodium was replaced by potassium. The nature of this phenomenon is unknown; however, the same behavior was observed in *Synechococcus* 6301 (*41*) where sodium-free medium had to be used for calcium depletion in vivo.

To further investigate the effect of these mutations on PS II electron transport we measured the fluorescence characteristics of the mutants in a PS I-less background. After a single flash, much faster charge recombination between Q_A^- and Y_Z^{ox} is expected when Y_Z^{ox} is not reduced rapidly by the OEC (*58*). Alternatively, recombination kinetics can be much slower if the S_2/S_1 midpoint potential is decreased. This stabilization of the S_2 state has been observed in the ΔOEE-1 mutant (*15*, *59*). The presence of photooxidizable manganese ions in reaction centers can be probed by following reoxidation kinetics of Q_A^- after illumination for several seconds in the presence of DCMU. If Y_Z^{ox} is reduced during the illumination by reductants other than the OEC, the stability of Q_A^- will be significantly increased (*58*). In our D440-P447 combinatorial mutants the fluorescence induction and decay after a single flash or continuous illumination in the presence of DCMU were not significantly changed, indicating normal donor side characteristics of PS II.

The fluorescence induction in the presence of DCMU and of a high concentration of hydroxylamine (20 mM) was more slow in the wild type than in the mutants. Hydroxylamine inactivates the oxygen-evolving complex by formation of unstable reduced intermediates of the Mn tetramer and by release of Mn ions from OEC (*60*). Concurrently it functions as an electron donor by reducing Y_Z^{ox} (*61*), trapping Q_A^- in the presence of DCMU. In most of the mutants hydroxylamine clearly is a better electron donor than in the control strain, which may be caused by easier access of hydroxylamine to its site of donation. It has been shown that also the donor side of the OEE-1 mutant is more accessible to hydroxylamine and other exogenous reductants (*16*, *40*, *62*). It is probable that both the OEE-1 and CP47 mutations destabilize the assembled manganese cluster by rendering the active site more accessible to endogenous or exogenous reductants and to inhibitors like ammonia (*62*); also, the mutations may decrease the affinity for calcium and/or chloride. Indeed, we have found that a cytochrome c_{550} deletion mutant is unable to grow photoautotrophically at increased light intensity [70 μE/(m^2•s)] and in a medium containing 5 mM ammonia (Tichy, M., and Vermaas, W., unpublished observations). This effect is even stronger in several combinatorial D440-P447 mutants after subsequent deletion of cytochrome c_{550}.

In summary, the results of this study show that the combinatorial approach used to complement PS II deficient mutant is efficient to study functional requirements in a protein domain. A variety of substitutions in this region were observed despite the fact that mutated residues are very highly conserved among all cyanobacterial and plant proteins. The spectrum of mutants obtained varied in their photoautotrophic growth, PS II content, oxygen evolution, and rate of photoinactivation. The increased requirement for chloride in many of our mutants indicates that the region as a whole has a role in chloride binding. To better define the apparent interaction of this region with the oxygen-evolving site, experiments are underway on one of the combinatorial mutants with a simultaneous deletion of the cytochrome c_{550} or OEE-1.

REFERENCES

1. Bricker, T. M., and Ghanotakis, D. F. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., Eds.) pp 113−136, Kluwer Academic Publishers, Dordrecht, The Netherlands.
2. Britt, R. D. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., Eds.) pp 137−164, Kluwer Academic Publishers, Dordrecht, The Netherlands.
3. Vermaas, W. F. J., Ikeuchi, M., and Inoue, Y. (1988) *Photosynth. Res. 17*, 97−113.
4. Rögner, M., Chrisholm, D. A., and Diner, B. (1991) *Biochemistry 30*, 5387−5395.
5. Vermaas, W. F. J., Williams, J. G. K., and Arntzen, C. J. (1987) *Plant Mol. Biol. 8*, 317−326.
6. Bricker, T. M. (1990) *Photosynth. Res. 4*, 1−13.
7. Bricker, T. M., and Frankel, L. K. (1987) *Arch. Biochem. Biophys. 256*, 295−301.
8. Bricker, T. M., Odom, W. R., and Queirolo, C. B. (1988) *FEBS Lett. 231*, 111−117.
9. Enami, I., Satoh, K., and Katoh, S. (1987) *FEBS Lett. 226*, 161−165.
10. Enami, I., Kaneko, M., Kitamura, N., Koike, H., Sonoike, K., Inoue, Y., and Katoh, S. (1991) *Biochim. Biophys. Acta 1060*, 224−232.
11. Odom, W. R., and Bricker, T. M. (1992) *Biochemistry 31*, 5616−5620.
12. Ohta, H., Yoshida, N., Sano, M., Hirano, M., Nakezato, M., and Enami, I. (1995) in *Photosynthesis: from Light to Biosphere, Vol. II* (Mathis, P., Ed.) pp 361−364, Kluwer Academic Publishers, Dordrecht, The Netherlands.
13. Frankel, L. K., and Bricker, T. M. (1989) *FEBS Lett. 257*, 279−282.
14. Koike, H., and Inoue, Y. (1985) *Biochim. Biophys. Acta 807*, 64−73.
15. Philbrick, J. B., Diner, B. A., and Zilinskas, B. A. (1991) *J. Biol. Chem. 266*, 13370−13376.
16. Burnap, R. L., and Sherman, L. A. (1991) *Biochemistry 30*, 440−446.
17. Shen, J.-R., Vermaas, W., and Inoue, Y. (1995) *J. Biol. Chem. 270*, 6901−6907.
18. Shen, J.-R., Burnap, R. L., and Inoue, Y. (1995) *Biochemistry 34*, 12661−12668.
19. Eaton-Rye, J. J., and Vermaas, W. F. J. (1991) *Plant Mol. Biol. 17*, 1165−1177.
20. Haag, E., Eaton-Rye, J. J., Renger, G., and Vermaas, W. F. J. (1993) *Biochemistry 32*, 4444−4454.
21. Gleiter, H. M., Haag, E., Shen, J.-R., Eaton-Rye, J. J., Inoue, Y., Vermaas, W. F. J., and Renger, G. (1994) *Biochemistry 33*, 12063−12071.
22. Gleiter, H. M., Haag, E., Shen, J.-R., Eaton-Rye, J. J., Seeliger, A. G., Inoue, Y., Vermaas, W. F. J., and Renger, G. (1995) *Biochemistry 34*, 6847−6856.
23. Putnam-Evans, C., and Bricker, T. M. (1992) *Biochemistry 31*, 11482−11488.
24. Putnam-Evans, C., and Bricker, T. M. (1994) *Biochemistry 33*, 10770−10776.
25. Putnam-Evans, C., Wu, J., Burnap, R., Whitmarsh, J., and Bricker, T. M. (1996) *Biochemistry 35*, 4046−4053.
26. Putnam-Evans, C., Wu, J., and Bricker, T. M. (1996) *Plant Mol. Biol. 32*, 1191−1195.
27. Wu, J., Putnam-Evans, C., and Bricker, T. M. (1996) *Plant Mol. Biol. 32*, 537−542.
28. Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.
29. Ono, T.-A., Zimmerman, J.-L., Inoue, Y., and Rutherford, A. W. (1986) *Biochim. Biophys. Acta 851*, 193−201.
30. Lindberg, K., Vänngård, T., and Andréasson, L.-E. (1993) *Photosynth. Res. 38*, 401−408.

31. Yachandra, V. K., DeRose, V. J., Latimer, M. J., Mukerji, I., Sauer, K., and Klein, M. P. (1993) *Science 260*, 675−679.
32. Babcock, G. T. (1995) in *Photosynthesis: from Light to Biosphere, Vol. II* (Mathis, P., Ed.) pp 209−215, Kluwer Academic Publishers, Dordrecht, The Netherlands.
33. Rutherford, A. W. (1989) *Trends Biochem. Sci. 14*, 227−232.
34. Bebeneck, K., and Kunkel, T. A. (1989) *Nucleic Acids Res. 17*, 5408.
35. T7-GEN in Vitro Mutagenesis Kit Protocol, United States Biochemical, Product No. 74500.
36. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
37. Shen, G., and Vermaas, W. F. J. (1994) *J. Biol. Chem. 269*, 13904−13910.
38. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) *J. Gen. Microbiol. 111*, 1−61.
39. Vermaas, W., Charité, J., and Shen, G. (1990) *Biochemistry 29*, 5325−5332.
40. Engels, D. H., Lott, A., Schmid, G. H., and Pistorius, E. K. (1994) *Photosynth. Res. 42*, 227−244.
41. Brand, J. J., and Becker, D. W. (1988) *Methods Enzymol. 167*, 280−285.
42. Reidhaar-Olson, J. F., and Sauer, R. T. (1988) *Science 241*, 53−57.
43. Robles, S. J., and Youvan, D. C. (1993) *J. Mol. Biol. 232*, 242−252.
44. Kless, H., and Vermaas, W. (1995) *J. Mol. Biol. 246*, 120−131.
45. Kless, H., and Vermaas, W. (1996) *Biochemistry 35*, 16458−16464.
46. Delagrave, S., Goldman, E. R., and Youvan, D. C. (1995) *Protein Eng. 8*, 237−242.
47. Hermes, J. D., Blacklow, S. C., and Knowles, J. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 696−700.
48. Bowie, J. U., and Sauer, R. T. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2152−2156.
49. Barten, R., and Lill, H. (1995) *FEMS Microbiol. Lett. 129*, 83−88.
50. Waldbutger, C., and Susskind, M. M. (1994) *J. Mol. Biol. 235*, 1489−1500.
51. Logan, K. M., and Knight, K. L. (1993) *J. Mol. Biol. 232*, 1048−1059.
52. Shen, J.-R., Ikeuchi, M., and Inoue, Y. (1992) *FEBS Lett. 301*, 145−149.
53. Burnap, R. L., Qian, M., and Pierce, C. (1996) *Biochemistry 35*, 874−882.
54. Fine, P. L., and Frasch, W. D. (1992) *Biochemistry 31*, 12204−12210.
55. Lindberg, K., and Andréasson, L.-E. (1995) in *Photosynthesis: from Light to Biosphere, Vol. II* (Mathis, P., Ed.) pp 401−404, Kluwer Academic Publishers, Dordrecht, The Netherlands.
56. Boussac, A., and Rutherford, A. W. (1994) *Biochem. Soc. Trans. 22*, 352−358.
57. Yachandra, V. K., DeRose, V. J., Latimer, M. J., Mukerji, I., Sauer, K., and Klein, M. P. (1991) *Photochem. Photobiol. 53S*, 98s−99s.
58. Chu, H.-A., Nguyen, A. P., and Debus, R. J. (1994) *Biochemistry 33*, 6137−6149.
59. Nixon, P. J., Trost, J. T., and Diner, B. A. (1992) *Biochemistry 31*, 10859−10871.
60. Cheniae, G. M., and Martin, I. F. (1971) *Plant Physiol. 47*, 568−575.
61. Bennoun, P. (1970) *Biochim. Biophys. Acta 216*, 357−363.
62. Tamura, N., Inoue, H., and Inoue, Y. (1990) *Plant Cell Physiol. 31*, 469−477.

BI9723818