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Site-Directed Mutagenesis of the *psb*C Gene of Photosystem II: Isolation and Functional Characterization of CP43-less Photosystem II Core Complexes†

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ABSTRACT: Two mutants of *Synechocystis* PCC 6803 lacking the *psb*C gene product CP43 were constructed by site-directed mutagenesis. Analysis of cells and thylakoid membranes of these mutants indicates that PS II reaction centers accumulate to a concentration of about 10% of that of WT cells. PS II core complexes isolated from mutants lacking the CP43 subunit show light-driven electron transfer from the secondary electron donor Z to the primary quinone electron acceptor Q_A with a quantum yield similar to that of wild type, indicating that CP43 is not required for binding or function of Q_A. The use of mutants for the removal of CP43 thus avoids the loss of Q_A function associated with biochemical extraction of CP43 from intact core complexes. Both absorbance and fluorescence emission maxima of the mutant complexes show a blue shift in comparison to the WT PS II core complex, indicating that the absorbance spectrum of CP43 is red-shifted relative to that of the remainder of the core complex. The antenna size of these CP43-less complexes is about 70% of that of WT, indicating that approximately 15 chlorophyll molecules are bound by CP43. The molecular mass of the PS II complex, including the detergent shell, shifts from 310 ± 15 kDa in WT to 285 ± 15 kDa in the CP43-less mutants.

The photosystem II reaction center is the site of one of the two primary photoreactions of the oxygenic electron-transport chain of photosynthesis [see Hansson and Wydrzynski (1990) for a recent review]. The redox components responsible for the primary charge separation are bound by two polypeptides, D1[1] and D2 (Nanba & Satoh, 1987). These form part of a larger ensemble, the oxygen-evolving core complex which may be isolated through detergent extraction techniques [see, for example, Ghanotakis et al. (1987)]. This complex retains the ability to evolve oxygen in the presence of artificial quinone electron acceptors and is composed of some 20 polypeptides (Ikeuchi et al., 1990; Koike et al., 1990).

The two major chlorophyll–protein complexes of the PS II core complex are CP47 and CP43 (Delepelaire & Chua, 1979; Camm & Green, 1980; Bricker, 1990), the apoproteins of which are ~56 and 50 kDa, respectively, as deduced from the known nucleotide sequences of the corresponding genes, *psb*B and *psb*C. Both are thought to contain six transmembrane α-helices and homologous histidine residues near the stromal

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[1] Abbreviations: β-DM, dodecyl β-D-maltoside; bp, basepair(s); Chl, chlorophyll; CP43, one of two major chlorophyll–protein complexes of the PS II core complex, and the *psb*C gene product; CP47, one of two major chlorophyll–protein complexes of the PS II core complex and the *psb*B gene product; cyt *b*559, cytochrome *b*-559; D1, one of two polypeptides that coordinate the primary photoreactants of PS II and the *psb*A gene product; D2, one of two polypeptides that coordinate the primary photoreactants of PS II and the *psb*D gene product; DCMU, Diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, dichlorophenolindophenol; kb, kilobase(s); kDa, kilodalton(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; NH_2OH, hydroxylamine; P680, primary electron donor; PCC, Pasteur culture collection; PCR, polymerase chain reaction; PQ, plastoquinone; PQ^-, plastosemiquinone anion; PS I, photosystem I; PS II, photosystem II; Q_A, primary quinone electron acceptor; Q_B, secondary quinone electron acceptor; RC, reaction center; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WT, wild type; Z, secondary electron donor identified as tyrosine-161 in polypeptide D1.

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and lumenal surfaces of these proteins (Vermaas et al., 1987; Bricker, 1990). These are likely sites for chlorophyll binding.

CP43, the subject of this paper, shows a high degree of homology on comparing amino acid sequences deduced from a comparison of cyanobacterial and plant *psb*C genes. Sixty-seven percent of the residues are identical, and the homology rises to 80% if conservative replacement of amino acids is allowed (Bricker, 1990). Its major role is thought to be one of light collection for the associated reaction center. However, other roles have been suggested through the characterization of core complexes from which CP43 has been extracted and through the study of mutants impaired in the synthesis of CP43.

All of the biochemical extraction techniques that remove CP43 have been reported to impair photoreduction of the primary quinone electron acceptor, Q_A (Yamagishi & Katoh, 1985; Akabori et al., 1988; Yamaguchi et al., 1988; Petersen et al., 1990). These observations have led to the suggestion that CP43 might play a role in the stabilization of Q_A binding (Petersen et al. 1990).

A considerable number of mutants have also been isolated that affect the accumulation of CP43 (Rochaix et al., 1989; Dzelzkalns & Bogorad 1989; Carpenter et al., 1990). These have all shown that the inability to accumulate CP43 results in reduced levels of CP47, D1, and D2. These observations indicate a role for CP43 in the stabilization of the other large subunits of the PS II core complex. Contrary to those mutants that are unable to express CP47, D1, or D2 and which as a consequence show no evidence of reaction center formation (Jensen et al., 1986; Vermaas et al., 1988a; de Vitry et al., 1989; Yu & Vermaas, 1990; Nixon et al., 1991), there is some evidence for the formation of trace concentrations of photoreactive reaction centers in CP43-less mutants (Vermaas et al., 1988a; de Vitry et al., 1989). It is the isolation and biophysical characterization of PS II core complexes from two such mutants of *Synechocystis* 6803 that form the subject of this paper. We show below that the primary and secondary electron-transfer reactions, including the photoreduction of Q_A, remain intact despite the absence of CP43. As the quantum yield of primary charge separation is unchanged with respect to the intact complex, we are able to establish that CP43 accounts for 30% of the light-harvesting capacity of the core complex.

Materials and Methods

Cyanobacterial Strains and Construction of Mutant Strains. All genetic manipulations were performed on a glucose-tolerant (Williams, 1988) and phycocyanin-deficient "olive" strain of *Synechocystis* PCC 6803 (Rögner et al., 1990). The cloning and manipulation of *Synechocystis* PCC 6803 *psb*D and *psb*C genes have been previously described (Williams & Chisholm, 1987; Chisholm & Williams, 1988). Genetic transformation of *Synechocystis* PCC 6803 was as described in Williams (1988).

Southern Hybridizations and Nucleotide Sequencing. Southern blotting and hybridization were performed by using a GeneScreen membrane (NEN–DuPont) following manufacturer's recommendations. Direct sequencing of the cyanobacterial genome was done by amplifying a 500 bp region using the polymerase chain reaction. The amplified DNA was purified by electrophoresis through low-melting agarose, followed by phenol extraction of the agarose and final concentration on a Centricon 100 spin filter (Amicon, Danvers, MA). Sequencing was carried out by using standard Sequenase (United States Biochemicals, Cleveland, OH) reaction conditions.

Preparation of Thylakoid Membranes and PS II Particles. Cells of the mutant strains were grown photoheterotrophically for 4–6 days at 30 °C in 18-L carboys in BG 11 medium in the presence of 5 mM glucose (Williams, 1988). Membranes were prepared according to Rögner et al. (1990). The washed membranes were stirred in the dark at 4 °C with 2 M NaBr for 30 min to release the ATPase, diluted with buffer A [20 mM MES, pH 6.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 M mannitol, and 0.03% β-DM (Calbiochem)], pelleted by centrifugation (125000*g* at r_{av}, for 30 min, Beckman 45 Ti rotor), and resuspended in buffer B (buffer A + 20% glycerol) to give a final chlorophyll concentration of 1 mg/mL. Extraction of the membranes, sucrose density gradient centrifugation, and separation of PS I and PS II by HPLC (anion-exchange and hydroxyapatite column) were done as in Rögner et al. (1990).

After purification on the hydroxyapatite column, the PS II complexes were immediately desalted by passage through gel filtration columns (Econo-pak 10 DG, Bio-Rad) equilibrated with buffer A, frozen in liquid nitrogen, and stored at −80 °C.

SDS–PAGE and Immunoblotting. Electrophoresis and immunoblotting were carried out as in Rögner et al. (1990). Antiserum specific for the D2 polypeptide was obtained by immunization against a synthetic peptide corresponding to the last 12 amino acids of the carboxy terminus of D2 (Nixon et al., 1990). Antisera specific for the apoproteins of either CP47 or CP43 of *Chlamydomonas reinhardtii* were kindly made available by N.-H. Chua (Chua et al., 1979).

Spectroscopic Methods. Absorbance spectra were recorded on a Perkin Elmer Lambda 9 spectrophotometer. The chlorophyll concentration was determined by using an extinction coefficient of 74000 M^{-1} cm^{-1} at 673 nm for PS II core complexes.

Light-induced absorbance changes were measured with a flash detection spectrophotometer similar to that originally described by Joliot et al. (1980). The concentration of photoreducible Q_A was determined by pretreating PS II core complexes in the dark with 10 μM ferricyanide in 20 mM HEPES, pH 7.5, to assure complete oxidation of Q_A. Q_A was then fully photoreduced by giving a series of 15 saturating flashes (18 Hz) 1 min after the addition of 2 mM NH_2OH. The reduced-minus-oxidized difference spectrum allowed quantitation of Q_A using an extinction coefficient of 12500 M^{-1} cm^{-1} at 320 nm for plastosemiquinone anion–plastoquinone (van Gorkom, 1974).

Flash saturation curves for photoreduction of Q_A were performed on PS II core complexes at 325 nm. Flashes (600-ns duration) provided by a pulsed dye laser (SLL 250 Candela) containing Rhodamine 6G (λ_{max} = 584 nm) were attenuated with neutral density filters.

Fluorescence emission spectra were recorded with a SLM 8000C spectrofluorometer.

Oxygen Measurements. Oxygen evolution was measured at 25 °C in a cuvette fitted with a Clark-type O_2 electrode (Hansatech Instruments Ltd.). Saturating white light was filtered through a heat-reflecting filter. Cells at a concentration of 2–5 μM Chl were taken from liquid culture and assayed in the presence of the electron acceptors 1 mM ferricyanide and 0.3 mM 2,6-dichloro-*p*-benzoquinone.

Results

Characterization of Mutant Cells Lacking the psbC Gene Product. Two mutant strains were constructed in which the *psb*C gene product, CP43, was not expressed. Mutant Tol 1357 has a 4.2-kb deletion that removes the *psb*D1–*psb*C gene cluster along with some flanking sequence, without disturbing

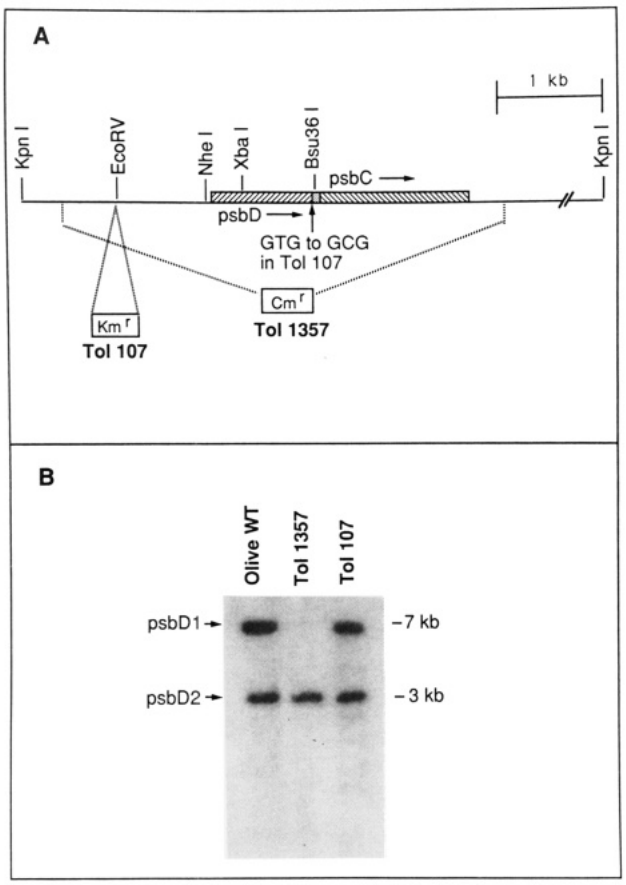

FIGURE 1: (A) Composite map indicating mutations within the *psb*D1 and *psb*C genes and relevant flanking regions within the cyanobacterial chromosome. Insertions or deletions in specific mutants are indicated by dotted lines. Tol 107: insertion of kanamycin resistance gene at *Eco*RV site about 800 bp upstream of *psb*D1 translation start site and replacement of GTG codon with GCG at *psb*C start site. Tol 1357: deletion of region extending approximately 1400 bp upstream of *psb*D1 start and 304 bases downstream of *psb*C stop codon, with insertion of chloramphenicol resistance gene. (B) Southern autoradiogram of *NheI–KpnI* digests of olive *Synechocystis* PCC 6803 wild-type and mutant genomic DNA probed with the *Xba*I–*Bsu*36I fragment of *psb*D1.

the *psb*D2 gene. The deletion was marked with a chloramphenicol resistance gene derived from pACYC184 (Chang & Cohen, 1978). We also constructed mutant Tol 107, which contains a single base change altering the *psb*C start codon from GTG to GCG, with the intention of disabling the translation of the *psb*C transcript while retaining two functional *psb*D genes. This mutant contains a 1.2-kb kanamycin resistance gene (Taylor & Rose, 1988) inserted upstream of *psb*D1. Figure 1A shows the restriction map of the *psb*D1-C gene cluster for wild-type *Synechocystis* 6803 and the two mutants Tol 107 and Tol 1357. Figure 1B shows the corresponding Southern blot of DNA isolated from these mutants and from wild type. The wild-type DNA shows two bands of hybridization, one to the overlapping *psb*D1 and *psb*C genes and one to the *psb*D2 gene. Mutant Tol 1357 shows only one band, due to the deletion of the *psb*D1-C operon. Mutant Tol 107 has a single base substitution in the *psb*C start codon and consequently shows the same pattern of hybridization as wild type. Direct genomic sequencing, following PCR amplification (results not shown), confirmed that the putative GTG start codon had been changed to GCG in Tol 107.

In order to investigate which reaction center polypeptides were still present in Tol 107, thylakoid membranes were probed with antibodies specific for the D2 protein (Figure 2, lanes 1 and 2), the CP43 apoprotein (lanes 3 and 4), and the CP47

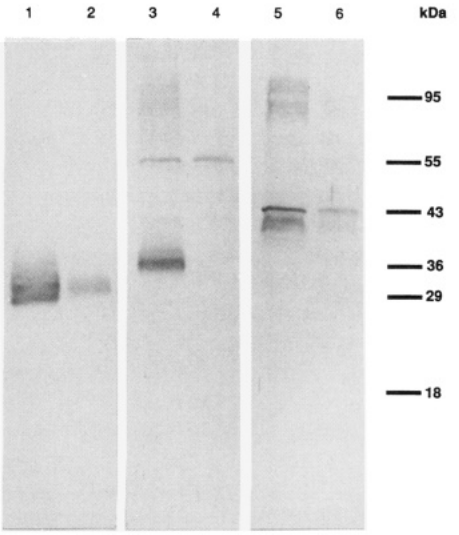

FIGURE 2: Immunoblot of thylakoid membranes from WT cells (lanes 1, 3, and 5) and mutant Tol 107 cells (lanes 2, 4, and 6); membranes were solubilized with 3% SDS at room temperature for about 1 h prior to application of 8 μg of chlorophyll per lane on an SDS/urea–PAGE (see Materials and Methods). Lanes 1 and 2 were probed with antibodies specific for the *psb*D gene product (D2 polypeptide), lanes 3 and 4 for the *psb*C gene product (CP43 apoprotein), and lanes 5 and 6 for the *psb*B gene product (CP47 apoprotein).

apoprotein (lanes 5 and 6). The WT membranes (lanes 1, 3, and 5) contain all three proteins, while the membranes of Tol 107 do not contain the CP43 apoprotein (lane 4), as expected from the genetic construct. As an equivalent amount of chlorophyll from both WT and Tol 107 mutant was loaded in each lane, this immunoblot indicates a much lower content of D2 and CP47 apoprotein in the mutant membranes in comparison to WT. Similar blots of the Tol 1357 mutant (see below) also confirmed the absence of the *psb*C gene product in this mutant.

Neither mutant was able to grow photoautotrophically or to show detectable oxygen evolution.

Isolation of PS II(–CP43) Core Complexes. In order to see if a core complex of PS II was still assembled in thylakoid membranes in the absence of CP43, detergent extracts of thylakoids from both mutants were applied to an anion-exchange column. Figure 3 shows, for comparison, the elution profile of an extract from WT thylakoids (a) and from thylakoids of mutant Tol 1357 (b). As previously shown (Rögner et al., 1990), the first peak contains free pigments, the second and third contain PS I core complexes, and the last main peak contains PS I core aggregates. The major difference between the Tol 1357 and WT extracts is the amplitude of peak 4, which has been previously shown in WT cells to consist of the PS II core complex (Rögner et al., 1990). On the basis of the amount of protein-bound chlorophyll extracted from the membranes, the mutant cells contain only about 10% of the PS II core complex of WT cells. The ratio of PSII core complex to total chlorophyll is 10 times smaller that that of WT. An equivalent amount of PS II core complex was found for mutant Tol 107. This result is corroborated by fluorescence induction measurements of whole cells in the presence of DCMU (not shown) which indicate about the same concentration of PS II reaction center complex per cell (~10% of WT). Within a factor of 2, there appears to be no difference in the amount of PS II core complex in a mutant carrying only one (Tol 1357) or both copies (Tol 107) of the *psb*D gene.

After enrichment of the PS II(–CP43) core complex by sucrose density gradient centrifugation and preparative anion-exchange HPLC (Rögner et al., 1990), residual PS I was

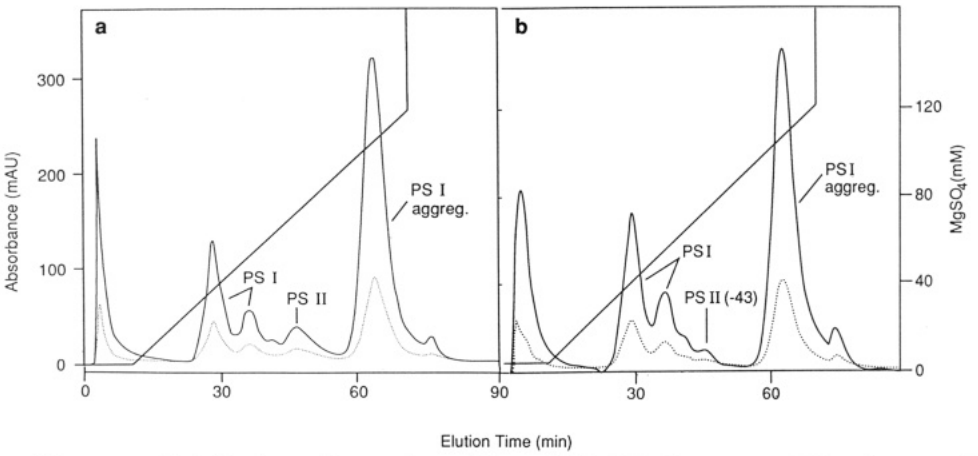

FIGURE 3: Elution profile on an analytical anion-exchange column (Mono Q HR 5/5) of an extract of *Synechocystis* olive WT (a) and Tol 1357 mutant (b) before sucrose density gradient centrifugation. Elution involved a linear $MgSO_4$ gradient at a flow rate of 0.4 mL/min. Absorbance in milliabsorbance units (mAU) was recorded at 280 nm (dotted line) and 435 nm (solid line).

removed by a second HPLC step on hydroxyapatite according to the method previously described by Rögner (1990). Upon application of a linear P_i gradient, an unpigmented protein, sometimes mixed with free pigment, was eluted after 30–40 min, followed by the PS II CP43-less core complex at about 55 min, and the PS I core complex (at ~80–90 min). As the separation of PS I core complex from the PS II(–CP43) complex is even greater than from WT PS II core complex under the same chromatographic conditions (Rögner, 1990), this second HPLC step allows the isolation of PS II(–CP43) core complexes completely free of cytochrome b_6/f complex and PSI core complex (see below and Figure 5B). These purification steps are sufficient to allow us to isolate a complex suitable for spectroscopic studies free of pigmented contaminants and of contaminating light-induced absorbance changes. They are not sufficient, given the extremely low levels of PS II reaction center in the CP43-less mutants, to purify the core complex to homogeneity.

Characterization of the Purified PS II(–CP43) Core Complexes. (*a*) *Immunoblotting.* Figure 4 shows immunoblots of hydroxyapatite–HPLC-purified PS II(–CP43) core complex from Tol 1357 (lanes 1 and 4), of PS II core complexes from olive WT (lanes 2 and 5), and of membranes from Tol 1357 (lanes 3 and 6). Lanes 1–3 have been probed with antibodies against the CP43 apoprotein and lanes 4–6 with antibodies against the CP47 apoprotein. CP43 could be detected in neither PS II particles nor membranes isolated from the Tol 1357 mutant. Antibody binding to components of higher molecular mass (>50 kDa) has previously been observed with PS II preparations from *Synechocystis* (Rögner et al., 1990) and assumed to be due to subunit aggregation.

(*b*) *Absorbance and Fluorescence Spectra.* Figure 5A shows the absorbance spectrum of the PS II(–CP43) hydroxyapatite-purified core complex isolated from Tol 107 and, for comparison, a similarly purified PS II core complex of WT. The mutant core complex shows an absorbance maximum at about 672.5 nm, a blue shift of 0.5–1 nm relative to the WT peak. In addition, the Soret band peak at 416 nm due to pheophytin is markedly increased in the mutant PS II, indicating a higher pheophytin to chlorophyll ratio than in the WT PS II. This is reflected, too, in a slightly increased peak at 540 nm also due to pheophytin. Similar absorbance spectra have also been obtained from a PS II core complex isolated from the Tol 1357 mutant.

The small blue shift seen in the absorbance spectrum of PS II(–CP43) core complex from Tol 107 relative to WT PS II is more pronounced in the fluorescence emission spectra of both

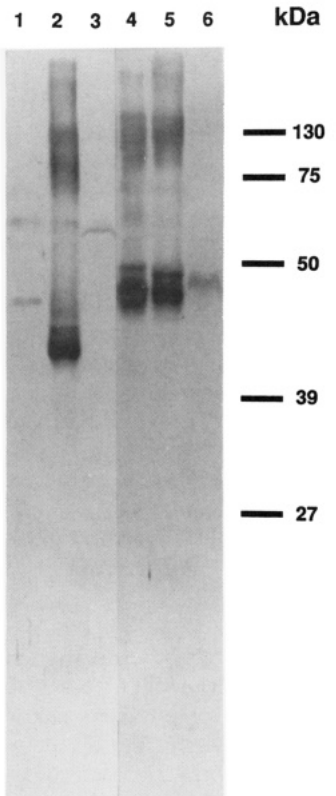

FIGURE 4: Immunoblot of hydroxyapatite–HPLC PS II(–CP43) complex from mutant Tol 1357 (lanes 1 and 4), PS II core complex from olive WT (lanes 2 and 5), and thylakoid membranes from Tol 1357 (lanes 3 and 6). Lanes 1–3 were probed with antibodies specific for the apoprotein of CP43 and lanes 4–6 with antibodies specific for the apoprotein of CP47.

core complexes recorded at 77 K. Figure 5B shows, upon excitation at 430 nm, the emission peak of PS II(–CP43) core complex at 685 nm, whereas WT PS II peaks at 689 nm. It should be noted that the peaks of all isolated particles are blue-shifted by about 5 nm relative to those measured in whole cells (data not shown). This shift is observed for PS II, PS II(–CP43), and PS I core complexes and may be a consequence of detergent solubilization. The peak of 720 nm is due to some minor contamination of PS I in the WT preparation; no such contamination is present in the PS II(–CP43) complexes.

(*c*) *Absorbance Difference Spectra.* Flash-induced difference spectra were recorded for the isolated PS II(–CP43) core

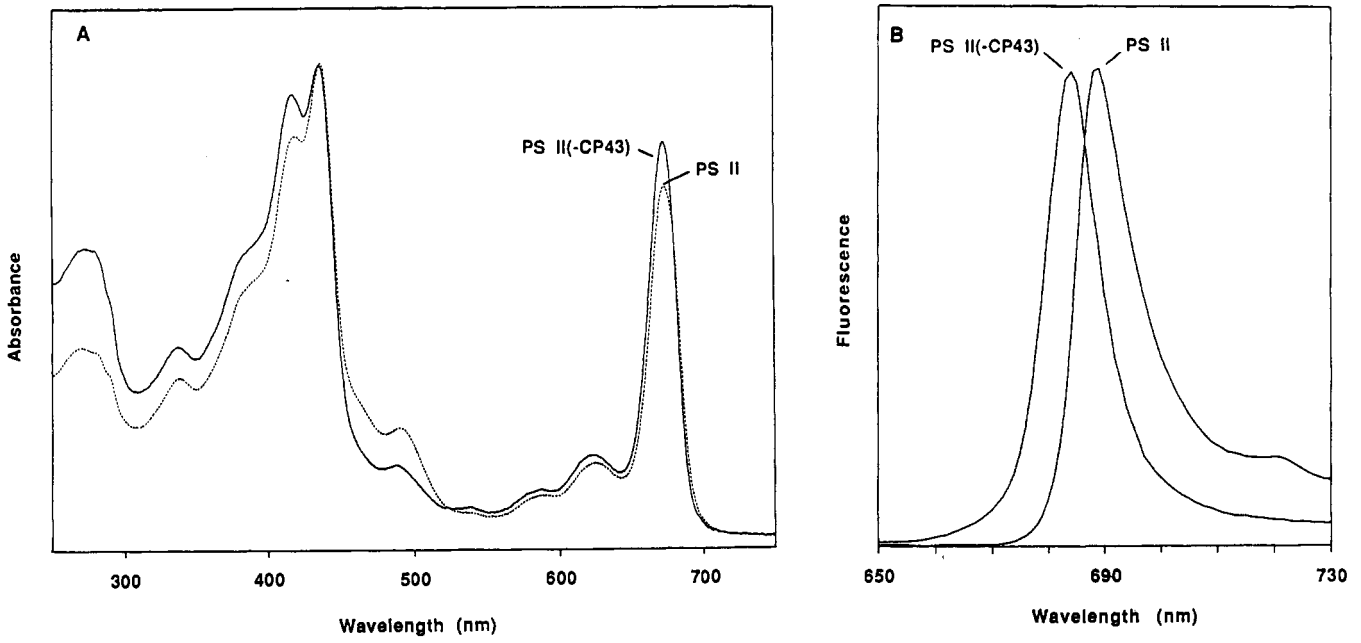

FIGURE 5: (A) Absorbance spectrum of PS II core complex (dotted line) isolated from hydroxyapatite-purified olive WT and PS II(–CP43) core complex (solid line) isolated from strain Tol 107. The spectra were normalized at their maximum absorption and are plotted in relative absorbance units. (B) Fluorescence emission spectra at 77 K of these same complexes at an excitation wavelength of 430 nm. These spectra were normalized at their emission maxima and are plotted in relative units.

complexes in order to investigate the presence of a functional secondary donor, Z, tyrosine-161 of the D1 polypeptide (Debus et al., 1988a,b; Metz et al., 1989), and acceptor, Q_A, a plastoquinone (van Gorkom, 1974). Figure 6 shows the two components which contribute to the flash-induced difference spectra measured at 0.5 ms after a saturating actinic flash for the core complex isolated from mutant Tol 107. The solid circles represent the spectrum of $Q_A^- - Q_A$ in $\Delta I/I$ measured at 270 ms after the actinic flash and multiplied by a factor of 1.64 to give the amplitude of this spectrum at 0.5 ms. This multiplicative factor was determined by measuring the decay of $\Delta I/I$ from 0.5 to 270 ms (a loss of 39%) at 325 nm. This wavelength is a peak in the $Q_A^- - Q_A$ difference spectrum and isosbestic for $Z^+ - Z$ and $Chl^+ - Chl$. At 270 ms after the actinic flash, the oxidized donor has been completely reduced by 20 μM benzidine ($t_{1/2}$ = 5 ms), leaving only the reduced acceptor. Absorbance changes arising from benzidine oxidation and ferricyanide reduction are negligible in this spectrum (Diner & de Vitry, 1984). This spectrum (open circles) is identical with the difference spectrum for $Q_A^- - Q_A$ which we reported earlier for WT core complexes of *Synechocystis* (Metz et al., 1989) and with that reported by Schatz and van Gorkom (1985) for PS II particles from *Synechococcus*. The conservation, in the CP43-less core complex (Figure 6), of the WT difference spectrum of the reaction center pigment electrochromic shifts in the blue region of the spectrum and their ratio to the UV absorbance changes arising from PQ^- – PQ (van Gorkom, 1974) indicates that we are indeed observing reduction of PQ in the Q_A binding site ($Q_A^- - Q_A$) and not the reduction of plastoquinone at any other site. There is no functional Q_B in core complexes isolated either from WT or from the CP43-less mutants.

The spectrum of $Q_A^- - Q_A$ at 0.5 ms after the actinic flash was subtracted from the total $\Delta I/I$ measured at 0.5 ms (not shown) to yield the spectrum with open circles in Figure 6. This spectrum is identical with that of $Z^+ - Z$ originally observed in *Chlamydomonas* (Diner & de Vitry, 1984) and in spinach (Dekker et al., 1984) and Metz et al. (1989) in *Synechocystis* 6803. Similar difference spectra were also obtained with PS II core complexes from the Tol 1357 mutant,

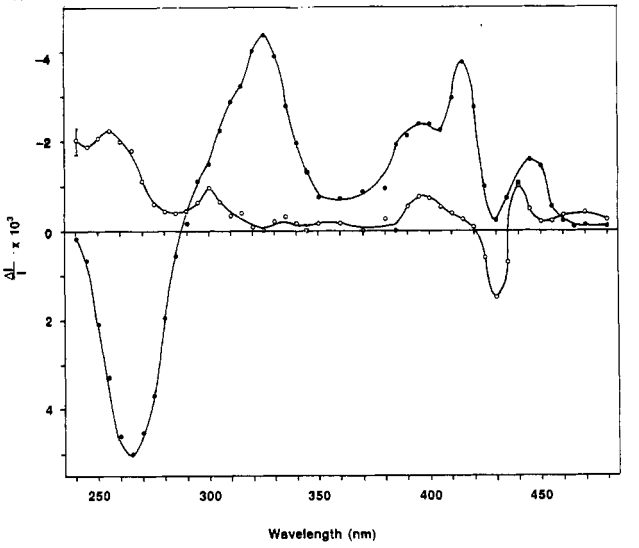

FIGURE 6: Saturating flash-induced absorbance changes measured after 0.5 ms from 240 to 480 nm in PS II(–CP43) complexes isolated from mutant Tol 107. The $\Delta I/I$ of $Q_A^- - Q_A$ was measured at 270 ms after saturating laser flash excitation of samples (5 μM Chl) in the presence of 20 μM benzidine, 10 μM ferricyanide and 20 mM HEPES, pH 7.5. Shown is the spectrum multiplied by a factor of 1.64 to give the amplitude at 0.5 ms after the actinic flash (●). This $\Delta I/I$ $Q_A^- - Q_A$ spectrum at 0.5 ms was subtracted from the total $\Delta I/I$ spectrum at 0.5 ms (not shown) to yield the spectrum of $Z^+ - Z$ at 0.5 ms (○).

indicating that the isolated PS II(–CP43) core complexes from both mutants contain both secondary donor, Z, and acceptor, Q_A, and that these particles are capable of stabilized charge separation. That this spectrum arises from $Z^+ - Z$ (D1-Tyr161; Debus et al., 1988a,b; Metz et al., 1989) and not from $D^+ - D$ (D2-Tyr160; Debus et al., 1988a,b; Vermaas et al., 1988a,b), which would be expected to have a similar difference spectrum, is supported by the arguments below:

We have shown in PS II core complexes from WT that D^+ is stable and that Z undergoes reversible oxidation and reduction in the light (Metz et al., 1989). The signal we attribute here to $Z^+ - Z$ is formed reversibly upon flash excitation, as

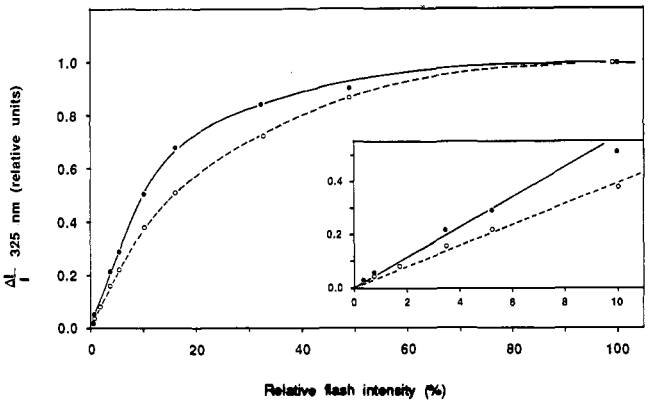

FIGURE 7: Flash saturation curve of PS II core complexes isolated from olive WT (closed circles) and PS II(−CP43) complexes from mutant Tol 107 (open circles). The assay contained 10 μM Chl, 20 mM HEPES, pH 7.5, 20 μM benzidine, and 10 μM $K_3Fe(CN)_6$; absorbance change was measured at 325 nm, 0.5 ms after the actinic flash. The actinic flash (λ_{max} = 584 nm) intensity was varied by using calibrated neutral density filters. The two curves were normalized to the same amplitude for the maximum signal obtained with the unattenuated laser flash.

in WT, decaying by recombination with Q_A^- with a $t_{1/2}$ of 70–100 ms, only slightly faster than in WT ($t_{1/2}$ = 150–200 ms) under the same conditions (pH 7.5).

Both benzidine and NH_2OH are effective donors to Z^+ (Babcock et al., 1983; Metz et al., 1989). In WT core complexes, 20 μM benzidine will reduce Z^+ with a $t_{1/2}$ of 10–20 ms, and 2 mM NH_2OH will block 80–90% of Q_A^- in the reduced state by reducing Z^+ following a single saturating flash. In core complexes from mutant D1-Tyr161Phe, where Z is absent, but D is still present, there is no evidence of photoactive tyrosine upon flash excitation in the presence of 20 μM benzidine (Metz et al., 1989). In the same material, in the presence of 2 mM NH_2OH, a single saturating flash will stabilize ≤30% of Q_A^- In whole cells of the D1-Tyr161Phe mutant, both benzidine and NH_2OH are ineffective in blocking charge recombination between Q_A^- and the donor side. These observations mean that both benzidine and NH_2OH are poor donors to $P680^+$. They also mean that in the absence of Z, either these donors cannot reduce D^+ and consequently there can be no reduction of $P680^+$ other than by back-reaction with Q_A^- or they can reduce D^+ and D is a slow donor to $P680^+$ relative to the rate of charge recombination.

In PS II core complexes from the CP43-less strains, the addition of 20 μM benzidine accelerates the disappearance, following flash excitation, of the signal we attribute to $Z^+ - Z$. Under these conditions, this signal disappears with a $t_{1/2}$ of ~5 ms. Also, a single saturating flash given in the presence of 2 mM NH_2OH (pH 7.5) blocks 70–80% of the CP43-less core complexes in the Q_A- state.

We conclude that the reversibility of oxidation–reduction that we observe for the signal we attribute to $Z^+ - Z$, its rapid rate of disappearance by charge recombination with Q_A^- and its reactivity toward benzidine and NH_2OH are all consistent with what we have observed for Z in WT and not at all with that of D.

(*d*) *Flash Saturation Curves.* Flash saturation curves were recorded for PS II core complexes from both WT and the Tol 107 mutant (Figure 7) to determine the relative antenna size of the PS II core complex lacking the CP43 subunit. We determined the relative antenna size of the mutant complexes with respect to WT from the slopes in the linear region of the flash saturation curves, shown in the insert. The mutant shows a slope at the origin 70% of that of WT if we normalize to the same amplitude signal for the unattenuated flash. If we assume that the chlorophylls of CP47 and CP43, which bind most of the chlorophyll of the PS II core complex, have similar extinction coefficients, then the decrease of 30% in the antenna size of the mutant corresponds to a reduction of about 15 chlorophyll molecules from the WT antenna of 48 ± 4 Chl/Q_A (Rögner et al., 1990) to ~33 Chl/Q_A. This is a reasonable assumption as there are strong structural homologies between these two chlorophyll–protein complexes (Bricker 1990). Independent measurements of flash-induced Q_A^- reduction at 320 nm ($\Delta\epsilon$ = 12.5 mM^{-1} cm^{-1}) using hydroxylamine as a donor also gave 33 ± 3 Chl/Q_A. Agreement between the relative antenna size of the mutant core complex as determined by the flash saturation curve and the measure of the number of chlorophylls per photoreducible Q_A compared to WT indicates that the quantum yield of Q_A photoreduction is the same in the mutant and WT core complexes and is independent of the presence of CP43. These measurements also indicate that there are no inactive centers in the preparation of the CP43-less core complexes. A similar reduced antenna size was also found for mutant Tol 1357. We attribute, therefore, the 15 chlorophyll difference between mutant and wild type to CP43.

(*e*) *Determination of Molecular Mass.* The molecular mass of the PS II(−CP43) core complexes was determined by applying them to a TSK 4000 SW gel filtration column as described by Rögner (1990). The average molecular mass of PS II(−CP43) core complexes including the detergent shell of about 50 kDa (Dekker et al., 1988) was determined to be 285 ± 15 kDa, i.e., about 25 kDa smaller than the corresponding core complex from WT (Rögner et al., 1990). The chromatogram (not shown) also demonstrated that the preparation procedure outlined above yields particles which are homogeneous in size, i.e., without aggregates or breakdown products.

Discussion

We show here that, in the absence of CP43, photoactive core complexes are still assembled in the thylakoid membrane of *Synechocystis* 6803. This observation is consistent with observations of trace activities of light-driven electron transfer from diphenylcarbazide to DCPIP in membranes isolated from a mutant of *Synechocystis* PCC 6803 containing an interruption in the *psb*C gene (Vermaas et al., 1988a,b) and with the reported assembly of low levels of complex containing D1, D2, and CP47 in a mutant of *Chlamydomonas reinhardtii* lacking CP43 (de Vitry et al., 1989). No activity measurements were made on the *Chlamydomonas* complex. We show here that the assembled complex isolated from *Synechocystis* not only contains all of the primary and secondary electron donors and acceptors that are present in core complexes that contain the CP43 subunit but also that these redox components function with quantum yields of charge separation equivalent to those of the more intact complex and, according to the optical difference spectra, in the same environments. This is the smallest complex isolated that is still capable of light-driven electron transfer from secondary donor Z to the primary quinone acceptor, Q_A.

Contrary to reports using biochemical means to extract CP43 (Yamagishi & Katoh, 1985; Akabori et al., 1988; Petersen et al., 1990), no Q_A plastoquinone is lost here from the isolated core complex. While biochemical extraction experiments have been interpreted to indicate a requirement for CP43 in stabilizing the binding of Q_A (Petersen et al., 1990), the present results indicate that CP43 is not required either for Q_A binding or for Q_A photoreduction with wild-type

quantum yields. Yamaguchi et al. (1988) reported some loss (30%) of Q_A photoreduction upon KSCN extraction of CP43; however, they still observe in those centers that remain active, flash-induced absorbance changes in the 540–550-nm region that are characteristic of PQ^- formation in the Q_A binding site (C550). The severity of the extraction conditions for the removal of the CP43 subunit may have removed a fraction of the bound Q_A.

The rates of charge recombination between Q_A^- and Z^+ in the WT and CP43-less core complexes are similar, differing by little more than a factor of 2 ($t_{1/2}$ of 150–220 and 70–100 ms, respectively) at pH 7.5. Boska et al. (1986) attribute decay half-times of 20 and 7 ms, respectively, for this reaction at pH 5.5. While the rates are a factor of 10 faster at the lower pH, the ratio of the rates of WT/CP43-less complex is the same at both pHs. These observations would imply that the loss of CP43 has only a minor effect on the free energy difference between the Q_A/Q_A^- and Z^+/Z redox couples.

Preliminary experiments indicate that electron transfer from Q_A to Q_B is slow in intact cells of the CP43-less mutants and that DCMU does not influence the kinetics of Q_A^- oxidation. Furthermore, Carpenter et al. (1990) have reported a loss of DCMU binding in mutants lacking CP43. Together these observations imply that there may be some impairment in Q_B function even in intact cells of mutants lacking CP43. Such cells do not evolve oxygen either. While an impaired donor side cannot be ruled out, blocked electron transfer on the acceptor side could explain the loss of oxygen-evolving activity.

The $Q_A^- - Q_A$ and $Z^+ - Z$ difference spectra are identical in the Tol 107 strain and in WT. As the blue regions of both of these spectra arise from charge-induced electrochromic shifts of reaction center pigments, we conclude that CP43 is not an integral part of the reaction center and that its loss has little effect on the organization of the reaction center pigments or on the sites at which Q_A is reduced and Z oxidized. This observation contrasts with recent studies (Yamaguchi et al., 1988) in CP43-less core complexes obtained with detergents and KSCN in which the EPR spectrum of Z^+ (EPR signal IIf) in continuous light was reported to have been altered such that the hyperfine lines were no longer visible. Another such complex was reported to produce $P680^+$ upon continuous illumination but not EPR signal II (Takahashi & Katoh, 1986). On the other hand, Boska et al. (1986) and Petersen et al. (1990) have reported an EPR signal II with a normal hyperfine structure upon illumination of CP43-extracted core complexes in the presence of ferricyanide, but no sign of a dark-stable signal II or of signal II in the absence of ferricyanide (Petersen et al., 1990). Petersen el al. (1990) suggest that in the absence of ferricyanide there is photoaccumulation of the pheophytin anion, I^-, in those centers that lack Q_A. The EPR signals observed by Takahashi and Katoh (1986) and by Yamaguchi et al. (1988) may have a similar origin, though the latter authors do see flash-induced optical changes consistent with some Z oxidation and both groups see some Q_A photoreduction. It is likely that the detergent-extracted CP43-less PS II core complexes mentioned above are heterogeneous with respect to the presence of Q_A. Those centers that lack Q_A will show, in the absence of ferricyanide, the EPR signal reported by Petersen et al. (1990) in continuous light. Those that do contain Q_A will probably show flash-induced absorbance changes showing both $Q_A^- - Q_A$ and $Z^+ - Z$ as we observe here. We have not examined the EPR spectrum of Z^+ in the core complexes from Tol 1357 and Tol 107.

As CP43 is though to function as part of the core antenna, its removal from PS II core complexes should result in a smaller remaining antenna size, with a considerably lower Chl/Q_A ratio than in PS II core complexes from WT. However, with the exception of Yamaguchi et al. (1988), all reported Chl/Q_A ratios of PS II(–CP43) core complexes obtained through biochemical treatment were, because of inactivation of Q_A reduction, actually higher than that of the original CP43-containing PS II core complexes (Yamagishi & Katoh, 1985; Akabori et al., 1988; Petersen et al., 1990). Our work describes a CP43-less core complex with a Chl/Q_A^- ratio considerably lower than that of the original PS II core complex. The chlorophyll contribution of CP43 to intact core complexes was determined by comparative flash saturation measurements to be about 30%, or 15 Chl, bound per CP43. This leaves about 70%, or 33 Chl, for the complex containg D1/D2/CP47. Depending on the amount of Chl per D1/D2 complex, with values varying between 6 and 12 (Dekker et al., 1989; Gounaris et al., 1989; Kobayashi et al., 1990), this would mean 21–27 Chl/CP47. An antenna size of about 33 Chl/RC in the absence of CP43 agrees with the conclusions of Dekker et al. (1990), based on the presence of 2 cyt *b*-559 per PS II core complex. Similarly, Yamaguchi et al. (1988) found 35 Chl/Q_A in PS II core complexes stripped of CP43, down from 51Chl/Q_A in the presence of CP43. The difference of 16 Chl was attributed to CP43. These numbers are very similar to ours. However, these authors then correct their numbers by assuming that 30% of the centers were inactivated by their treatment with KSCN to remove CP43, thus giving 25 Chl per complex in the absence of CP43 and 26 Chl/CP43. It may be that their correction is unnecessary if the nondenaturing gel purification these authors used to purify the CP43-less PSII core complexes removed the inactive centers. The two methods that we use to determine Chl/Q_A allow us to exclude the presence of inactive centers in our PS II(–CP43) core preparations.

The absence of the *psb*C gene product reduces the concentration of core complex to about 10% of WT levels as determined by HPLC of extracted membranes (Figure 3), by light-induced changes in the fluorescence yield of chlorophyll in whole cells in the presence of DCMU (not shown), and by immunoblot detection of the CP47 and D2 subunits in the membrane (Figure 2). This is consistent with the finding in *Chlamydomonas reinhardtii* that the absence of any of the PS II core polypeptides leads to an increased rate of turnover of the other core proteins and to a severe deficiency of the PS II core (Rochaix & Erickson, 1988; de Vitry et al., 1989). It is also consistent with the work of Yu and Vermaas (1990), who, using immunoblotting, have shown that interruption of *psb*C in *Synechocystis* 6803 results in significantly reduced levels of CP47, D1, and D2. We find that CP47 is present at roughly the same level as that of the D2 subunit on a per mole basis. In agreement with our results, Dzelzkalns and Bogorad (1988) report only trace concentrations of D2 in a mutant of *Synechocystis* 6803 (PS3) with a partly deleted *psb*C gene. In contrast to our findings, however, Dzelzkalns and Bogorad (1989) report a concentration of CP47 80% of WT in the same mutant. It is possible that in their case the synthesis of a truncated but unstable CP43 might permit the accumulation of higher levels of CP47.

Estimation by HPLC of the amount of PS II(–CP43) core complex extracted from the thylakoid membranes of mutants Tol 1357 and Tol 107 (not shown) indicates that the presence of either *psb*D2 alone or *psb*D1 and *psb*D2, respectively, has little influence on the amount of PS II(–CP43) in the thylakoid membrane and that the *psb*D2 gene is fully functional in the absence of *psb*D1. We now find that *psb*D1 and *psb*D2 encode

identical gene products. The correct sequence is that earlier reported for the *psb*D2 gene product (Williams & Chisholm, 1987) except that residue 231 is a threonine. Yu and Vermaas (1990) have reported that a strain of *Synechocystis* 6803 in which *psb*D2 was deleted was capable of photoautotrophic growth. The ability of either copy of the gene to express functional D2 polypeptide is in agreement with results obtained by using the photoautotrophic cyanobacterium *Synechococcus* PCC 7942 (Golden et al., 1989). Our results concerning mutant Tol 107 also agree with those of Carpenter et al. (1990), who showed that the start codon for *psb*C in *Synechocystis* PCC 6803 was GTG and that altering the codon resulted in loss of expression of CP43.

The fluorescence emission of the isolated PS II(−CP43) complex (at 77 K) peaks at 685 nm, which is 4–5 nm blue-shifted in comparison to WT core complexes. This observation does not agree with the attribution of fluorescence emission peaks at 687 nm to CP43 and at 691 nm to CP47, respectively, for *Synechocystis* cells (Dzelzkalns & Bogorad, 1989). We have found in Tol 107 and in mutant cells of *Synechocystis* depleted of reaction centers (Nilsson et al., 1990; Peter Nixon, personal communication) that upon excitation at 580 nm, there appears at 77 K an emission band at 685–689 nm significantly larger than that of PS I at 725 nm. In WT cells, emission in the 685–695-nm region arising from PS II is much smaller than that at 725 nm under the same conditions. The enhanced emission in the 685–689-nm region, therefore, cannot arise from PS II as these are depressed in the mutants and most likely comes from allophycocyanin-B in phycobilisomes no longer coupled to reaction centers (Elmorjani et al., 1986).

The average molecular mass determined by size-exclusion HPLC for our PS II(−CP43) complexes suggests a molecular mass only about 25 kDa smaller than that for WT PS II core particles. After correction for the detergent contribution of about 50 kDa (Dekker et al., 1988), our estimated mass of 235 ± 15 kDa is about 60 kDa larger than the average size of a CP43-less PS II particle obtained from spinach PS II core complexes by detergent treatment (Dekker et al., 1990). Considering the elliptical top view of isolated PS II monomers as seen in electron micrographs (Rögner et al., 1987), our results suggest that CP43 does not greatly contribute to the hydrodynamic radius of these particles, indicating that it may be located at the short axis of this complex.

In conclusion, the results reported in this paper demonstrate that by using site-directed mutants, it is possible to isolate PS II complexes completely devoid of CP43 that still retain functional secondary donor and acceptor electron transfer. The quantum yield for primary charge separation is unchanged compared to the core complex in which CP43 is present, which makes this complex a suitable material for studying the contribution of the chlorophyll binding subunits to excitation-transfer processes.

In addition to a role in light harvesting, the drastically reduced amount of PS II in the CP43-less mutants indicates that CP43, although dispensable for primary photochemistry and stabilization of Q_A binding, fulfills a stabilizing role for the PS II complex in general and that its absence diminishes the steady-state level of other subunits of the PS II complex, and the ability of the core complex to carry out tertiary electron donor and acceptor reactions.

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Conformational Changes in Sensory Rhodopsin I: Similarities and Differences with Bacteriorhodopsin, Halorhodopsin, and Rhodopsin†

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ABSTRACT: FTIR difference spectra have been obtained for the $sR_{587} \rightarrow S_{373}$ phototransition of sensory rhodopsin I (sR-I), a signal-transducing protein of *Halobacterium halobium*. The vibrational modes of the sR_{587} chromophore have frequencies close to those of the bacteriorhodopsin bR_{568} chromophore, confirming that the two chromophores have very similar structures and environments. However, the sR-I Schiff base C=N stretch frequency is downshifted relative to bR, consistent with weaker hydrogen bonding with its counterion(s). The carboxyl (COOH) stretch modes of sR-I and halorhodopsin (hR) are at the same frequencies. On the basis of sequence homologies, these bands can be assigned to Asp-106 in helix D and/or Asp-201 in helix G. In contrast, no band was found that could be assigned to the protonation of Asp-76. In bR, the homologous residue Asp-85 serves as the acceptor group for the Schiff base proton. Bands appear in the amide I and II regions at similar frequencies in sR-I, hR, and bR, indicating that despite their different functions they all undergo closely related structural changes. Bands are also detected in the C–H stretch region, possibly due to alterations in the membrane lipids. Similar spectral features are also observed in the lipids of rhodopsin-containing photoreceptor membrane upon light activation.

Sensory rhodopsin I (sR-I)[1] functions as one of two signal-transducing proteins involved in control of phototaxis of *Halobacterium halobium* (Spudich & Bogomolni, 1988). It mediates both an attractant response and a repellent response due to photoreactions of sR-I's resting state (sR_{587}) and its long-lived photoproduct (S_{373}), respectively (Spudich & Bogomolni, 1984). Like rhodopsin, the light receptor in vision, sR-I has seven transmembrane helical segments, as deduced

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[1] Abbreviations: PM, purple membrane; sR-I, sensory rhodopsin I; bR, bacteriorhodopsin; hR, halorhodopsin; FTIR, Fourier transform infrared; au, absorbance units; λ_{max}, wavelength of maximum visible absorption; MPP-I, methyl-accepting phototaxis protein I; sR_{587}, the thermally stable attractant form of sR-I with λ_{max} = 587 nm; S_{373}, the photointermediate (repellent form) of sR-I with λ_{max} = 373 nm (Spudich & Bogomolni, 1984).

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