Truncation of the COOH-Terminal Domain of the *psb*E Gene Product in *Synechocystis* sp. PCC 6803: Requirements for Photosystem II Assembly and Function†

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ABSTRACT: The COOH-terminal domain of the 80-residue cytochrome b_{559} α-subunit (*psb*E gene product) in *Synechocystis* sp. PCC 6803 was sequentially truncated in order to determine the minimum polypeptide length needed for function and assembly. A stop codon was introduced into the Arg-50, Arg-59, or Tyr-69 codons of the *psb*E gene, generating mutants truncated by 31, 22, and 12 residues, respectively. Removal of 12 residues caused a decrease of 20% in PSII function. Truncation of 22 or 31 residues caused a large decrease (60–85%) in the photoautotrophic growth rate, the rate of O_2 evolution, and the amplitude of the 77 K 696-nm fluorescence, and a concomitant increase in the constant yield fraction (F_0/F_{max}) of the chlorophyll fluorescence. The level of residual activity in the Arg50-stop mutant was 10–20% of the wild type, which was reflected in a similar low level of immunochemically detected D2 polypeptide. Quantitation of the PSII reaction center stoichiometry of the Arg50-stop mutant by analysis of [^{14}C]DCMU binding also showed a 5-fold decrease (1:910 Chl in wild type and 1:5480 Chl in R50) in the PSII reaction center concentration. However, the K_D value for DCMU in the residual 15% of the complexes to which it bound was approximately equal to that (25 nM) of the wild type. Northern blot analysis showed no decrease in the b_{559} *psb*E mRNA level. Chemical difference spectral analysis of heme content indicated that the level of native cytochrome b_{559} heme in the Arg50-stop mutant (1:640 Chl) was 80% that of wild type (1:510 Chl). It was concluded that removal of 12 residues from approximately 38 in the lumen-side COOH-terminal domain of the cytochrome b_{559} α-subunit had only a small effect on function and assembly. Removal of 31 residues caused a decrease of 80–90% in reaction center function and in the content of assembled PSII reaction centers, without greatly affecting the assembly of cytochrome b_{559}. The existence of a small fraction (ca. 15%) of active PSII complexes in the R50-stop mutant would imply that the COOH-terminal 31 residues of the α-subunit are not required for functions essential for O_2 evolution such as the binding of manganese.

The folding pattern within the membrane of the D1–D2 core polypeptides of photosystem II of oxygenic photosynthesis has been predicted, and the functional roles for individual amino acids have been proposed, on the basis of similarity in primary structure and analogy to the L and M subunits of the reaction centers from the bacteria *Rhodopseudomonas viridis* (Deisenhofer & Michel, 1989) and *Rhodobacter sphaeroides* (Feher et al., 1989). A major difference between the bacterial reaction centers and those functioning in oxygenic photosynthesis is the presence of cytochrome b_{559} in the latter. Cytochrome b_{559} contains two polypeptides, α and β, consisting of 82 and 38 residues in spinach chloroplasts (Herrmann et al., 1984) and of 80 and 43 residues in the cyanobacterium *Synechocystis* sp. PCC 6803 (Pakrasi et al., 1988). The absolute orientation of the α-subunit has been obtained, and its NH_2- and COOH-termini lie on the stromal and lumen sides of the membrane, respectively (Tae et al., 1988; Vallon et al., 1989).

The COOH-terminal 35–40 residues of the b_{559} α-subunit extend into the lumen space (Tae et al., 1988). Shielding of the COOH-terminus from protease action by the extrinsic 33-kDa polypeptide of the oxygen-evolving complex suggested a possible role of the α-subunit COOH-terminus in the water-splitting function of photosystem II (PSII)[1] (Tae & Cramer, 1989). The presence of 5 conserved charged amino acids and 15 identical residues in the COOH-terminal segment of cytochrome b_{559} from 10 different plant, algal, and cyanobacterial sources [summarized in Tae (1991)] suggested a possible role of the COOH-terminal domain in the binding of ions such as manganese or calcium critical to O_2 evolution, and possibly in the assembly of the reaction center complex. Insertional inactivation of the *psb*A gene (D1) family in *Synechocystis* sp. PCC 6803 (Jansson et al., 1987) caused the loss of some reaction center polypeptides, D2 and CP-47, whereas cytochrome b_{559}, the CP-43 polypeptide, and the 33-kDa extrinsic polypeptide accumulated to almost wild-type levels (Nilsson et al., 1990). These results show that cytochrome b_{559} can be maintained in the thylakoid membrane despite the absence of the reaction center D1–D2 heterodimer and may itself be essential for PSII assembly.

Molecular genetic techniques have been developed to introduce specific mutations in photosystem II of the photoheterotrophic cyanobacterium *Synechocystis* sp. PCC 6803 (Williams, 1988) in order to analyze structure, function, and biogenesis of the PSII complex [e.g., see Debus et al. (1988), Metz et al. (1989), and Vermaas et al. (1990)]. The role of

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[1] Abbreviations: *A*, absorbance; R, arginine; Y, tyrosine; bp, base pair(s); Chl, chlorophyll; cyt, cytochrome; DAD, diaminodurol; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MES, 2-(*N*-morpholino)ethanesulfonic acid; MV, methyl viologen; NTP, nucleotide 5′-triphosphate; OEC, oxygen-evolving complex; PCR, polymerase chain reaction; PSI and PSII, photosystems I and II; TBS, Tris-buffered saline (10 mM Tris-HCl, pH 7.5, and 0.15 M NaCl).

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the COOH-terminal domain of the cytochrome b_{559} α-subunit was investigated in *Synechocystis* sp. PCC 6803 through its progressive truncation by mutation. Removal of 21 residues of the COOH-terminal domain of the cytochrome b_{559} α-subunit significantly decreased the fraction of active PSII complexes in the thylakoid membrane. However, even after truncation of 31 residues of the COOH-terminal domain, a small fraction of the PSII reaction centers could be correctly assembled.

Materials and Methods

Growth and Manipulation of Cyanobacterial Strains. Wild-type and mutant strains of *Synechocystis* sp. PCC 6803 were grown either in liquid media (BG-11) or in agar plates under constant illumination [20–30 μEinstein/(m^2·s)] at 30 °C (Rippka et al., 1979; Williams, 1988). The 2-fold-concentrated BG-11 medium and 3% (w/v) Bacto-agar (Difco, Detroit, MI) were separately autoclaved and mixed, and supplements such as 5 mM glucose, 20 μg/mL spectinomycin, and 20 μg/mL kanamycin were added when the solution was cooled to 55–60 °C. Truncation and substitution mutants of the cytochrome b_{559} α-subunit and the *psb*EF T1297 deletion mutant were propagated and maintained on plates containing 5 μg/mL spectinomycin and 20 μg/mL kanamycin, respectively. All the strains of *Synechocystis* sp. PCC 6803 were stored at −70 °C in the presence of 20% glycerol.

Thylakoid Membrane Preparations of Synechocystis sp. PCC 6803. Cells were grown in BG-11 medium containing 5 mM glucose, harvested in late log phase, and resuspended at 500 μg of chlorophyll/mL in 50 mM MES/NaOH, pH 6.0, 0.8 M sucrose, 1 mM PMSF, 2 mM benzamidine, and 2 mM ϵ-aminocaproic acid, the latter three components added as protease inhibitors (Widger et al., 1984). The cells were added to a Beadbeater (Biospec Products, Bartlesville, OK) chamber (250 mL) filled to two-thirds of capacity with 0.1-mm-diameter glass beads, and broken in 7–8 cycles of 30-s pulses separated by 15-min intervals. After centrifugation (8000*g*, 10 min), most of the thylakoid membranes remain in the supernatant and were sedimented following the addition of 40 mM $CaCl_2$ by a second centrifugation step (25000*g*, 30 min). The resulting sediment was resuspended in 25% glycerol, 20 mM MES/NaOH, pH 6.0, 20 mM $CaCl_2$, and 20 mM $MgCl_2$, centrifuged again at 45000*g* for 30 min, and resuspended in the same buffer (Noren et al., 1991). The chlorophyll concentration was then measured according to Lichtenthaler (1987).

Immunoblot Analysis. The mixture of proteins to be blotted was separated on 15–20% (w/w) gradient SDS–PAGE containing 6 M urea. The gel was incubated in 30 mM Tris-HCl buffer, pH 8.0, including 17 mM boric acid, 2 mM SDS, and 20% methanol for 10 min at room temperature. Polypeptides were transferred to nitrocellulose paper (pore size: 0.45 μm; Hybond-C, Amersham) with a semi-dry transfer blotter (130-mA constant current, 30 min) (Model TE70, Hoefer Scientific Instruments). The paper was washed in TBS buffer (0.15 M NaCl and 10 mM Tris-HCl, pH 7.5), incubated in a "Seal-a-Meal" bag with 10% milk casein on a nutator (2 h, 25 °C), removed from the bag, and washed in TBS buffer. After nutation with antibody in TBS buffer containing 0.5% (w/v) bovine serum albumin (BSA) for 2–3 h, and washing in TBS buffer containing 0.1% (w/v) Nonidet-40, the paper was again incubated with a second antibody [goat anti-rabbit IgG conjugated with horseradish peroxidase (type VI, Accurate Chemical and Scientific Corp.)] in TBS buffer containing 0.5% BSA. The paper was nutated for 1–2 h, washed in TBS buffer containing 0.1% (w/v) Nonidet-40, and stained for 10 min with 0.017% 4-chloro-1-naphthol and hydrogen peroxide in TBS buffer.

Construction of the Recombinant Plasmid pCT901. The 2.2-kb *Hin*dIII/*Eco*RI fragment of the pKW1261 plasmid (kindly provided by R. Burnap and L. A. Sherman), which carried the *psb*E and *psb*F genes encoding the cytochrome b_{559} α- and β-subunits of *Synechocystis* sp. PCC6803, was cleaved by restriction digestion and ligated into the linearized pBluescript II SK^+ phagemid which carries the ampicillin resistance gene and T7 promoter, as well as the T4 promoter (Stratagene, La Jolla, CA). The ligation mixture was transformed directly into the *Escherichia coli* JM101 strain, and transformants were selected by blue color on agar plates that contained 50 μg/mL ampicillin, 80 μg/mL X-gal, and 20 mM IPTG. The candidate plasmids were extracted from individual colonies and screened by restriction digestion. The pBluescript II SK^+ containing the *psb*E and *psb*F genes was linearized with restriction enzyme, *Nhe*I, for which the cleavage site was located downstream of the *psb*F gene, in order to introduce compatible restriction digestion sites for ligation with the 2.0-kb *Xba*I fragment of spectinomycin-resistant (Sp^r) pRL463. After ligation and transformation into the JM101 strain, the new construct named pCT901 containing the *psb*E and *psb*F genes of *Synechocystis* sp. PCC 6803, as well as the spectinomycin resistance gene, was screened by spectinomycin and restriction digestion analyses.

Site-Directed Mutagenesis of the psbE Gene in pCT901. In order to introduce a stop codon at Arg-50, Arg-59, or Tyr-69 in the *psb*E gene of *Synechocystis* sp. PCC 6803, the synthetic oligonucleotides 5′-GCACTCCCTGACCCGATGA-3′,5′-CCAGACCTGACAAGAGTTG-3′, and 5′-AGGAACGCTAGGACATTAA-3′ were used as primers, respectively. Each of the synthetic phosphorylated oligonucleotides was annealed to the single-stranded DNA, extracted from M13KO7 phage, in 50 mM NaCl, 20 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.5 (70 °C, 2 min), and the annealing mixture was cooled to 30 °C in 20–30 min. After the altered oligonucleotide was annealed to the template, native T7 DNA polymerase, T4 DNA ligase, and 10-fold-concentrated synthesis buffer (5 mM each of dATP, dCTP, dGTP, and dTTP, 10 mM ATP, 50 mM $MgCl_2$, 20 mM DTT, and 100 mM Tris-HCl, pH 7.9) were added and incubated at 37 °C for 30 min to synthesize the mutated complementary DNA strand (Bebenek & Kunkel, 1989). The resulting closed heteroduplex DNA was transformed directly into the *E. coli* MV1190 strain, in which dUTPase and uracil *N*-glycosylase were active. Several colonies were picked and plasmids isolated from individual colonies. The mutants were selected by direct DNA sequencing of the plasmid (Tabor & Richardson, 1987; Bebenek & Kunkel, 1989).

Isolation of the Chromosomal DNA from Synechocystis sp. PCC 6803. One hundred milliliters of a late-log-phase cell culture grown at 30 °C was harvested by centrifugation (5000*g*, 10 min), washed once with 5 mM EDTA and 50 mM Tris-HCl, pH 7.5, and resuspended in saturated NaI (5 mL). Cells were heated to 65 °C for 20 min and centrifuged (10000*g*, 10 min). The sediment was washed once with the Tris–EDTA buffer and resuspended in 10 mL of Tris–EDTA containing lysozyme (4 mg/mL). After incubation (37 °C, 45 min), 2% (w/v) SDS, 2% (w/v) *N*-laurylsarcosine, and 100 μg/mL proteinase K (745723, Boehringer) were added and incubated (50 °C, 1–2 h). The lysate was extracted with an equal volume of phenol/chloroform (1 h) and then with chloroform (1 h). The upper phase was precipitated with 0.1 volume of 7.5 M ammonium acetate, pH 7.8, and 2 volumes

of 100% ethanol. The chromosomal DNA dried under vacuum was redissolved in TE (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) buffer (200 μL) and 1 M $MgCl_2$ (4 μL), digested with 100 μg/mL RNase (10 mg/mL, 1 h, room temperature), twice extracted with phenol/chloroform and then twice with chloroform, and dried under vacuum.

Transformation of the Mutated psbE Gene in pBluescript II SK⁺ into the psbEF Deletion Mutant. A 1.5-mL sample of the culture grown to (2–5 × 10^8 cells/mL (A_{730} = 0.25 corresponds to 10^8 cells/mL) was harvested by centrifugation at 4500*g* (room temperature, 10 min) and resuspended in 200 μL of fresh BG-11 medium with 5 mM glucose. Two to five microliters of plasmid DNA (0.2 μg/μL) was added and incubated for 4 h in a sterile Falcon tube under standard temperature and light conditions. The transformation mixture was incubated another 18 h after addition of 1 mL of BG-11 including 5 mM glucose and spread on Petri dishes containing glucose and selective antibiotics. Colonies of transformed cells could be seen in 10–20 days.

Identification of Mutation Sites by the Polymerase Chain Reaction. The *psb*E gene carrying mutations was amplified from the cyanobacterial genome by use of PCR with *Taq* DNA polymerase (Saiki et al., 1985) in order to ascertain that the correct mutations had been introduced into the cyanobacterium. For PCR of the R50-stop, R59-stop, and Y69-stop mutants, as well as the wild type, two synthetic oligonucleotide 30-mers were used as primers for DNA polymerization: (i) 5′-CCGAATTCGGTCAGGGACTACCGGCGAGCG-3′ (forward primer; G+C content, 60%; *Eco*RI site at 5′ end) to hybridize the complementary strand upstream of the *psb*E gene including the start codon; (ii) 5′-GAAGCTTCCCAACACA GCCACCAACAATAG-3′ (reverse primer; G+C content, 52%; *Hin*dIII site at 5′ end), which was complementary to the region in the middle of the coding strand of the *psb*L gene, which is located immediately downstream of the *psb*E and F genes. Twenty-five PCR cycles (each 60 s at 94 °C for denaturation of the double-stranded DNA followed by 60 s at 55 °C and 90 at 72 °C for the hybridization and chain elongation, respectively) in a reaction medium (Promega, M1862) including 0.8 mM dNTP, 1 μM forward primer, 1 μM reverse primer, 0.5 μg of chromosomal DNA, and 2 units of *Taq* DNA polymerase were sufficient to generate several micrograms of the amplified 0.5 kb DNA fragment between the hybridization sites of the oligonucleotides.

DNA fragments amplified by PCR from the R50-stop, R59-stop, and Y69-stop mutants as well as wild type were treated with Klenow fragment in the presence of dNTP and ligated into the overexpression vector (pT7-7) (Tabor & Richardson, 1985; Studier & Moffatt, 1986) using the restriction enzyme sites for *Eco*RI and *Hin*dIII. The ligation mixture was transformed directly into the BL21 strain, and candidate plasmids were extracted from individual colonies and screened by restriction digestion. The mutation sites on the cyanobacterial genome were directly confirmed by sequencing (Tae, 1991) using a modified dideoxynucleotide chain termination method (Tabor & Richardson, 1987; Bebenek & Kunkel, 1989).

Northern Blot Analysis. The total RNA purified from the wild-type, R50-stop, and T1297 strains of *Synechocystis* sp. PCC 6803 according to Reddy et al. (1990) was loaded onto a 1.2% (w/v) formaldehyde/agarose gel, separated at constant voltage (75 V) in MOPS/EDTA buffer (20 mM MOPS, 5 mM sodium acetate, pH 7.0, and 1 mM EDTA), and transferred to a Duralon-UV membrane (Stratagene, La Jolla, CA). The pCT901 plasmid was nick-translated with [^{32}P]dCTP and used to detect [X-ray film, X-OMAT (Kodak), −70 °C, 48 h] mRNA (0.8 kb) from the *psb*EFLJ gene cluster (Pakrasi et al., 1991).

PSII Function. (A) Electron Transport Activity and O_2 Evolution. Steady-state electron transport activities of PSII and PSI of the wild-type and truncation mutants were measured at 25 °C using a Clark-type O_2 electrode and a saturating actinic light intensity of 2000–2500 μEinstein/(m^2·s). Measurements of O_2 evolution were performed with cells (10 μg of Chl/mL) in BG-11 growth medium containing 0.25 mM DCBQ and 0.25 mM ferricyanide. PSI activity was measured with 0.1 mM MV, 0.2 mM DAD, and 2 mM sodium ascorbate added to BG-11 growth medium containing 20 μM sodium azide and 10 μM DCMU.

(B) DCMU Binding. In order to quantitate the stoichiometry of functional PSII complexes of the R50-stop, R59-stop, and Y69-stop mutants on the basis of chlorophyll concentration, [^{14}C]DCMU binding to cells was measured (Vermaas et al., 1990). Various (4.5–91.3 nM) concentrations of radiolabeled DCMU (243 μCi/mg; Amersham, kindly donated by W. F. J. Vermaas) were added to 1 mL of cells (25 μg of Chl/mL) in BG-11. To correct for the amount of [^{14}C]-DCMU bound to cells at sites other than the PSII complex, control experiments were performed in which [^{14}C]DCMU binding was measured in the presence of 40 μM unlabeled atrazine, which is a specific inhibitor of the quinone or DCMU binding site in the PSII reaction center. After incubation for 15 min at room temperature in dim light, cells were sedimented in a microcentrifuge, and 0.8 mL of the supernatant was mixed with scintillation cocktail and assayed.

(C) Fluorescence Yield. Fluorescence was excited with weak chopped (270 Hz) monochromatic 438-nm light of intensity ~0.1 μEinstein/(m^2·s). The fluorescence excited by this light was defined as the F_0 level. Emission at wavelengths longer than 680 nm was measured with a Hamamatsu (type R562, S20 red sensitive) photomultiplier tube (blocked with Corning filters 9830 and 2030), connected to an Ithaco Lock-In amplifier tuned to the chopping frequency with an output filter of 3 s (Cramer & Butler, 1969). The fluorescence level of cells (10 μg of Chl/mL) excited by a 650-nm actinic beam [150 μEinstein/(m^2·s)] in BG-11 medium containing 20 μM DCMU was defined as F_{max}.

(D) Fluorescence Emission Spectra at 77 K. Mid-log-phase cells were harvested by low-speed centrifugation at room temperature, resuspended in fresh BG-11 medium containing 5 mM glucose at a concentration of 75 μg of Chl/mL, and placed on a rotary shaker while measurements were carried out. Samples were directly frozen as a thin coating on the outside of a liquid nitrogen chilled NMR (2 mm) sample tube and used immediately for measurement in an SLM 8000C spectrofluorometer fitted with a liquid nitrogen optical Dewar, with excitation at 435 nm and fluorescence emission measured with a 3-s time constant.

(E) Cytochrome Difference Spectra. Chemical difference spectra were measured at room temperature on an Aminco dual-wavelength monochromator with a computer (IBM AT)-controlled stepper motor (High-Sync AC synchronous/DC stepper motor; Bodine Electric Co.). The amplifier output time constant (0.67 ms) was digitized and interfaced to an IBM PC in which data were stored. The measuring beam (half-bandwidth, 2 nm), arranged to pass vertically through the cuvette, was blocked by a Balzer's DT-Gruen filter to prevent any interference from fluorescence and detected by an S11 EMI 9524 photomultiplier. Difference spectra were

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 1                                                              15
 S   G   T   T   G   E   R   P   F   S   D   I   V   T   S
TCA GGG ACT ACC GGC GAG CGT CCA TTT TCC GAT ATT GTC ACC AGC

16                      *                                      30
 I   R   Y   W   V   I   H   S   I   T   I   P   M   L   F
ATT CGC TAC TGG GTG ATC CAC AGC ATC ACC ATC CCG ATG TTG TTT

31                                                             45
 I   A   G   W   L   F   V   S   T   G   L   A   Y   D   A
ATT GCT GGT TGG TTG TTT GTC AGC ACG GGC TTA GCC TAC GAT GCT

46                                                             60
 F   G   T   P   R   P   D   E   Y   F   T   Q   T   R   Q
TTT GGC ACT CCC CGC CCC GAT GAA TAT TTC ACC CAG ACC CGT CAA

61                                                             75
 E   L   P   I   L   Q   E   R   Y   D   I   N   Q   E   I
GAG TTG CCC ATT CTC CAG GAA CGC TAC GAC ATT AAT CAG GAA ATT

76
 Q   E   F   N   Q   *
CAA GAG TTT AAT CAA TAA AACATTTAATTGTTCTTTTTTAGTTGGTAA
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FIGURE 1: Nucleotide and amino acid sequences of the cytochrome b_{559} α-subunit in *Synechocystis* sp. PCC 6803. The amino acid sequence is written in one-letter code. The central hydrophobic domain of 24 amino acid residues is underlined. The single His residue is noted (*). Nucleotide sequences, CGC, CGT, and TAC, in boxes, for Arg-50-stop, Arg-59-stop, and Tyr-69-stop truncation mutants, respectively, were changed to stop codons TGA, TGA, and TAG, respectively.

obtained by subtracting the stored spectrum of the initial state from that arising from further additions, and deconvoluted using the Peakfit program (Jandel Scientific, Corte Madera, CA) into two components (for cytochrome b_{559}, peak, 559 ± 0.5 nm; half-bandwidth, 10 ± 1 nm; for cytochrome b_6, peak, 563 ± 0.75 nm; half-bandwidth, 10 ± 0.5 nm). Samples were mixed with a magnetic stirrer that was turned off before measurement of a spectrum. Other parameters are as in Furbacher et al. (1989).

RESULTS

Truncation Mutants. In order to examine the function of the COOH-terminal domain of the cytochrome b_{559} α-subunit, mutants truncated by 31, 22, or 12 residues were generated by introducing a stop codon at Arg-50 (R50-stop), Arg-59 (R59-stop), and Tyr-69 (Y69-stop) of the *psb*E gene (Figure 1), in which the codons for R50 (CGC), R59 (CGT), and Y69 (TAG) were changed to TGA, TGA, and TAG, respectively. These mutated genes together with the spectinomycin resistance (Sp^r) gene were transformed into the *psb*EF deletion mutant T1297 (Pakrasi et al., 1988) in order to remove the background activity of cytochrome b_{559}.

Photoautotrophic Competence. The mutants were screened by the ability to grow on plates with spectinomycin. The growth rate of these mutants was similar to that observed for wild type under conditions in which PSII activity is not required (data not shown; Tae, 1991). However, under photoautotrophic growth conditions, the growth rates of the R50-stop and R59-stop truncation mutants were significantly slower, whereas that of the Y69-stop mutant was similar to that observed for wild type (Figure 2; Table IA). In order to examine whether the reduced growth rate of the R50- and R59-stop mutants was related to the presence of the Sp^r marker downstream of the *psb*EF gene, the plasmid carrying the wild-type *psb*E and *psb*F genes as well as the Sp^r gene was transformed into the T1297 mutant. The photoautotrophic growth rate and O_2 evolution activity of the resulting revertant carrying the Sp^r gene were similar to those observed in wild type (data not shown).

Electron Transport Activities. The rate of O_2 evolution (H_2O to DCBQ–ferricyanide) of cells of the R50- and R59-stop mutants was reduced to 15 and 40% of the wild-type activity, respectively, whereas that of the Y69-stop mutant was a larger fraction (75–80%) of that observed for wild type

Table I: Summary of Relative Rates of (A) Photoautotrophic Growth, (B) O_2 Evolution, and (C) Photosystem I Electron Transport and of the (D) Relative Fluorescence Yield of Cytochrome b_{559} α-Subunit Truncation Mutants

	WT	α-Y69-stop	α-R59-stop	α-R50-stop
(A) normalized growth rate	1.00	0.83	0.25	0.21
(B) O_2 evolution[a]	1.00	0.78	0.40	0.15
(C) PSI e^- transport[a] (DAD/Asc → MV)	1.00	1.00	1.03	1.07
(D) fluorescence of constant yield (F_0/F_{max})[b,c]	0.53	0.62	–[d]	0.83

[a] Control rates of the wild type were 271 ± 6 and 1040 for O_2 evolution and PSI activity, respectively; units of O_2 evolution (B) or uptake (C) were micromoles of O_2 per milligram of chlorophyll per hour. The standard deviation was calculated from three independent measurements; the O_2 evolution rate of the T1297 *psb*EF deletion mutant that is completely deficient in cytochrome b_{559} was zero. [b] Fluorescence yield values are the average of two measurements; the increase in the F_0 value of the wild type after addition of 20 μM DCMU was approximately 12%; F_{max} levels were measured 20 s after turning on the actinic light. [c] F_0/F_{max} = 0.96 for the T1297 cytochrome b_{559} deletion mutant of Pakrasi et al. (1988). [d] Not measured.

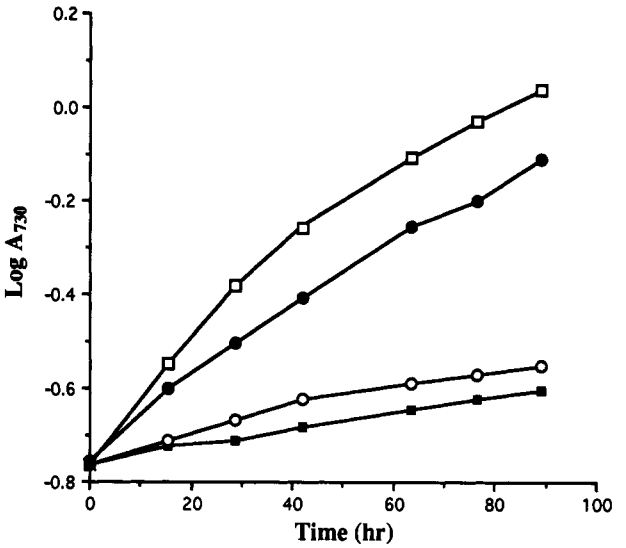

FIGURE 2: Photoautotrophic growth curves of *Synechocystis* sp. PCC 6803 wild-type and truncation mutants. Cells of wild type (□) and truncation mutants [α-R50-stop (■), α-R59-stop (○), and α-Y69-stop (●)] were grown in BG-11 medium without glucose under constant illumination [Rippka et al., 1979; Williams, 1988]; growth rates were monitored at 730 nm.

(Table IB). The photosystem I electron transport activity of the truncated mutants, measured from DAD/ascorbate to methyl viologen, was the same as wild type (Table IC), indicating that the PSI complex of all truncated mutants was not impaired.

Fluorescence Yield. Minimum and maximum levels of the fluorescence yield of a given cell strain correspond to fully oxidized and reduced levels, respectively, of the quinone quencher, Q_A (Cramer & Butler, 1969). The level of constant fluorescence yield, F_0, measured in the weak modulated measuring beam relative to the fluorescence yield, F_{max}, in the presence of a strong actinic light was used as an assay of the loss of quencher Q_A in mutant strains deficient in the PSII reaction center. The ratio of the fluorescence of constant yield to the maximum fluorescence, F_0/F_{max}, of the wild-type, Y69-stop, R50-stop, and T1297 deletion mutants was 0.53, 0.62, 0.83, and 0.96 (Table ID). The comparison of the 0.83 value obtained with the R50-stop mutant with 0.96 for the total deletion mutant, T1297, is another indicator of a low level of residual PSII electron transport activity in the R50-stop mutant.

Low-Temperature Fluorescence Emission Spectra. Excitation of intact *Synechocystis* sp. PCC 6803 cells frozen at

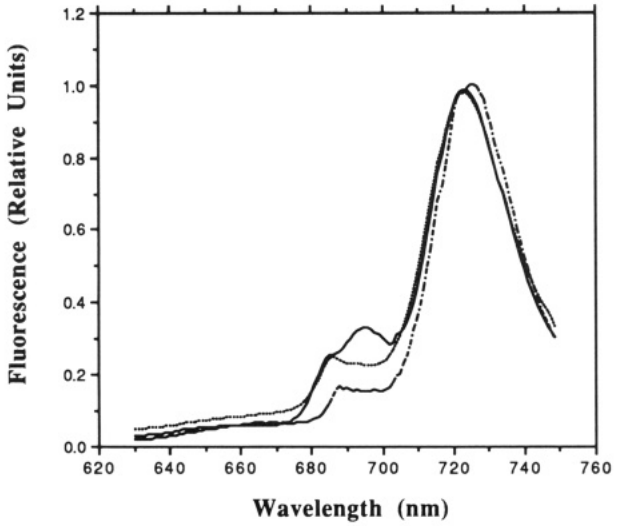

FIGURE 3: Low-temperature fluorescence emission spectra of wild-type and truncation mutants of the cytochrome b_{559} α-subunit. Cells in BG-11 medium (75 μg of Chl/mL) containing 5 mM glucose were directly frozen at 77 K and excited at 435 nm. Fluorescence emission spectra were measured as described under Materials and Methods and normalized to the emission band at 725 nm. Emission spectra of wild-type, R50-stop, and T1297 deletion mutants are represented as solid, dotted, and dot–dashed lines, respectively.

77 K gives rise to three emission peaks at 685 (F685), 696 (F696), and 725 nm (F725). The F685 and F696 bands are believed to arise from the PSII core complex, including the D1, D2, cytochrome b_{559}, CP-47, and CP-43 polypeptides (Satoh et al., 1983; Bricker et al., 1985), and allophycocyanin (Elmorjani et al., 1986; Rögner et al., 1991), whereas the F725 emission originates from PSI (Goedheer, 1972). The low-temperature fluorescence emission spectra, normalized to the peak at 725 nm, of the wild-type and truncation mutants of cytochrome b_{559} α-subunit indicated that the amplitude of the F696 emission was significantly reduced in the R50 truncation mutant relative to wild type (Figure 3). The F696 component of the Y69 mutant was similar to that of the wild type, and that of the R59 mutant was much less than wild type, and slightly larger than that of the R50 mutant (data not shown).

D2 Polypeptide Level. The decrease in PSII activity and the increase in the fluorescence of constant yield of the R50-stop mutant imply a decrease in concentration of functional D1–D2–b_{559} reaction center complexes. The level of D2 polypeptide in the R50 mutant detected by Western blotting was much smaller ($\sim$10%) than that of the wild type (Figure 4). The level of D2 in the R50 mutant is, however, greater than that of the T1297 deletion mutant, where D2 is not detectable. The levels of CP-47 and extrinsic 33-kDa (*psb*O) polypeptides were comparable to those of the wild type (data not shown).

Cytochrome b_{559} Content. It was thought that insertion of a translation stop codon in the *psb*E gene could have destabilized and inhibited transcription of the multicistronic *psb*EFLJ mRNA that encodes the *psb*E and *psb*F gene products associated with cytochrome b_{559}. The transcript level of the R50 mutant was found to be identical to that of the control (Figure 5, lanes 1 and 2), and zero in the T1297 *psb*EF deletion mutant, as shown by Pakrasi et al. (1991). Chemical difference spectra of cytochromes b_{559} and b_6 in thylakoid membranes were obtained by the sequential addition of menadiol (0.5 mM) and dithionite (2 mM) following oxidation by ferricyanide and reduction of cytochrome *f* by hydroquinone. Because cytochrome *f* in these membranes has a reduced α-band maximum of 556–557 nm (Ho & Krogmann, 1980) that is close to that of high-potential cytochrome b_{559}, the midpoint potential of cyt b_{559} was shifted to low potential by heat treatment and incubation in dilute Triton X-100 (Cramer & Whitmarsh, 1977) in order to separate it from cytochrome *f* on the redox scale. Spectra of the wild type (Figure 6A,B) and the R-50 mutant (Figure 6C,D) are shown,

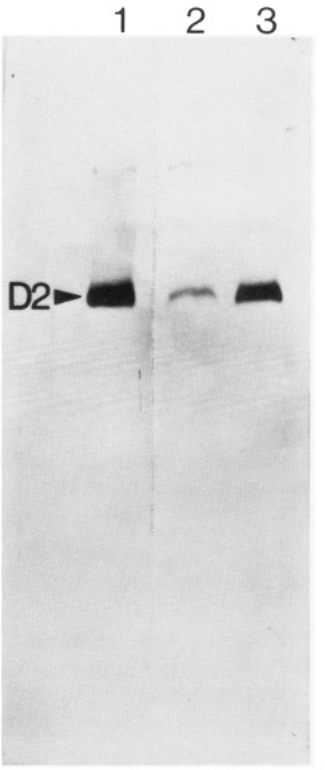

FIGURE 4: Western blot analysis of thylakoid membranes from *Synechocystis* sp. PCC 6803 probed with antibody against the D2 protein in (1) wild type and (2) R50 and (3) Y69 cyt b_{559} truncation mutants. Thylakoid membranes were separated as described under Materials and Methods and separated on 15–20% gradient SDS–PAGE including 6 M urea. Each lane was loaded with the membrane equivalent of 10 μg of Chl. The gel was incubated in 30 mM Tris-HCl buffer, pH 8.0, including 17 mM boric acid and 2 mM SDS (10 min, 25 °C) and transferred (130-mA constant current, 30–40 min) to nitrocellulose paper (pore size, 0.45-μm diameter) with a semidry blotter (Hoefer Scientific Instruments, Model TE-70). Color reactions were performed with 0.017% 4-chloro-1-naphthol and 0.05% H_2O_2 in TBS.

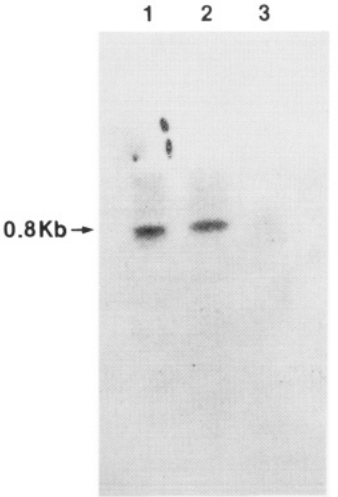

FIGURE 5: Northern blot analysis of RNA from *Synechocystis* sp. PCC 6803 wild type (lane 1), R50-stop (lane 2), and T1297 (lane 3) probed with the plasmid pKW1261 nick-translated with [^{32}P]dCTP. Each lane was loaded with 10 μg of RNA and separated on a 1.2% formaldehyde/agarose gel. Other details of the procedure are described under Materials and Methods.

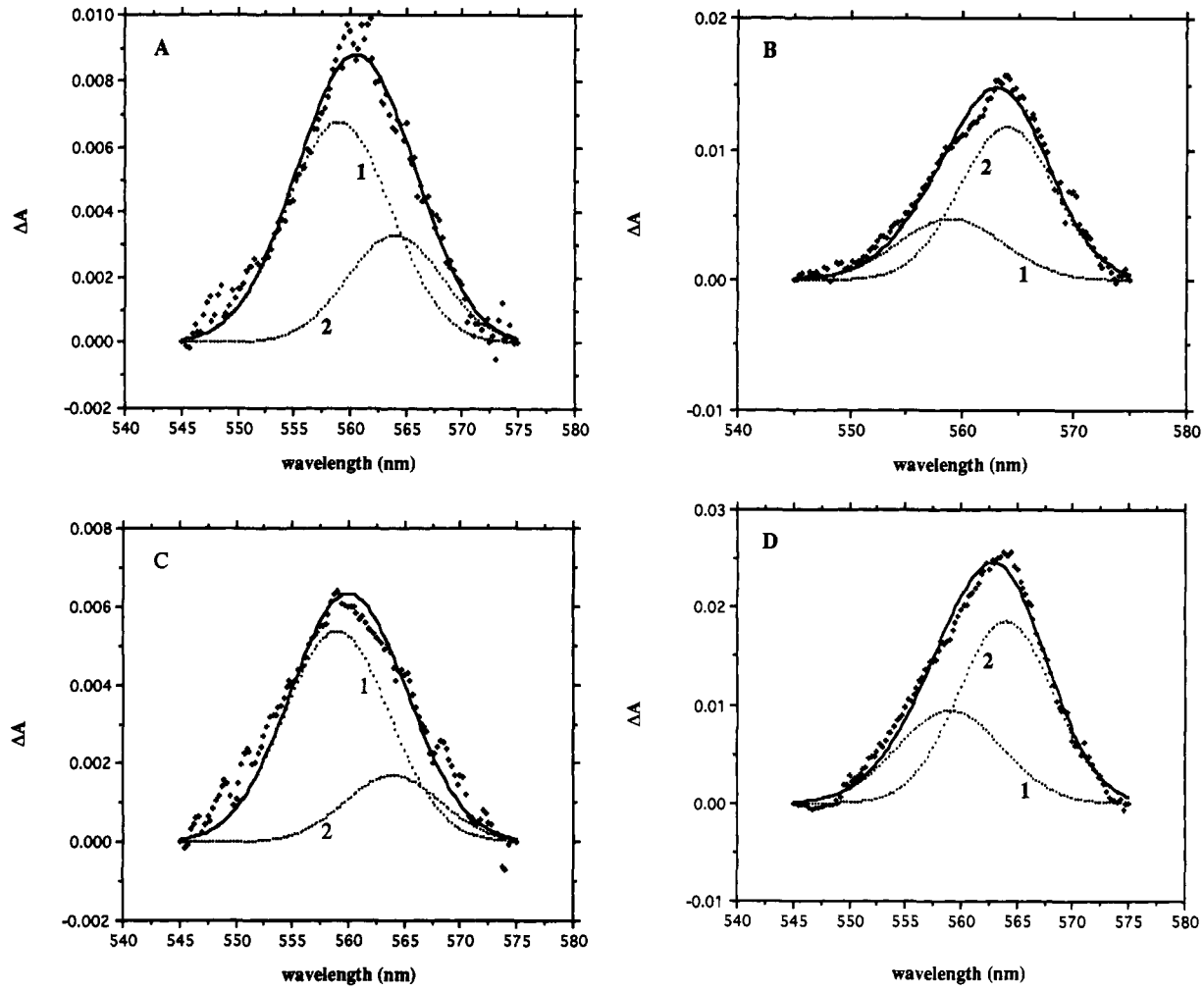

FIGURE 6: *Difference spectra of cytochromes in the thylakoid membranes from the wild type and the R50-stop truncation mutant.* Membranes were incubated at 50 °C for 5 min and then incubated in 0.01% Triton X-100 to convert all high-potential b_{559} to a low-potential state. Chemical oxidation–reduction difference spectra of cytochrome b_{559} (1) and cytochrome b_6 (2) were measured in thylakoid membranes of the wild type (A, B) and the R50-stop mutant (C, D) by the sequential addition of menadiol (0.5 mM) (A, C) and dithionite (ca. 2 mM) (B, D) after oxidation by ferricyanide (50 μM) and reduction of cytochrome *f* by hydroquinone. Spectra were deconvoluted into cyt b_{559} (1) and cyt b_6 (2) components as described under Materials and Methods. The correlation coefficient, r^2, in panels A–D is 0.98, 0.96, 0.95, and 0.99, respectively. The cyt b_{559} heme:Chl (mol/mol) ratio of menadiol-reducible cytochrome b_{559} heme (curve 1) in the wild type and R50-stop mutant was 1:510 and 1:640, respectively, and the heme:Chl ratio of dithionite (total area) minus menadiol-reducible cyt b_6 (curve 2) in wild type and R50 mutant was 1:210 and 1:120, respectively, using a millimolar extinction coefficient of 21.5 (reduced α-band peak minus trough at 572–575 nm) (Cramer et al., 1986).

with the menadiol minus hydroquinone difference spectra in Figure 6A,C and the dithionite minus menadiol spectra in Figure 6B,D. The spectra were deconvoluted into cyt b_{559} and cyt b_6 components (curves 1 and 2, respectively, in Figure 6A–D) using the spectral parameters described under Materials and Methods. Cytochrome b_{559} is the major spectral component in the spectra obtained with menadiol as the reductant (Figure 6A,C). The ratio of menadiol-reducible cyt b_{559} heme to chlorophyll is 1:510 and 1:640, respectively, for wild type and R50-stop mutant. The total (dithionite minus menadiol-reducible) cytochrome b_6 heme content (heme:Chl ratio) reducible by menadiol and dithionite in wild type and mutant is 1:210 and 1:120, respectively. If there are two hemes per PSII reaction center, the ratio of cyt b_{559} complex to PSII reaction center would be 1:1020 and 1:1280, respectively. The stoichiometry of menadiol-reducible cytochrome to chlorophyll in the mutant was approximately 80% that of the wild type. The normalization of the cytochrome must be made relative to chlorophyll or another component in the membrane such as cyt b_6, because with the PSII deficiency in the R50-stop mutant it is not possible to use a marker intrinsic to PSII. The nature of the cytochrome b_{559} molecule in the truncated mutant is different from that in the wild type, as determined from the inability of antibody made to the wild-type *psb*E gene product to interact with this product in membranes from the R50-stop or T1297 mutants (data not shown). This is consistent with the main epitope of the α-subunit being in the COOH-terminal region (cf. spinach chloroplast case; Tae et al., 1988; Vallon et al., 1989).

An additional cytochrome b_{559}-like component can be resolved by deconvolution from the short-wavelength side of the cyt b_6 spectrum (Figure 6B,D). It is not known whether this component arises from cyt b_{559} or from asymmetry in the normal cyt b_6 spectrum. If the dithionite-reducible b_{559}-like component is added to the menadiol-reducible cyt b_{559}, then the heme:Chl ratio in wild type and mutant would be 1:300 and 1:230, respectively.

Stoichiometry of Functional PSII Reaction Centers. The low PSII activity of the R50-stop mutant activity might be ascribed to (i) reduced activity and impaired assembly of all centers or (ii) completely competent assembly and activity of a small fraction (10–20%) of the centers. The stoichiometry of binding of the quinone analogue competitive inhibitor of the quinone (Q_B) binding site, DCMU, can be used to assay

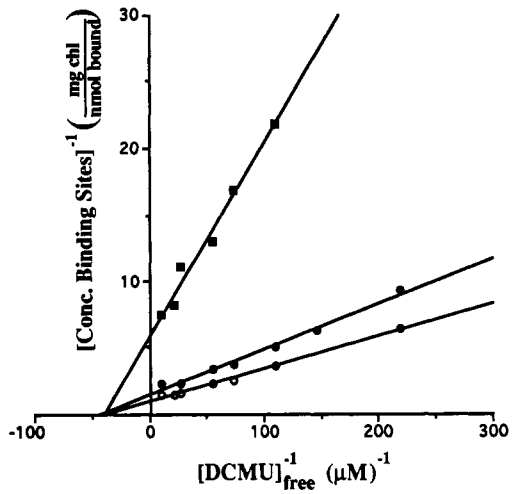

FIGURE 7: [^{14}C]DCMU binding of wild-type (○), R-50 (■), and Y69 (●) mutants of the cytochrome b_{559} α-subunit in *Synechocystis* sp. PCC 6803. 4.5–91.3 nM radiolabeled DCMU was used to determine the total number of PSII complexes in the cell in the presence of 40 μM unlabeled atrazine. Inverted values of x and y intercepts indicate the dissociation constant for DCMU and the number of the PSII complexes, respectively.

the concentration and functional state of the PSII reaction center (Vermaas et al., 1990). The binding of [^{14}C]DCMU to cyanobacterial cells as a function of DCMU concentration was assayed to quantitate the number of PSII complexes in vivo in wild-type, R50, and Y69 truncation mutants. Double-reciprocal plots of [^{14}C]DCMU binding to wild-type, R50, and Y69-stop mutant cells, grown under the same conditions, showed from the values of the respective y intercepts that the R50-stop mutant has a smaller number of functional PSII complexes per unit chlorophyll (1:5480) than does the wild type (1:910), whereas the stoichiometry of PSII complexes in the Y69 truncation mutant is slightly smaller (1:1410) than the wild type (Figure 7). The dissociation constant (K_D) of DCMU, calculated from the inverse of the x-axis intercept, of both R50 and Y69 truncation mutants was approximately the same (ca. 25 nM) as the wild type.

Arg-59, Gln-60, Pro-63, and Arg-68 are highly conserved residues in 10 cyt b_{559} sequences [summarized in Tae (1991)] in the truncated COOH-terminal domain of the α-subunit in the Y69-stop mutant that are removed in the R50 and R59 mutants. In order to examine the contribution of specific charges on the effect of truncation of the α-subunit, site-specific mutations were made at Arg-59 or Arg-68 to Gln-59 and Gln-68, respectively, for which the mutation sites were confirmed by sequencing of chromosomal DNA using PCR. Two single and one double mutant (Q59, Q68) showed almost the same O_2 evolution activity (Table II), photoautotrophic growth rates similar to the wild type, and similar low-temperature emission spectra (data not shown). It was concluded that the Arg-59 and/or Arg-68 residues in the COOH-terminal domain are not essential for the correct assembly of the PSII reaction center complex.

Discussion

Extrapolating from the determination of the orientation of the cytochrome b_{559} α-polypeptide in spinach chloroplasts (Tae et al., 1988; Vallon et al., 1989), the 80-residue α-subunit of cytochrome b_{559} in the photosystem II reaction center of *Synechocystis* sp. PCC 6803 is assumed to be oriented in the thylakoid membrane with a single hydrophobic α-helical span extending approximately from Trp-19 to Ala-42 (Figure 1). Then, the COOH-terminal domain from Tyr-43 to Gln-80

Table II: Photosynthetic Electron Transport Activities of Wild-Type, R59→Q, R68→Q, and R59,68→Q Mutant Cells of *Synechocystis* sp. PCC 6803

		cyanobacterial mutants		
	WT	R59→Q	R68→Q	R59,68→Q
O_2 evolution[a]	268 ± 9	274 ± 10	271 ± 14	254 ± 19

[a] Steady-state electron transport activities of PSII were measured from water to DCBQ with a Clark-type oxygen electrode using whole cells (10 μg of Chl/mL in BG-11) in the presence of 250 μM ferricyanide. The units of O_2 evolution were micromoles of O_2 per milligram of Chl per hour. The standard deviation was calculated from three independent measurements.

(Figure 1) would extend into the thylakoid lumen space. The presence of cytochrome b_{559} in the PSII reaction center core complex (Nanba & Satoh, 1987), and the proximity of the COOH-terminal domain to the 33-kDa OEC polypeptide (Tae & Cramer, 1989), suggested that this domain might be essential for the water-splitting reaction, perhaps by serving as a ligand for necessary metal ions.

Properties of Truncation Mutants. Truncation of the last 12 residues of the cytochrome α-subunit in *Synechocystis* that include 3 nonconserved carboxylate residues had a relatively small (20–25%) effect on the photoautotrophic growth rate and function. Deletion of 22–31 residues from the COOH-terminus had a much larger (60–85%) inhibitory effect. The effect of the 31-residue deletion was studied in greater detail in the R50-stop mutant. Photoautotrophic growth and O_2 evolution were inhibited by 80–85% in this mutant while the fluorescence of constant yield (F_0) increased to approximately 85% of F_{max}.

Fraction of Active Centers in the R50-Stop Mutant. There are two alternative possibilities for the decrease in the PSII function between P680 and Q_B in these mutants: (i) all PSII reaction centers are altered with a consequent decrease in PSII function; (ii) most of the PSII complexes are damaged, degraded, inactivated, or incorrectly assembled, but a small fraction (10–20%) is correctly assembled and stable and has an electron transfer rate between P680 and Q_B similar to that of the wild type. Binding of [^{14}C]DCMU to cyanobacterial cells can be used to quantitate the number of PSII reaction centers (Vermaas et al., 1990) and the structural integrity of the quinone binding site (Q_B) on the D1 polypeptide of the PSII reaction center. Purified D1–D2–cytochrome b_{559} complex has a lower affinity for DCMU (Chapman et al., 1988). A double-reciprocal plot of DCMU binding to the Q_B site in cells of the wild type and the R50-stop and Y69-stop mutants indicated that the content of PSII complexes is much smaller (1:5480 Chl) in the R50-stop mutant compared to 1:910 in the wild type and approximately the same value in the Y69-stop mutant (Figure 7). However, the R50- and Y69-stop mutants have K_D values (ca. 25 nM) for DCMU binding that are very similar to the wild type. These results indicate that truncation of 31 residues from the COOH-terminal domain of the α-subunit caused a decrease of approximately 80–90% in the PSII complexes to which [^{14}C]DCMU can bind but it did not affect the K_D value for DCMU of the remaining functional fraction. These results imply that the R50-stop truncation mutant can assemble intact and functional PSII reaction centers, although the concentration of these centers in the membrane is much smaller than in the wild type. It should be noted that *Synechocystis* 6803 also assembles a similar small fraction of PSII centers in the absence of the *psb*C gene product ("CP43" polypeptide) (Rögner et al., 1991).

An alternative explanation of the 10–20% residual active and functional PSII centers could be a low-level genetic sup-

pression allowing synthesis of 10–20% intact *psb*E cyt b_{559} gene product. No such polypeptide could be seen in the immunoblot of the R50 mutant, but the strength of the antibody reaction with the wild type did not preclude the presence of the cytochrome at the 15% level in lane 2 (data not shown). This alternative seems less likely because the presence of the wild-type cytochrome would convey a large selective advantage (~6-fold greater photoautotrophic growth rate, Table IA) and the phenotype of the R50 mutant would tend to be unstable. However, it has been stable at a level of 10–20% of wild-type activity for more than 1 year.

Incomplete Assembly Except for Cytochrome b_{559}. Evidence for incomplete assembly of PSII came from immunochemical assay of the D2 polypeptide, determination of DCMU binding affinity that showed loss of 80–85% of the Q_B binding sites, and the increased F_0 level of fluorescence, implying the absence of the quencher, Q_A, from approximately 80% of the centers. The finding that the cytochrome b_{559} heme content of the R50 mutant (1 heme:640 Chl) was similar to that of the wild type (1 heme:510 Chl) was not necessarily expected because of several examples of pleiotropic loss of PSII core polypeptides: thylakoids from a *C. reinhardtii* mutant lacking the gene encoding the D1 polypeptide (Bennoun et al., 1986), as well as mutants from *Chlamydomonas reinhardtii* or *Synechocystis* sp. PCC 6803 impaired in the *psb*D gene encoding the D2 polypeptide (Erickson et al., 1986; Vermaas et al., 1988), are depleted in the CP-47 and CP-43 polypeptides, as well as D2 or D1, respectively. However, there is substantial precedent for cytochrome b_{559} remaining at levels close to the wild type after genetic inactivation of the D1 protein and concomitant loss of D2 and CP-47 (Nilsson et al., 1990), and for cytochrome b_{559} showing a "core" or "anchor" property in the reaction center by its appearance first among the reaction center polypeptides during PSII biogenesis (Westhoff et al., 1990). An excess of the b_{559} α-subunit relative to D1 and D2 has been noted for the PSII components in bundle sheath chloroplasts that have partially assembled PSII units (Oswald et al., 1990).

Cytochrome b_{559} Pool. Cytochrome b_{559} is present to a much larger extent (1 heme:640 Chl or 2 hemes:1280 Chl, depending on the number of cyt b_{559} hemes per reaction center) in the R50-stop mutant compared to the Q_B content (1:5480 Chl), as determined by DCMU binding. The large excess of cyt b_{559} indicates a nascent pool of this protein that is potentially available for assembly. Much of the low-potential cytochrome b_{559} that is present in spinach chloroplasts in excess of the high-potential component also appears to constitute such a pool (unpublished data).

Cyt b_{559} Lumenal Segment—Search for Critical Residues. The conclusion that the extrinsic lumenal segment of cytochrome b_{559} is critical for assembly of the PSII complex is consistent with a role in the assembly process for electrostatic interactions between the peripheral segments of the proteins of the complex, as has been inferred for the large polypeptides of the cytochrome b_6f complex (Szczepaniak et al., 1991). Because R59 and R68 are present in the Y69-stop mutant that has high activity and are deleted in the R59-stop mutant with much lower activity, it was thought that these two charged residues might have an important role in electrostatic interactions critical for assembly. However, mutagenesis of one or both of these residues had no significant effect on activity.

Conclusions. The conclusions that follow from these data are the following: (i) removal of 12 residues from the COOH-terminal domain of the cytochrome b_{559} α-subunit has relatively little effect on function and assembly; (ii) removal of 31 residues decreases the net probability or efficiency of correct assembly of the PSII complex by a factor of 6, either by decreasing the rate of assembly or by increasing the rate of degradation and turnover. PSII is assembled correctly at a low level of about 15% in the R50-stop mutant. The possibility that this residual activity can be ascribed to a low level of genetic suppression seems less likely, but cannot be excluded. The existence of the 15% level of assembly and function in the R50 mutant would imply that 31 residues of the COOH-terminus of the cytochrome b_{559} α-subunit are not crucial for functions related to O_2 evolution, such as the binding of essential metal ions (e.g., Mn) of the water-splitting enzyme complex. (iii) A role for cytochrome b_{559} as a "core" or "anchoring" subunit of the PSII reaction center is suggested by its presence at relatively high levels when other reaction center polypeptides are missing.

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Registry No. cyt b_{559}, 9044-61-5.

References

Bebenek, K., & Kunkel, T. A. (1989) *Nucleic Acids Res. 17*, 5408.

Bennoun, P., Spierer-Herz, M., Erickson, J., Girard-Bascou, J., Pierre, Y., Delosme, M., & Rochaix, J.-D. (1986) *Plant Mol. Biol. 6*, 151–160.

Bricker, T. M., Pakrasi, H. B., & Sherman, L. A. (1985) *Arch. Biochem. Biophys. 237*, 170–176.

Chapman, D. J., Gounaris, K., & Barber, J. (1988) *Biochim. Biophys. Acta 933*, 423–431.

Cramer, W. A., & Butler, W. L. (1969) *Biochim. Biophys. Acta 172*, 503–510.

Cramer, W. A., & Whitmarsh, J. (1977) *Annu. Rev. Plant Physiol. 28*, 133–172.

Cramer, W. A., Theg, S. M., & Widger, W. R. (1986) *Photosynth. Res. 10*, 393–403.

Debus, B. A., Barry, B. A., Sithole, I., Babcock, G. T., & McIntosh, L. (1988) *Biochemistry 27*, 9071–9074.

Deisenhofer, J., & Michel, H. (1989) *Science 245*, 1463–1473.

Elmorjani, K., Thomas, J.-C., & Sebban, P. (1986) *Arch. Microbiol. 146*, 186-191.

Erickson, J. M., Rahire, M., Malnoe, P., Girard-Bascou, J., Pierre, Y., Bennoun, P., & Rochaix, J.-D. (1986) *EMBO J. 5*, 1745–1754.

Feher, G., Allen, J. P., Okamura, M. Y., & Rees, D. C. (1989) *Nature 339*, 111–116.

Furbacher, P. N., Girvin, M. E., & Cramer, W. A. (1989) *Biochemistry 28*, 8990–8998.

Goedheer, J. C. (1972) *Annu. Rev. Plant Physiol. 23*, 87–112.

Herrmann, R. G., Alt, J., Schiller, B., Widger, W. A., & Cramer, W. A. (1984) *FEBS Lett. 176*, 239–244.

Ho, K. K., & Krogmann, D. W. (1980) *J. Biol. Chem. 255*, 3855–3861.

Jansson, C., Debus, R. J., Osiewacz, H. D., Gurevitz, M., & McIntosh, L. (1987) *Plant Physiol. 85*, 1021–1025.

Lichtenthaler, H. K. (1987) *Methods Enzymol. 148*, 350–382.

Metz, J. G., Nixon, P. J., Rögner, M., Brudvig, G. W., & Diner, B. A. (1989) *Biochemistry 28*, 6960–6969.

Nanba, O., & Satoh, K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 109-112.
Nilsson, F., Andersson, B., & Jansson, C. (1990) *Plant Mol. Biol. 14*, 1051-1054.
Noren, G. H., Boerner, R. J., & Barry, B. (1991) *Biochemistry 30*, 3943-3950.
Oswald, A., Streubel, M., Ljungberg, U., Hermans, J., Eskins, K., & Westhoff, P. (1990) *Eur. J. Biochem. 190*, 185-194.
Pakrasi, H. B., Williams, J. G. K., & Arntzen, C. J. (1988) *EMBO J. 7*, 325-332.
Pakrasi, H. B., De Ciechi, P., & Whitmarsh, J. (1991) *EMBO J. 10*, 1619-1627.
Reddy, K. J., Webb, R., & Sherman, L. A. (1990) *Biotechniques 8*, 250-251.
Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979) *J. Gen. Microbiol. 30*, 5387-5395.
Rögner, M., Chisholm, D. A., & Diner, B. A. (1991) *Biochemistry 30*, 5387-5395.
Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R. G., Horn, T. T., Mullis, K. B., & Erlich, H. A. (1985) *Science 230*, 1350-1354.
Satoh, K., Nakatani, H. Y., Steinback, K. E., Watson, J., & Arntzen, C. J. (1983) *Biochim. Biophys. Acta 724*, 142-150.
Studier, F. W., & Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113-130.
Szczepaniak, A., & Cramer, W. A. (1991) *EMBO J. 10*, 2757-2764.
Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074-1078.
Tabor, S., & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4767-4771.
Tae, G.-S. (1991) Ph.D. Thesis, Purdue University.
Tae, G.-S., & Cramer, W. A. (1989) *FEBS Lett. 259*, 161-164.
Tae, G.-S., Black, M. T., Cramer, W. A., Vallon, O., & Bogorad, L. (1988) *Biochemistry 27*, 9075-9080.
Vallon, O., Tae, G.-S., Cramer, W. A., Simpson, D., Hoyer-Hansen, G., & Bogorad, L. (1989) *Biochim. Biophys. Acta 975*, 132-141.
Vermaas, W. F. J., Ikeuchi, M., & Inoue, Y. (1988) *Photosynth. Res. 17*, 97-113.
Vermaas, W. F. J., Charite, J., & Shen, G. (1990) *Biochemistry 29*, 5325-5332.
Westhoff, P., Shrubar, H., Oswald, A., Streubel, M., & Offerman, K. (1990) *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. III, pp 483-490, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Widger, W. R., Cramer, W. A., Hermodson, M., Meyer, D., & Gullifor, M. (1984) *J. Biol. Chem. 259*, 3870-3876.
Williams, J. G. K. (1988) *Methods Enzymol. 167*, 766-778.

Inactivation of Calcium Uptake by EGTA Is Due to an Irreversible Thermotropic Conformational Change in the Calcium Binding Domain of the Ca^{2+}-ATPase†

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ABSTRACT: Calcium uptake by rabbit skeletal sarcoplasmic reticulum (SR) is inhibited with an effective inactivation temperature (T_I) of 37 °C in EGTA with no effect on ATPase activity. Since the Ca-ATPase denatures at a much higher temperature (49 °C) in EGTA, this suggests that a small or localized conformational change of the Ca-ATPase at 37 °C results in inability to accumulate calcium by the SR. Using a fluorescent analogue of dicyclohexylcarbodiimide, *N*-cyclohexyl-*N'*-[4-(dimethylamino)-α-naphthyl]-carbodiimide (NCD-4), the region of the calcium binding sites of the SR Ca-ATPase was labeled. Steady-state and frequency-resolved fluorescence measurements were subsequently performed on the NCD-4-labeled Ca-ATPase. Site-specific information pertaining to the hydrophobicity and segmental flexibility of the region of the calcium binding sites was derived from the steady-state fluorescence intensity, lifetime, and rotational rate of the covalently bound NCD-4 label as a function of temperature (0-50 °C). A reversible transition at ~15 °C and an irreversible transition at ~35 °C were deduced from the measured fluorescence parameters. The low-temperature transition agrees with the previously observed break in the Arrhenius plot of ATPase activity of the native Ca-ATPase at 15-20 °C. The high-temperature transition conforms well with the conformational transition, resulting in uncoupling of Ca translocation from ATP hydrolysis as predicted from the irreversible inactivation of Ca uptake at 31-37 °C in 1 mM EGTA. We conclude that an irreversible conformational change in the region of the calcium binding sites, of considerable lesser magnitude than unfolding, is responsible for the thermal inactivation of Ca uptake and uncoupling of Ca transport from ATP hydrolysis at elevated temperatures in EGTA.

The Ca transport function of the purified membrane-bound calcium adenosinetriphosphatase (Ca-ATPase) from muscle sarcoplasmic reticulum (SR) is quite sensitive to temperature with a significant inactivation of ATP-dependent Ca uptake of the Ca-ATPase at temperatures in excess of 30 °C when heated in the absence of calcium and in the presence of EGTA (McIntosh & Berman, 1978; Lepock et al., 1990). An inactivation temperature (T_I) of 37 °C is found for Ca uptake in 1 mM EGTA. Here T_I is defined as the temperature of

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