

Construction and Characterization of Genetically Modified *Synechocystis* sp. PCC 6803 Photosystem II Core Complexes Containing Carotenoids with Shorter π -Conjugation than β -Carotene*

Received for publication, May 5, 2005, and in revised form, August 17, 2005 Published, JBC Papers in Press, September 12, 2005, DOI 10.1074/jbc.M504953200

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β -Carotene has been identified as an intermediate in a secondary electron transfer pathway that oxidizes Chl_z and cytochrome *b*₅₅₉ in Photosystem II (PS II) when normal tyrosine oxidation is blocked. To test the redox function of carotenoids in this pathway, we replaced the ζ -carotene desaturase gene (*zds*) or both the *zds* and phytoene desaturase (*pds*) genes of *Synechocystis* sp. PCC 6803 with the phytoene desaturase gene (*crtI*) of *Rhodobacter capsulatus*, producing carotenoids with shorter conjugated π -electron systems and higher reduction potentials than β -carotene. The PS II core complexes of both mutant strains contain approximately the same number of chlorophylls and carotenoids as the wild type but have replaced β -carotene (11 double bonds), with neurosporene (9 conjugated double bonds) and β -zeacarotene (9 conjugated double bonds and 1 β -ionylidene ring). The presence of the ring appears necessary for PS II assembly. Visible and near-infrared spectroscopy were used to examine the light-induced formation of chlorophyll and carotenoid radical cations in the mutant PS II core complexes at temperatures from 20 to 160 K. At 20 K, a carotenoid cation radical is formed having an absorption maximum at 898 nm, an 85 nm blue shift relative to the β -carotene radical cation peak in the WT, and consistent with the formation of the cation radical of a carotenoid with 9 conjugated double bonds. The ratio of Chl⁺/Car⁺ is higher in the mutant core complexes, consistent with the higher reduction potential for Car⁺. As the temperature increases, other carotenoids become accessible to oxidation by P₆₈₀⁺.

A carotenoid molecule is an extended polyene chain with a variety of end groups and has been thought of as a "molecular wire," because, in the one-electron oxidized form, or carotenoid radical cation, the hole is delocalized over the entire conjugated π -system. Carotenoid radical cations have recently been observed in several photosynthetic pigment-

protein complexes, including PS II,³ bacterial light-harvesting complexes LHC II, and recently in plant PsbS where they may have a role in non-photochemical energy quenching (1). Among photosynthetic reaction centers, carotenoid photooxidation is unique to PS II, which uses light energy to catalyze the oxidation of water to molecular oxygen. The process of water oxidation involves highly oxidizing species that can also oxidize a carotenoid molecule. Carotenoid radical cations have been identified as intermediates in the secondary electron transport pathway of PS II (2–6).

Detergent-solubilized PS II core complexes contain up to 25 different integral membrane and extrinsic polypeptide subunits, including the D1/D2 complex and light-harvesting pigment-protein complexes CP43 (PsbC) and CP47 (PsbB). Light energy is absorbed by the chlorophyll-containing light-harvesting antenna and transferred to the primary donor chlorophyll(s) coordinated by the D1/D2 polypeptides. The term "PS II reaction centers" describes a structure that includes the D1 and D2 polypeptides, cytochrome *b*₅₅₉ (Cyt *b*₅₅₉), PsbI, and protein-bound cofactors that include six chlorophyll *a* (Chl *a*) molecules, two pheophytin *a* (Pheo *a*) molecules, a tightly bound plastoquinone (Q_A) and an exchangeable plastoquinone (Q_B). The primary photochemical reactions in PS II are initiated by the absorption of light energy by the primary chlorophyll donor (most likely chlorophyll *B_A*) (7–9). The excited state of this chlorophyll drives charge separation, producing a reduced pheophytin (Pheo_A[−]) and a chlorophyll radical cation species, P₆₈₀⁺. The charge separation is stabilized by transfer of the electron from Pheo_A[−] to Q_A, forming the state, P₆₈₀⁺ Q_A[−]. Under normal physiological conditions, the very high potential oxidant, P₆₈₀⁺, is reduced by electrons from the oxygen-evolving complex via a redox-active tyrosine (Y_Z). However, there are several conditions known, some of them of physiological relevance, in which the donation of an electron from the oxygen-evolving complex to P₆₈₀⁺ is inhibited or retarded, allowing the highly oxidizing P₆₈₀⁺ (*E_M* ~ 1.1–1.3 V) (10) to abstract an electron from other sources. Chlorophyll_z (Chl_z), Cyt *b*₅₅₉, and

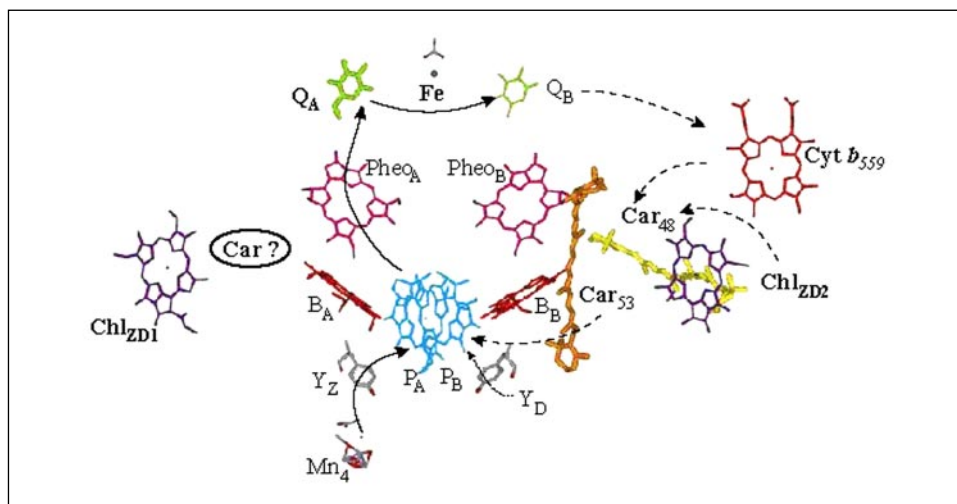
* The project was supported by the National Research Initiative of the U. S. Dept. of Agriculture Cooperative State Research, Education and Extension Service Grant 2003-35318-13589 (to B. A. D.), the Dept. of Energy, Office of Basic Energy Sciences, Division of Chemical Sciences Grant DE-FG02-01ER15281 (to G. W. B.), and National Institutes of Health Predoctoral Traineeship GM08283 (to C. A. T.). This paper is contribution 8614 of the Central Research and Development Dept. of E. I. du Pont de Nemours & Co. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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³ The abbreviations used are: PS II, photosystem II; β -DM, *n*-dodecyl- β -D-maltoside; Car⁺, carotenoid radical cation; Car, carotenoid; Chl, chlorophyll; Chl_z, an oxidizable chlorophyll located at the periphery of the PS II reaction center; CP43, chlorophyll-binding protein encoded by *psbC*; CP47, chlorophyll-binding protein encoded by *psbB*; CrtI, purple bacterial phytoene desaturase (encoded by *crtI*); HPLC, high pressure liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; P₆₈₀, first stable electron donor of PS II; Pds, cyanobacterial phytoene desaturase (encoded by *crtP* or *pds*); Q_A, primary quinone electron acceptor; Q_B, secondary quinone electron acceptor; WT, wild type; Zds, cyanobacterial ζ -carotene desaturase (encoded by *crtZ* or *zds*); Cyt *b*₅₅₉, cytochrome *b*₅₅₉.

FIGURE 1. **Electron transfer pathways of Photosystem II.** The principal electron-transfer pathway is shown in *solid arrows* involving the photoactive oxidized electron donor chlorophyll P_{680}^{+} (the cation is localized primarily on P_A), tyrosine Y_Z , and the tetra-manganese cluster (Mn_4). An alternate electron-transfer pathway, shown using *dashed arrows*, involves β -carotene (*Car*), the accessory Chl_{ZD2} (in the D2 subunit), and cytochrome b_{559} (*Cyt b₅₅₉*). The chlorophyll and pheophytin tails have been removed for clarity. This figure is adapted from the x-ray crystallographic structure described in Ref. 14 (PDB code 1S5L).



carotenoid (*Car*) have been identified as electron donors to P_{680}^{+} and are thought to comprise an alternate electron transfer pathway that has been suggested to protect PS II against uncontrolled oxidative reactions of the strong oxidant P_{680}^{+} .

Cytochrome b_{559} and the Chl_Z accessory chlorophylls (Chl_{ZD1} and Chl_{ZD2}) are located at the periphery of the PS II reaction center (Fig. 1). Because these species can be oxidized by P_{680}^{+} despite their location $\geq 30\text{\AA}$ away, it has been postulated that β -carotene enables long distance electron transfer in PS II from $Cyt b_{559}$ to P_{680}^{+} by acting as an electron wire, forming an intermediate cation radical. Earlier studies suggested that the carotenoid molecule is the primary donor to P_{680}^{+} at low temperature, followed by subsequent oxidation of Chl_Z and $Cyt b_{559}$ (3, 5). Recently, evidence has been obtained for the involvement of two carotenoid molecules in this pathway, most likely the all-*trans*- β -carotenes in the reaction center (6, 11). Although four crystal structures of PS II core complexes have been determined, the location of the carotenoids in the D1 and D2 polypeptides remains uncertain because of the limited resolution of the diffraction data, 3.2–3.8 $\text{\AA}$ (12–15). Because of the snake-like structure of carotenoids, they are difficult to identify in the electron density, although a carotenoid was modeled in a very similar location in the D2 polypeptide in two of the structures. In two of the x-ray crystal structures, a second carotenoid was modeled in the D2 polypeptide, but with different orientations. No carotenoids have been modeled in the D1 polypeptide even though the accessory Chl ligated to His-118 in D1 (Chl_{ZD1}) can be photooxidized (16), and a carotenoid located in the D1 subunit would supply a feasible pathway for electron transfer. However, a pathway for electron transfer from Chl_{ZD1} to P_{680}^{+} through intermediate chlorophylls in CP43 has been suggested (17).

The uncertainty in the location of the carotenoid molecules in the structure of PS II also means that the exact pathway of the alternate electron transfer is unknown. To increase our understanding of the role that carotenoids play in this electron transfer pathway, we decided to alter the carotenoid reduction potential and to study the effect of this alteration on the alternate pathway. The PS II reaction center binds two β -carotene molecules, which have 11 conjugated double bonds each (18, 19). It is known that, as the number of double bonds of the polyene backbone increases, the wavelength maximum of the S2–S0 optical transition increases, and the molecule is easier to oxidize. Therefore, by decreasing the number of conjugated double bonds the carotenoid will be more difficult to oxidize, likely altering electron transfer through the

secondary electron transfer pathway. There have been recent suggestions indicating that the reduction potential of P_{680}^{+}/P_{680} might be higher than previously thought (20, 21). Replacing β -carotene with a carotenoid that is more difficult to oxidize would also test the oxidative capacity of P_{680}^{+} .

Exchanging cofactors in proteins has been demonstrated for many systems and has been useful in determining energy differences between redox cofactors. Extraction of carotenoids from PS II reaction centers has been demonstrated (22). However, this extraction requires harsh conditions, resulting in the loss of Chl molecules. Reconstitution of carotenoids into solvent-extracted PS II and into carotenoid-less bacterial reaction centers has, however, been reported (23–25). A more gentle approach to this problem is to alter the carotenoid composition of PS II by changing the types of carotenoids available *in vivo* to be incorporated into the pigment-protein complex. As in green algae (26), we have found that cyanobacteria grown in the presence of carotenoid biosynthesis inhibitors do not assemble PS II, even in the presence of carotenoid supplements, presumably because the cells are unable to take up carotenoids from the growth medium. Therefore, this is not a viable method to replace β -carotene in PS II with another carotenoid.

We show here that we can produce carotenoids that are not native to *Synechocystis* sp. PCC 6803 by modifying the carotenoid biosynthetic pathway of this organism. We have learned what types of carotenoids can be incorporated into PS II. We have found that gene deletions that block β -carotene formation do not result in PS II formation (27). However, expressing phytoene desaturase from *Rhodobacter capsulatus* in *Synechocystis* in place of the endogenous ζ -carotene and phytoene desaturases, results in the accumulation of carotenoids that can be incorporated into PS II. In this way, PS II core complexes from *Synechocystis* sp. PCC 6803 can be genetically modified to produce carotenoids with fewer than 11 conjugated double bonds.

Herein, we report the construction of *Synechocystis* sp. PCC 6803 carotenoid mutants and characterization of PS II core complexes from the carotenoid mutant cells. Using low temperature visible and near-IR spectroscopy to detect the formation of new carotenoid radical cation species, we measure the formation and decay of the carotenoid and chlorophyll radical cations to probe the sequence of electron transfer steps in the secondary electron transfer pathway. Overall, these experiments provide new insight into the role of carotenoids in the secondary electron transfer pathways and assembly of PS II.

MATERIALS AND METHODS

Construction of Mutants

Deletion Mutants—The genes for phytoene desaturase (*pds*) and ζ -carotene desaturase (*zds*) were deleted separately in the genome of the wild type (WT) *Synechocystis* sp. PCC 6803 and replaced by a kanamycin resistance cassette. A PCR copy of the gene to be deleted (*zds* or *pds*) that included 500 bases upstream and downstream of the gene was ligated onto a pGEM-T plasmid vector (Promega A1360) using T4 DNA ligase and amplified in XL1-Blue *Escherichia coli*-competent cells (Stratagene 200249). These plasmids were named pA-*zds*_{Syn} or pB-*pds*_{Syn}, respectively. The 500 bases flanking each gene together with the ligated pGEM-T plasmid vector between them were copied by PCR using primers designed to give unique restriction enzyme sites to the termini of the PCR product. The PCR product was digested with the appropriate restriction enzymes and then ligated using T4 DNA ligase to a kanamycin resistance cassette with the same sticky ends as the PCR product. The final plasmid construct was then amplified in XL1-Blue *E. coli*-competent cells and used to transform the WT *Synechocystis* sp. PCC 6803 strain.

Single Replacement Mutant (Pdscap1)—The *zds* gene in the *Synechocystis* WT His tag strain (28) encodes ζ -carotene desaturase (Zds), which converts ζ -carotene to lycopene, converted in turn by lycopene cyclase to β -carotene. This gene was replaced by the *crtI* gene of *R. capsulatus*, which encodes a phytoene desaturase, which converts phytoene to neurosporene, converted in turn by lycopene cyclase to β -zeacarotene (see below). This gene replacement should result (Fig. 2) in the loss of β -carotene expression with 11-double bonds and its replacement by β -zeacarotene with 9 conjugated double bonds. Plasmid pA-*zds*_{Syn} above was copied by PCR, excluding the *zds* gene using primers that gave unique restriction sites to the termini of the PCR product. The primers introduced a KpnI site in place of the gene ATG start codon and a XbaI site in place of the TAA stop codon. This PCR product was digested with the appropriate restriction enzymes and then ligated to PCR-amplified *R. capsulatus crtI* designed with the same sticky ends as the PCR product. The *R. capsulatus crtI* was derived from plasmid pGABX2 (29). The primers introduced a KpnI site 100 bases upstream of the NCBI designated start codon for this gene and an XbaI site 24 bases downstream of the TGA stop codon. Following ligation, the new plasmid, called pA-*crtI*_{Rhod}, was then amplified in XL1-Blue *E. coli*-competent cells and digested with XbaI, which gave a single cut. A streptomycin resistance cassette with XbaI sticky ends was ligated into the cut plasmid using T4 DNA ligase. The final plasmid construct was then amplified in XL1-Blue *E. coli*-competent cells and used to transform the WT *Synechocystis* sp. PCC 6803 His tag strain.

Double Replacement Mutant (Pdscap2)—To more completely channel the conversion of phytoene to β -zeacarotene, the endogenous *Synechocystis pds* gene, still present in the single replacement mutant was also replaced with the *R. capsulatus crtI* gene. Plasmid pB-*pds*_{Syn} containing the *Synechocystis pds* gene was copied by PCR, excluding the *pds* gene, using primers that gave unique restriction sites to the termini of the PCR product. The primers introduced a KpnI site in place of the gene ATG start codon and an XbaI site in place of the TAA stop codon. This PCR product was digested with the appropriate restriction enzymes and then ligated to PCR-amplified *R. capsulatus crtI* as described above to give plasmid pB-*crtI*_{Rhod}. This plasmid was amplified in XL1-Blue *E. coli*-competent cells and digested with XbaI, which gave a single cut. A kanamycin resistance cassette with XbaI sticky ends was ligated into the cut plasmid using T4 DNA ligase. The final plasmid construct was then amplified in XL1-Blue *E. coli*-competent cells and

used to transform Pdscap1. This gave a double replacement mutant with two copies of the *R. capsulatus crtI* gene.

Transformation of *Synechocystis* sp. PCC 6803 WT (His-tagged)⁴ strain with the deletion and single replacement constructs made above was as described in Nixon *et al.* (30). Successful transformants were selected on Petri plates containing BG-11 plus 5 mM glucose with 50 μ g/ml kanamycin or 100 μ g/ml streptomycin and grown in very dim light ($\sim 0.1 \mu$ mol of photons $\text{m}^{-2}\text{s}^{-1}$) at 30 °C. Antibiotic-resistant colonies representing successful transformants were replated seven times in BG-11 plus 5 mM glucose plates with 200 μ g/ml kanamycin, for the deletion mutants or 100 μ g/ml streptomycin, for the single replacement mutant, to obtain full segregation. Deletion mutations and single replacement mutation were confirmed by extracting total pigments from the cells and analyzing for the complete absence of β -carotene by HPLC. The cells were also unable to produce β -carotene under non-selective conditions (without kanamycin or streptomycin) indicating complete segregation. For the double replacement mutant, the fully segregated single replacement strain was subjected to another round of transformation, this time using plasmid pB-*crtI*_{Rhod} with a kanamycin resistance cassette ligated 5 bases after the stop codon of *R. capsulatus crtI*. The transformation procedure was the same as above except that incubation of the fully segregated single replacement strain with the plasmid construct was done in dim light ($\sim 0.1 \mu$ mol photons $\text{m}^{-2}\text{s}^{-1}$).

PS II Preparation

Wild-type *Synechocystis* sp. PCC 6803 cells were grown in liquid BG-11 plus 5 mM glucose medium as described (31–33). The mutants were grown in BG-11 with 5 mM glucose in very dim light ($\sim 1 \mu$ mol of photons $\text{m}^{-2}\text{s}^{-1}$). Isolation of the thylakoid membranes from these cells was performed as previously described (31, 32). The thylakoid membranes were suspended at a concentration of 1 mg/ml chlorophyll in Buffer A (25% glycerol, 20 mM CaCl₂, 5 mM MgCl₂, 50 mM MES, pH 6.0). After addition of a one-eighth volume of 10% (w/v) *n*-dodecyl- β -D-maltoside (β -DM), the suspension was stirred for 20 min and then centrifuged at 40,000 rpm in a 45-Ti rotor (Beckman-Coulter) for 10 min. The supernatant was then loaded onto a nickel-nitrilotriacetic acid-agarose resin of ~ 40 ml pre-equilibrated with Buffer B (Buffer A containing 0.03% β -DM) in a fritted column. The mixture was made 5 mM in imidazole by adding appropriate volumes of a 2 M imidazole stock solution and nutated in the dark at 4 °C for 30 min. Afterward, the column was drained and washed with 700 ml of Buffer C (Buffer A containing 0.1% β -DM and 5 mM imidazole). His-tagged PS II was eluted with 160 ml of buffer D (Buffer B containing 100 mM imidazole). The eluant containing PS II was made 1 mM in EDTA and concentrated in a Millipore concentrator utilizing a YM 100 Diaflo ultrafiltration membrane. The concentrated sample was then desalted by passing it through a gel filtration column (Econo-Pac 10 DG, Bio-Rad) pre-equilibrated with Buffer B then further concentrated in an Amicon Ultra-4 concentrator (Millipore) with a 100,000 molecular weight cutoff. The samples were then frozen in liquid N₂ and stored at -80 °C until use.

Manganese Depletion

PS II core complexes were manganese-depleted for low temperature near-IR measurements. The complexes in Buffer B were diluted 1:1 with Buffer B containing 10 mM hydroxylamine and 2 mM EDTA, and incubated in the dark for 30 min. The samples were transferred to an Amicon Ultra-4 concentrator (Millipore) with a 100,000 molecular weight

⁴ His-tagged PS II refers to the presence of a heparhistidine tag located at the C terminus of CP47 (28).

PS II with Genetically Modified Carotenoid Composition

cutoff filter and centrifuged at $5,000 \times g$ to reduce the volume to $\sim 100 \mu\text{l}$. The sample was diluted twice to a volume of 4 ml with Buffer B containing 2 mM EDTA and concentrated to $100 \mu\text{l}$. The Mn^{2+} -depleted sample was diluted and mixed with glycerol and Buffer B to a final concentration of 63% glycerol, 20 mM CaCl_2 , 5 mM MgCl_2 , 0.03% β -DM, and 50 mM MES, pH 6.0.

Pigment Analysis

The chlorophyll content of the cells was determined by extraction with 100% methanol and using the 79.24 ml/mg extinction coefficient of Lichtenthaler (34) at 665 nm. For total pigment analysis, PS II core complexes were extracted with a 50:50 acetone/methanol mixture under dim light. The mixture was centrifuged for 2 min ($10,000 \times g$), and a $50\text{-}\mu\text{l}$ aliquot of the supernatant was immediately filtered ($0.45 \mu\text{m}$, Millex-LH polytetrafluoroethylene) and injected onto a reverse-phase HPLC column (Zorbax-ODS, $4.6 \text{ mm} \times 15 \text{ cm}$, Rockland Technologies) equilibrated with methanol:ethyl acetate (68:32, v/v). The separation was performed in isocratic mode using the same solvent system (35) at a flow rate of 0.25 ml/min for 60 min at room temperature. The relative chlorophyll and carotenoid concentrations were determined by measuring the area of their respective peaks in the HPLC chromatogram and dividing by the extinction coefficients according to Giorgi *et al.* (35) and Britton (36). The eluted carotenoids were identified by their absorbance spectra using the diode array detector of the HPLC (Agilent Series 1100) and by their mobility using β -carotene and zeaxanthin standards.

Visible Absorbance Spectra and Light-minus-dark Absorbance Difference Spectra at 77 K

PS II core complexes were diluted to $\sim 10 \mu\text{M}$ Chl in a buffer containing 20 mM MES/NaOH (pH 6.5), 5 mM MgCl_2 , 20 mM MgCl_2 , 0.02% β -DM, 2 mM ferricyanide, and 65% glycerol. The sample (1.5 ml) was placed in a plastic cuvette with a 1-cm path length, inserted into a liquid nitrogen bath cryostat (DN 1704 from Oxford), and dark-adapted. The cryostat was then cooled to 77 K with liquid nitrogen and centered in the measuring beam of a Cary 1E UV-visible spectrophotometer (Varian) using a home-built cryostat holder. Spectra were recorded with data intervals of 0.1 nm, a scan speed of 60 nm/min, and a spectral bandwidth of 1 nm. The difference spectra were obtained by subtracting the absorbance spectrum in the dark-adapted state from those measured after 2-min illumination of the sample with continuous white light from a 200-watt halogen lamp.

Low Temperature Near-IR Spectroscopy

PS II core complex samples were prepared in a buffer containing 63% glycerol, 20 mM CaCl_2 , 5 mM MgCl_2 , and 50 mM MES (pH 6.0) and transferred to a plastic cuvette (Sigma) with a path length of 1 cm. The samples were treated with $100 \mu\text{M}$ potassium ferricyanide to oxidize Cyt b_{559} , dark-adapted for 1 h, slowly frozen in a liquid nitrogen bath, and transferred to an Oxford Optistat cryostat mounted in a PerkinElmer Life Sciences λ -20 spectrophotometer. All experiments were performed on samples equilibrated at the specified temperature inside the Optistat for at least 1 h or until the baseline was stable.

Samples were illuminated by a specified number of flashes at 532 nm from the second harmonic of an Nd:YAG Q-switched laser LAB-150-10 (Spectra-Physics) operating at 10 Hz. A beam splitter was used to vary the laser power delivered to the sample. A 1064 nm filter was also used to further reduce the level of first harmonic present in the laser beam. Typically, the flash energy was 150–180 mJ per pulse. The laser was coupled to the cryostat by a fiber optic cable (Schott-Fostec). Illu-

mination of the sample by a series of pulses was achieved by manually triggering the laser Q-switch every 2–5 s, as specified below. In some cases, the laser Q-switch was triggered by a home-built laser controller device. The device is a counter that detects a transistor-transistor logic pulse from the laser indicating that the flash lamp has fired. After the specified number of flashes (or time since the laser operates at 10 Hz), the laser controller device triggers the Q-switch electronically. The total number of flashes and repetition rate ($<10 \text{ Hz}$) can be specified by binary input into the laser controller device. In some cases, the samples were illuminated by a 150-watt halogen lamp filtered by a 6-inch water bath and a heat-absorbing filter (Schott KG-5).

Scattered illumination light was filtered from the measuring beam of the PerkinElmer Life Sciences λ -20 spectrophotometer by a dichroic mirror that blocks 532 nm (Spectra Physics) and a high band pass filter with a 750 nm cutoff to reduce interference from chlorophyll fluorescence (Oriol 59550).

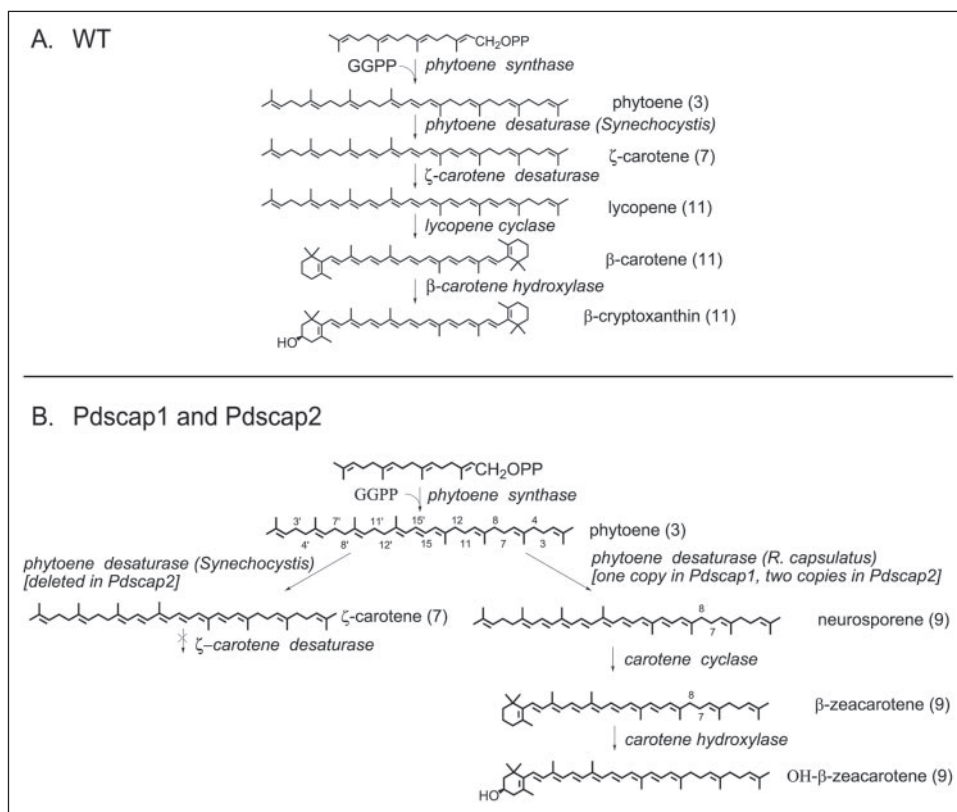
RESULTS

Modification of Carotenoid Biosynthesis Pathway in Synechocystis sp. PCC 6803—In WT *Synechocystis*, ζ -carotene is converted to lycopene (linear, 11 conjugated double bonds) by ζ -carotene desaturase, Zds, and both ends of lycopene are cyclized by lycopene cyclase to form β -carotene (2 β -ionylidene rings, 11 conjugated double bonds, see Fig. 2A). However, the lycopene cyclase gene has not yet been definitively identified in the *Synechocystis* sp. PCC 6803 genome. Because we could not delete this gene to prevent formation of β -carotene, we instead opted to delete other genes that would prevent the formation of lycopene in an attempt to modify the carotenoid content of PS II. We used three different strategies to modify carotenoid expression in *Synechocystis* sp. PCC 6803. Separately, we: 1) deleted the gene encoding phytoene desaturase (Δpds^S) or ζ -carotene desaturase (Δzds^S); 2) replaced the zds^S gene with the *crtI* gene of *R. capsulatus* ($\Delta zds^S crtI^{RC}$, strain called Pdscap1); and 3) replaced both the pds^S and zds^S genes with the *crtI* gene of *R. capsulatus* ($\Delta zds^S \Delta pds^S 2crtI^{RC}$, strain called Pdscap2).

Separate deletion of pds^S or zds^S produced cells that accumulate phytoene (3 conjugated double bonds) or ζ -carotene (7 conjugated double bonds), respectively, and no β -carotene (Fig. 2A). This strain required glucose and dim light for growth and contained functional Photosystem I (PS I) but no PS II, as indicated by the absence of a variable chlorophyll fluorescence yield upon illumination of dark-adapted cells (27). Failure to isolate PS II core complexes using chromatographic methods also confirmed the absence of PS II centers in this strain.

Because linear carotenoids with shorter conjugated chains like phytoene and ζ -carotene apparently do not substitute for β -carotene in PS II assembly, we employed an alternative strategy, that of replacing the zds gene with the *crtI* gene of *R. capsulatus*. In this strain, designated Pdscap1, much of the phytoene is diverted to the formation of neurosporene, a linear carotenoid with 9 conjugated double bonds, rather than to ζ -carotene (Fig. 2B). We expected that only one end of the neurosporene would be cyclized by lycopene cyclase because of the absence of a double bond in the C7–C8 position of neurosporene (37). The product of this cyclization reaction would be β -zeacarotene. That this is indeed the case is shown below. Because lycopene cyclase does not change the number of double bonds in a carotenoid, β -zeacarotene and neurosporene both have 9 conjugated double bonds and should differ little in their redox behavior. In the Pdscap1 strain, PS II accumulates, although to only $\sim 10\%$ of WT levels. That PS II accumulates at all is probably because β -zeacarotene more closely resembles the structure of β -carotene than does ζ -carotene or phytoene. However, the cells can tolerate brighter light conditions than the pds and zds deletion strains.

FIGURE 2. A, carotenoid biosynthetic pathway starting from geranylgeranyl pyrophosphate (GGPP) and producing β -carotene in WT *Synechocystis* sp. PCC 6803. B, modified biosynthetic pathway engineered in mutant strains Pdscap1 and Pdscap2. All molecules are depicted in the all-*trans* configuration. Standard numbering is shown for phytoene in B. The numbers of conjugated double bonds in each molecule are shown in parentheses.



The Pdscap1 strain can also grow on minimal media, but at a much slower rate than WT. In addition to β -zeacarotene, neurosporene and ζ -carotene were also detected in the cell extract. No β -carotene is detectable in this strain.

We reasoned that diverting more of the phytoene to β -zeacarotene, might well increase the number of PS II centers per cell. A mutant was, therefore, constructed from the Pdscap1 strain in which the native *pds* gene was also replaced by a copy of the *crtI* gene of *R. capsulatus*. This strain, Pdscap2, or $\Delta zds^S \Delta pds^S 2crtI^{Rc}$, has two copies of the *crtI* gene of *R. capsulatus*. Pdscap2 is able to produce slightly more PS II reaction centers per cell (~ 15 – 20% compared with WT levels) and is able to tolerate even brighter light conditions than Pdscap1 despite the complete absence of β -carotene. Surprisingly, however, this strain does not grow on minimal medium, which could arise from secondary effects associated with the loss of *pds*, possibly influencing the expression of *crtB* (phytoene synthase), which is located immediately downstream of *pds* (*crtP*) in the *Synechocystis* genome. Such secondary effects could include destabilization of other pigment-protein complexes. Examples include coupled transcription of *crtI* and *crtB* in *Rhodobacter sphaeroides* and its role in the assembly of light-harvesting complex 2 (38).

The visible absorbance spectra of the Pdscap2 and the WT PS II core complexes measured at 77 K show major differences in the carotenoid region (Fig. 3), where the carotenoid absorbance bands are shifted to shorter wavelengths, consistent with the replacement of β -carotene by carotenoids with shorter π -conjugation. There are also differences in the Qy region, where, compared with the WT, the Pdscap2 mutant shows the marked loss of the chlorophyll absorption peak at 677.5 nm and a small (~ 0.5 nm) shift to the red of the Qy absorbance maximum.

Pigment Analysis of Photosystem II Core Complexes—TABLE ONE summarizes the Chl *a* and carotenoid compositions of the His-tagged WT PS II core complexes, and the His-tagged PS II core complexes from Pdscap1 and Pdscap2 mutant strains of *Synechocystis* sp. PCC 6803. The

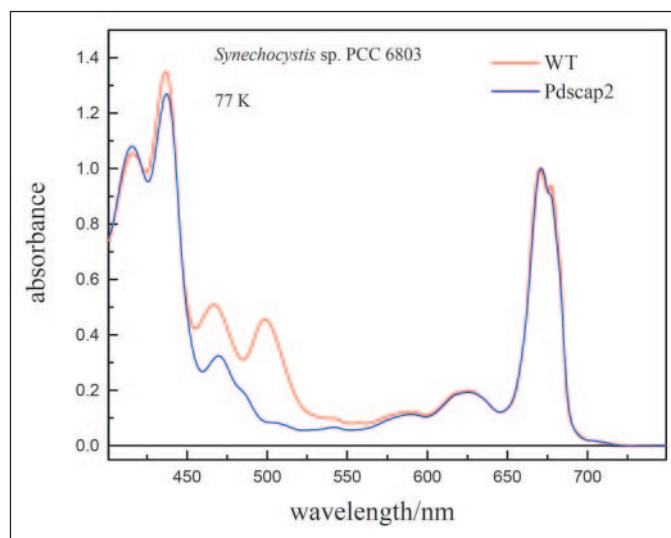


FIGURE 3. Visible range absorbance spectra of WT and Pdscap2 PS II core complexes measured at 77 K. The data were normalized at $\lambda_{\max}$ in the Qy region of the spectrum.

pigment compositions were normalized to two pheophytin *a* (Pheo *a*) molecules, because it is generally accepted that each PS II reaction center contains two such molecules (39). Both the WT and mutant core complexes contain ~ 38 Chl *a* molecules as reported by Tang and Diner (32) in their determination of the pigment content of non-His-tagged WT PS II core complexes.

HPLC analyses of carotenoids in WT PS II core complexes from *Synechocystis* sp. PCC 6803 cells have identified the main carotenoid as β -carotene and minor components as hydroxylated derivatives, including β -cryptoxanthin, zeaxanthin, and a trace amount of myxoxanthophyll or echinenone (4). Here, we find that the WT His-tagged PS II core

TABLE ONE

Chl *a* and carotenoid compositions of the purified His-tagged WT and carotenoid mutant PS II core complexes from *Synechocystis* sp. PCC 6803

Pigment component	Molar ratio
WT	
Pheo <i>a</i>	2
Chl <i>a</i>	37.9 ± 2.1
Carotenoid	12.6 ± 1.4
β-Carotene	11.3 ± 1.1
β-Cryptoxanthin/isocryptoxanthin	0.2 ± 0.1
Zeaxanthin	0.9 ± 0.2
Pdscap1	
Pheo <i>a</i>	2
Chl <i>a</i>	38.4 ± 1.6
Carotenoid	10.5 ± 0.8
β-Zeacarotene	4.7 ± 0.2
<i>cis</i> -β-Zeacarotene	2.1 ± 0.3
OH-β-zeacarotene	1.5 ± 0.1
Neurosporene	2.0 ± 0.1
ζ-Carotene	0.2 ± 0.1
Pdscap2	
Pheo <i>a</i>	2
Chl <i>a</i>	38.6 ± 1.4
Carotenoid	13.2 ± 1.3
β-Zeacarotene	8.3 ± 0.4
<i>cis</i> -β-Zeacarotene	0.7 ± 0.2
OH-β-zeacarotene	1.9 ± 0.1
Neurosporene	1.9 ± 0.4
ζ-Carotene	0.4 ± 0.2

complexes contain ~13 carotenoid molecules dominated by β-carotene. The other carotenoids present in the WT His-tagged PS II core complexes include β-cryptoxanthin and zeaxanthin. All three molecules have the same number of conjugated double bonds, 11, and have identical UV-visible spectra. Pigment analyses of PS II reaction center preparations have identified two β-carotene molecules bound to the D1 and D2 polypeptides (18, 19, 22). Both of these β-carotenes have been reported to be redox-active (6, 11).

HPLC analyses of the carotenoid content of the PS II core complexes from Pdscap1 and from Pdscap2 (Fig. 4 and TABLE ONE) show both the complete absence of β-carotene and the presence of a major carotenoid (peak 7, λ_{max} 406, 428, and 454 nm) that has an absorbance spectrum blue-shifted by ~16 nm and with less sharp features than neurosporene (peak 6, λ_{max} 416, 441, and 469 nm), both indicative of end cyclization (37). Either one or both ends of neurosporene might be cyclized to form β-zeacarotene or 7,8-dihydro-β-carotene, respectively, which have nearly identical spectra. To distinguish between these possibilities, two plasmids that in concert produce 7,8-dihydro-β-carotene, were used to transform *Escherichia coli* strain BL21(DE3) (Invitrogen). The first plasmid, pAC-NEUR (37), produces neurosporene, whereas the second plasmid, a full-length *Arabidopsis* β-cyclase cloned as an in-frame fusion in pBluescript SK- (40), transforms neurosporene to mainly 7,8-dihydro-β-carotene. Transformed *E. coli* cells were extracted with 50:50 (v/v) acetone/methanol mixture in the dark, after which the extract was run in the HPLC under the same conditions as the pigment extracts of the PS II core complexes (see "Materials and Methods"). We observed that the principal carotenoid peak in Pdscap2 (Fig. 4, peak 7) with a spectrum analogous to β-zeacarotene or 7,8-dihydro-β-carotene elutes much earlier (32 min) than the 7,8-dihydro-β-carotene

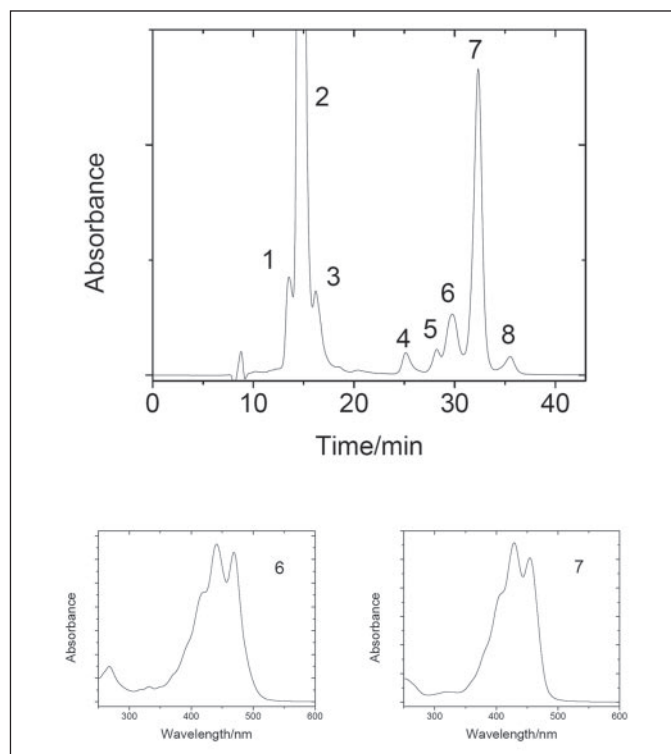


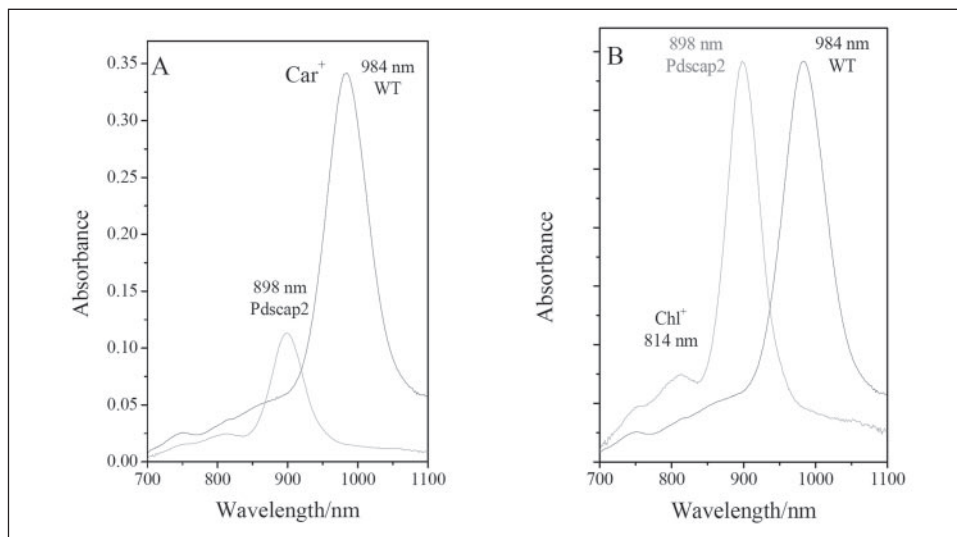
FIGURE 4. An HPLC chromatogram, detected at 430 nm, of a total pigment extract of the Pdscap2 core complexes, performed in isocratic mode using methanol:ethyl acetate (68:32, v/v). The components were identified as follows: 1, Chl derivative; 2, Chl; 3, OH-β-zeacarotene; 4, pheophytin; 5, *cis*-β-zeacarotene; 6, neurosporene; 7, β-zeacarotene; 8, ζ-carotene. The absorbance spectra are shown for components 6 and 7.

standard (eluting at 46 min, not shown) under the same HPLC conditions. As peak 7 could only be β-zeacarotene or 7,8-dihydro-β-carotene, we therefore assign to β-zeacarotene the principal carotenoid expressed. We conclude that in *Synechocystis* sp. PCC 6803, as in the case of *Synechococcus* sp. PCC 7942, only one end of neurosporene can be cyclized by lycopene cyclase, likely because of the absence of a double bond in the C7–C8 position of neurosporene (37).

The PSII core complex of the Pdscap2 strain contains about one-third more *cis*- plus *trans*-β-zeacarotene than does that of Pdscap1 (TABLE ONE), most probably because the former has two copies of the *crtI* gene of *R. capsulatus*. This observation is a reflection of the increased expression of β-zeacarotene on a per chlorophyll basis observed by HPLC analysis (at 430 nm) of total pigment extracts in whole cells of Pdscap2 compared with Pdscap1 (not shown). Other minor carotenoid components were also identified in the Pdscap1 and Pdscap2 PS II core complexes, including OH-β-zeacarotene, neurosporene, and ζ-carotene (Fig. 4). The presence of ζ-carotene (7 conjugated double bonds) can be readily explained in Pdscap1, because the native *pds* gene is still present in that strain where it catalyzes the formation of ζ-carotene from phytoene. However, there is still some ζ-carotene produced in the Pdscap2 strain where the native *pds* gene has been replaced by the *crtI* gene of *R. capsulatus*. It is conceivable that the dehydrogenation of phytoene by *R. capsulatus* CrtI is a two-step reaction: formation of ζ-carotene from phytoene, followed by the conversion of ζ-carotene to neurosporene. Some of the first reaction product may dissociate from the enzyme before the second reaction occurs. Consistent with this suggestion is the detection of trace amounts of β-zeacarotene in *R. capsulatus*.

Carotenoid and Chlorophyll Oxidation—PS II core complexes from the *Synechocystis* WT and Pdscap2 strains were manganese-depleted

FIGURE 5. Near-IR light-minus-dark difference spectrum of Pdscap2 PS II core complexes following a series of 160 laser flashes (0.033 Hz) at 20 K. The measurements were made on samples with the same Chl concentration, 0.3 mg Chl/ml (A) and normalized to the absorbance maximum of Car^+ (B).



(see “Materials and Methods”) and pre-treated with 100 μM potassium ferricyanide to pre-oxidize Cyt b_{559} . Carotenoid is oxidized by P_{680}^+ in PS II following the formation of $\text{P}_{680}^+ \text{Q}_\text{A}^-$ at low temperature under conditions where normal donor side electron transfer is blocked. At cryogenic temperatures, only one stable charge separation is possible per reaction center, because Q_A can only be reduced once to form the radical anion. Therefore, the maximum number of radical cations (Car^+ and Chl^+ combined) is one per reaction center. Fig. 5A shows the near-IR light-minus-dark difference spectrum of Car^+ and Chl^+ formed in both WT and Pdscap2 mutant core complexes, suspended at the same chlorophyll concentration, after a series of 160 saturating laser flashes (Nd:YAG laser at 532 nm, one flash/30 s) at 20 K, at which point the radical cation concentrations have reached a steady state. The Chl^+ largely contributes to the absorption at just above 800 nm with an extinction coefficient of about $6000 \text{ M}^{-1}\text{cm}^{-1}$ (41). The β -carotene Car^+ spectrum in the WT PS II core complex has an absorbance maximum at 984 nm, in agreement with previous results (2, 5, 11) and with a much higher extinction coefficient (ranging from 6.3×10^4 to $2.1 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$) (42–45). The Car^+ spectrum in the Pdscap2 mutant complex (Fig. 5), on the other hand, has an absorbance maximum at 898 nm, consistent with both the absence of β -carotene and the oxidation of a carotenoid species with nine conjugated double bonds. These observations are in agreement with the observed carotenoid composition of the sample (TABLE ONE).

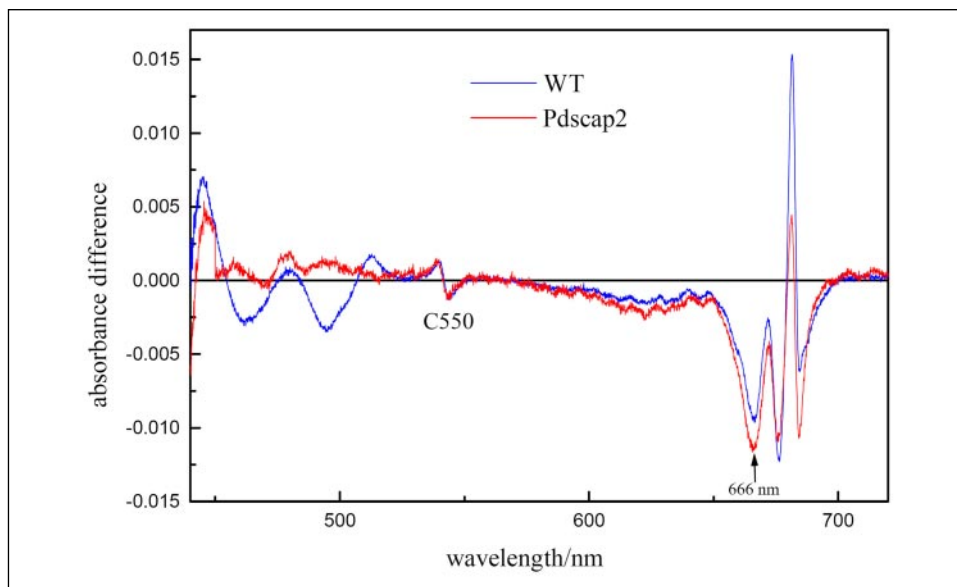
Fig. 5A, as well as experiments in a subsequent paper in preparation,⁵ show that the accumulation of Car^+ per center in the Pdscap2 cores is only ~25–35% of that of WT on a per chlorophyll basis. The apparently lower Car^+ yield in the mutant compared with WT can arise from a number of sources. 1) There may be a greater percentage of inactive centers in the mutant resulting in lower Car^+ formation on a per chlorophyll basis. 2) The extinction coefficient of the β -zeacarotene Car^+ cation radical could be less than that of β -carotene. 3) Carotenoid oxidation by P_{680}^+ in the mutant could be slower in the mutant, competing less well against $\text{P}_{680}^+ \text{Q}_\text{A}^-$ charge recombination, producing a lower Car^+ flash yield. 4) The oxidized carotenoid could, as a result of its higher reduction potential, oxidize other species, including chlorophyll, and so accumulate less Car^+ but more Chl^+ than in WT.

The following experiments and arguments provide some tests of these possibilities. 1) Single saturating flash measurements of photoreducible Q_A in PS II core complexes, measured in terms of $\text{Q}_\text{A}^-/\text{Chl}$, indicate a 20% lower ratio in the mutant than in WT. This observation implies that ~80% of the reaction centers in the mutant are active for charge separation, providing only a partial explanation for the deficiency in the formation of Car^+ in the Pdscap2 cores. The presence of some inactive centers in the mutant may either reflect an impairment of center assembly *in vivo* or a more facile loss of Q_A during purification of the centers. 2) There is very little information in the literature on the extinction coefficients of carotenoid cation radicals. However, based on a study of radical cations of linear polyenes (46), there should be a general tendency toward a decrease in extinction coefficient of the carotenoid cation radicals with decreasing extent of π -conjugation. Jeevarajan *et al.* (45) have compared, in the infrared, the extinction coefficients of the monocation radicals of 7',7'-dimethyl-7'-apo- β -carotene (10-conjugated double bonds) and β -carotene (11-conjugated double bonds). Although the extinction coefficient of the neutral form of the former is 11% less than that of the latter, the extinction coefficients of the cation radicals are similar ($6.3 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$). β -Zeaxanthin has one fewer conjugated double bonds than does 7',7'-dimethyl-7'-apo- β -carotene, it is likely therefore that the extinction coefficient of the cation radical of β -zeaxanthin is somewhat less than that of β -carotene. How much less cannot be estimated precisely, although certainly less than a factor of two based on a comparison with linear polyenes (46). 3) After correction for the inactive centers,⁵ the flash yield of stabilized charge separation ($[\text{Q}_\text{A}^-]/\text{flash}$) in a series of light flashes is identical for the WT and Pdscap2 strains indicating that the rates of oxidation of carotenoid by P_{680}^+ and of charge recombination are not perturbed by the change in carotenoid composition. 4) Experimental evidence that the difference in the carotenoid cation yield for the Pdscap2 mutant and WT core complexes is at least in part due to the enhanced oxidation of Chl at the expense of Car^+ in the mutant is provided below.

Fig. 5B shows, following normalization to the Car^+ absorption, that the ratio of Chl^+ (represented by the absorbance peak at 814 nm in both the WT and mutant) to Car^+ , generated upon illumination is about 2-fold higher in the mutant than in the WT. Also the absorbance peak at 814 nm is more marked in the mutant than in the WT. Assuming that the extinction coefficient of the radical cation of β -zeaxanthin is >50%

⁵ E. Schlodder, J. A. Bautista, C. A. Tracewell, F. X. Cunningham, B. W. Brudvig, and B. A. Diner, in preparation.

FIGURE 6. Continuous light-minus-dark difference spectra following illumination at 77 K for the WT and Pdscap2 PS II core complexes normalized to the C550 signal arising from Q_A^- .



of that of β -carotene, it is likely that at least some of the Car^+ is lost to the oxidation of Chl^+ . Fig. 6 shows a visible light-minus-dark difference spectrum of PS II core complexes recorded at 77 K for WT and the Pdscap2 mutant following illumination with continuous light for ~ 120 s in the presence of 2 mM $K_3Fe(CN)_6$. The spectra are normalized to the C550 signal, an electrochromic band shift of pheophytin that is a linear indicator of Q_A^- formation (47). As shown in Fig. 5B, the experiment indicates an enhanced oxidation of Chl_Z (~ 1.25 times at 666 nm) (48) in the mutant as compared with WT. Both of these observations indicate a displacement of the $Car^+Chl_Z \rightleftharpoons CarChl_Z^+$ equilibrium to the right in the Pdscap2 mutant, consistent with the presence in the mutant of shorter π -conjugated chain carotenoids with higher reduction potentials.

Variation of Car^+ Spectra with Temperature of Illumination—The near-IR absorbance spectra of the carotenoid cations of WT *Synechocystis* and Pdscap2 mutant PS II core complexes were also determined following a series of 160 laser flashes given at different temperatures (Fig. 7). The laser flash illumination was performed at the same temperature at which the spectra were recorded. The yield of Car^+ is observed to decrease as the temperature increases, which likely stems from the faster rate by which Car^+ is reduced at higher temperatures, by charge recombination with Q_A^- (5) and by reduction by Chl . The absorbance maximum of the Car^+ absorption band in the Pdscap2 sample is seen to red shift from 898 nm at 20 K, to 907 nm at 120 K, and to 913 nm at 160 K, whereas in the WT, at the same temperatures, the absorbance maximum increases from 984 nm, to 993 nm, and to 996 nm, respectively. The bandwidth (full width at half maximum) is also observed to increase with temperature. Previous studies of WT PS II core complexes have shown that the shift and broadening is due to two overlapping Car^+ species (11).

The shift in absorbance maximum as a function of temperature may be due to inhomogeneous broadening of a single carotenoid cation or to the generation of a different population of carotenoid cations. To test the origin of the shift with temperature, the Pdscap2 sample was illuminated at a higher temperature, 160 K, and then cooled to 20 K. Re-illumination at 20 K resulted in the formation of additional Car^+ having an absorbance maximum of 900 nm (data not shown). Therefore, the peak, which forms at warmer temperature, is an independent species from the peak formed at 20 K. The Pdscap2 PS II sample was also illuminated at 160 K by white light for initially 30 s followed by increasing time incre-

ments (see Fig. 7C). Gaussian deconvolution of the Pdscap2 spectrum at 160 K demonstrates that the carotenoid radical cation absorbance feature is composed of three absorbance bands centered near 900 nm, 940 nm, and 990 nm. For the interpretation of these spectra, see below.

Decay of Car^+ —The ability to generate Car^+ in PS II core complexes containing carotenoids with different reduction potentials provides a handle by which to investigate the mechanism of Car^+ reduction. The decay of the Car^+ species was measured in the dark at the Car^+ absorbance maximum following laser-induced Car^+ formation. Both the WT and Pdscap2 mutant PS II core complexes were studied as a function of temperature (Fig. 8 and replotted as a normalized comparison of mutant and WT in Fig. 9). Tracewell *et al.* (5) have shown in WT *Synechocystis* PS II core complexes, that Car^+ is reduced mainly by Q_A^- and that the rate of reduction increases with temperature. Fig. 8 shows that the decay profiles of both the WT and Pdscap2 samples are multiphasic, indicating inhomogeneity in the Car^+ environments. The faster decay rates at higher temperature indicate that Car^+ reduction is thermally activated, probably reflecting a charge reorganization that accompanies charge recombination and diversity in the locations of the carotenoids that are involved in recombination. Paradoxically, the carotenoid cation appears to decay more slowly in the Pdscap2 mutant strain than in WT.

DISCUSSION

Importance of the β -Ionylidene Ring—Although able to assemble PS I complexes, the Δpds and Δzds strains of *Synechocystis* sp. PCC 6803 are completely devoid of PS II. The absence of PS II in these deletion strains is most likely a consequence of the inability of the mutants to assemble PS II centers in the absence of β -carotene. It is likely that neither phytoene nor ζ -carotene can substitute for β -carotene in PS II assembly. However, both the Pdscap1 and the Pdscap2 strains are able to accumulate PS II with the Pdscap2 mutant strain producing more ζ -carotene and more PS II than strain Pdscap1. The carotenoid composition of PS II core complexes isolated from Pdscap2 is higher in β -zeacarotene, and we were also able to isolate more PS II cores per cell from this strain than from cells of Pdscap1. These results suggest that carotenoids play a structural role in PS II assembly and that the presence of a carotenoid containing at least one β -ionylidene ring is a necessary condition for such assembly. The chlorophyll to pheophytin ratio of the His-tagged Pdscap1 and Pdscap2 PS II core complexes is the same as that from the His-tagged WT strain. This observation suggests that the $Chl a$ mole-

FIGURE 7. Near-IR light-minus-dark spectra of WT (A) and Pdscap2 (B) illuminated at 20, 120, and 160 K under the same conditions as in Fig. 5. Pdscap2 illuminated at 160 K by white light from a 150-watt lamp for 30 s, 1 min, 2 min, 5 min, 10 min, 15 min, and 20 min shown with 5 Gaussian curves fit simultaneously to the spectra (C). The width and wavelength position of the 5 Gaussian curves are the same for all fits. Gaussian components of fit and sum of Gaussians for each spectrum are shown in D.

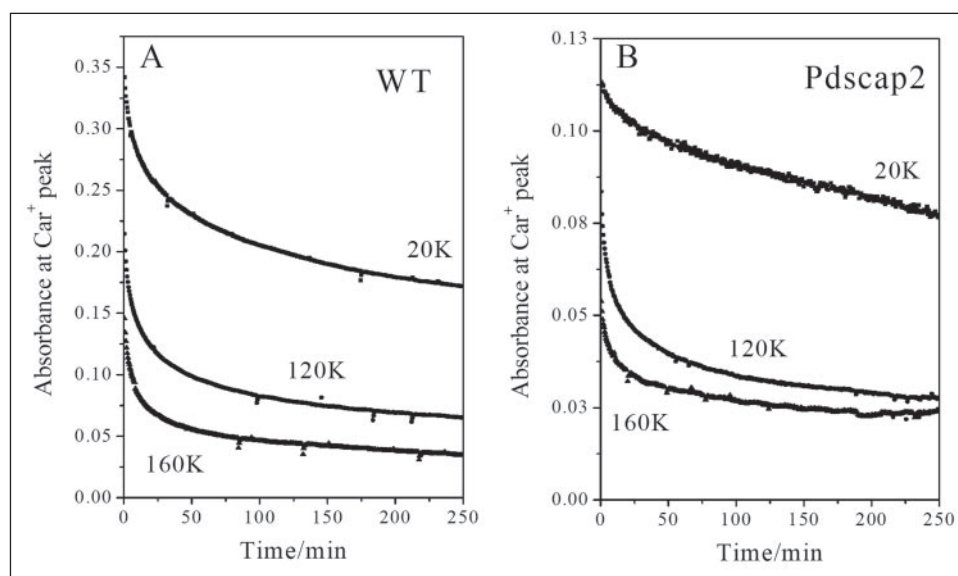
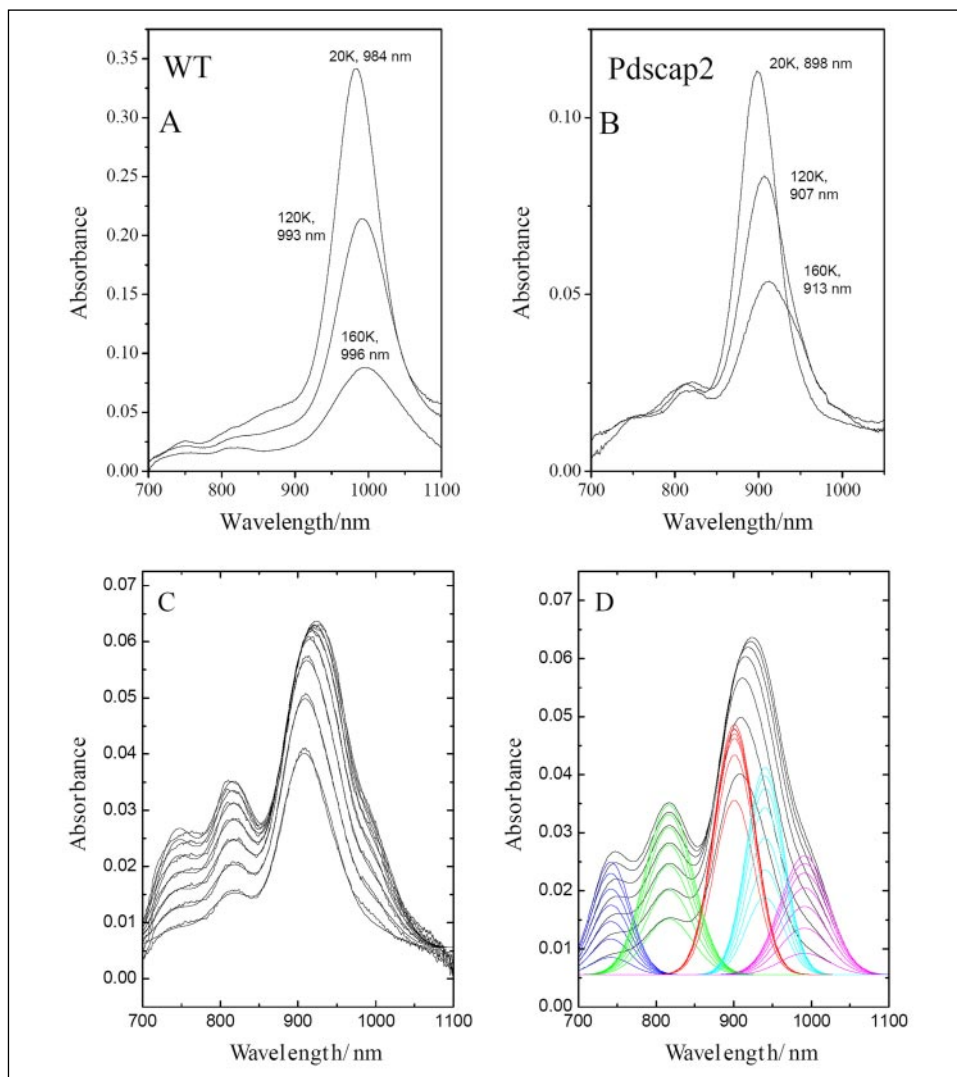
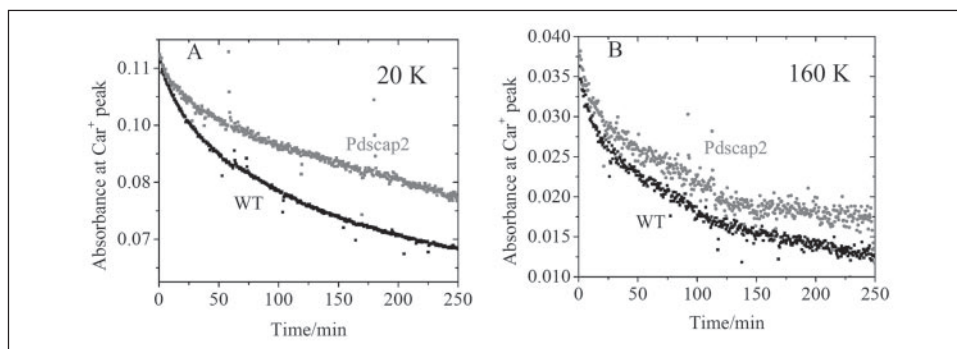


FIGURE 8. Decay of Car^+ in the dark after illumination by 160 flashes (0.033 Hz) at different temperatures for WT (A) and Pdscap2 (B) PS II core complexes. The sample was illuminated, and the spectra were collected at the specified temperatures. For A: $\lambda = 984$ nm at 20 K, 993 nm at 120 K, and 996 nm at 160 K. For B: $\lambda = 898$ nm at 20 K, 907 nm at 120 K, and 913 nm at 160 K. The measurements were made on samples with the same Chl concentration, 0.3 mg/ml.

cules are tightly bound in the PS II core complexes and that this tight association with the protein environment is not affected by the replacement of β -carotene with β -zeacarotene. Thirty-five to thirty-six chlo-

rophylls were reported in the x-ray crystallographic structures of the PS II core complexes from *Thermosynechococcus elongatus* and *vulcanus* resolved to 3.2–3.8 Å (12–15). This number is slightly smaller than the

FIGURE 9. Direct comparison of the decay kinetics of Car^+ in the WT and double replacement mutant at 20 K (A) and 160 K (B). For 20 K, WT $\lambda = 984$ nm and Pdscap2 $\lambda = 898$ nm, and for 160 K, the WT $\lambda = 996$ nm and Pdscap2 $\lambda = 913$ nm.



38 Chl *a*/PS II we measure, but within the range of uncertainty of the values reported here. The difference could reflect residual uncertainty in the x-ray structures, species differences, or different preparation methods. There are small differences in the low temperature absorbance spectra of the core complexes in the Q_Y region. A peak present in the WT complex at 677.5 nm is reduced to a shoulder in the mutant strains, suggesting that there is a change in the environment of a small fraction of the chlorophylls within the complex. The location of this chlorophyll or chlorophylls is not known. The overall similarity in structure of the His-tagged WT and Pdscap2 PS II core complexes as suggested by the cofactor content suggests that the PS II pigment-protein complex in the Pdscap2 cells is intact, allowing us to evaluate the consequences of the change in redox properties of the carotenoid cofactors on the secondary electron transfer pathways.

Blue-shifted Carotenoid Cation Spectrum—Illumination of the Pdscap2 PS II sample at 20 K results in the formation of a near-IR absorbance band at 898 nm instead of at 984 nm where the Car^+ peak is observed in WT PS II, a shift of 86 nm (see Fig. 5). In a study of chemically generated carotenoid cation radicals, Deng *et al.* (49) observe a blue shift of approximately ~ 48 nm for each incremental decrease in the total number of conjugated double bonds for a series of carotenoids having 12, 11, and 10 conjugated double bonds. This predicts an approximate 96 nm blue shift in the absorption max for a decrease in conjugation by two double bonds and implies that the Car^+ in the mutant has only 9 conjugated double bonds. Although the blue shift of the Car^+ peak observed here is 10 nm less than the predicted value, it is likely that the absorption spectrum of the carotenoid radical cation also depends on the dielectric environment and conformation of the carotenoid (degree of twist that reduces the π - π conjugation) in the protein. The near-IR absorption maxima of β -carotene radical cations (β - Car^+) observed in spinach and WT *Synechococcus* and *Synechocystis* PS II samples have been observed in the range of 980–1000 nm (11). The terminal double bonds of β -carotene are located within the β -ionylidene rings, which are forced out of the plane of conjugation with the rest of the polyene chain because of steric repulsion between the methyl groups on the ring and the polyene chain. The absorbance spectrum of β - Car^+ is thus less red-shifted than it would be if the terminal double bonds were not associated with the β -ionylidene rings (see Fig. 2).

According to the HPLC analysis (Fig. 4 and TABLE ONE), the Pdscap PS II core complexes contain two types of carotenoids that have 9 conjugated double bonds: neurosporene and β -zeacarotene. The initial carotenoid photooxidized is likely bound to the D1/D2 polypeptides and is known to be β -carotene in WT PS II reaction centers. The apparent selectivity of the carotenoid binding site for a β -ionylidene ring suggests that β -zeacarotene, which has one β -ring, and not neurosporene, which is linear, would be bound to the D1/D2 polypeptides.

Reduction Potentials for Carotenoid Oxidation—Reduction potentials for carotenoid radical cations have been reported by several groups

measured in different environments. The reduction potential for β - Car^+ has been reported for β -carotene dissolved in micelles prepared from mixtures of Triton X-100 and Triton X-450. This hydrophobic environment is similar to that of a membrane protein and results in values between 1.03 and 1.06 V *versus* normal hydrogen electrode (50, 51). Electrochemical oxidation has been measured in dichloromethane for a series of carotenoids containing 12, 11, or 10 conjugated double bonds. The reduction potential was found to decrease by ~ 45 mV per unit increase in the number of double bonds (49). Using these values, we estimate the reduction potential of a carotenoid radical cation containing nine conjugated double bonds to be between 1.12 and 1.15 V *versus* normal hydrogen electrode.

As mentioned above and in more detail elsewhere,⁵ the fraction of centers able to generate Car^+ plus Chl^+ on each of a series of light flashes is the same for PS II core complexes containing carotenoids having 9- or 11-conjugated double bonds. Earlier estimates of the reduction potential of P_{680}^+ were ~ 1.12 V (52), which is very close to the estimated value for the reduction potential of a carotenoid with 9 conjugated double bonds. More recently, a reassessment of the Pheo⁻/Pheo redox couple by Rappaport and coworkers resulted in an increase in the estimate of the reduction potential of P_{680}^+ to 1.26 V (20, 21). The independence of the amount of stabilized Car^+ plus Chl^+ radical to the degree of conjugation of the PS II carotenoids appears more consistent with the higher value.

Chlorophyll/Carotenoid Cation Equilibrium—A mixture of Car^+ and Chl^+ radical cations are formed in the Pdscap2 PS II core complexes, as observed previously in the WT. However, a striking difference between the WT and the Pdscap2 PS II core complex is the larger proportion of Chl^+ relative to Car^+ (Fig. 5) and of Chl^+ relative to Q_A^- (Fig. 6) observed in the mutant PS II sample. Although the higher $\text{Chl}^+/\text{Car}^+$ in the Pdscap2 mutant may be due in part to a lower extinction coefficient for Car^+ , the higher Chl^+/Q_A^- in Fig. 6 indicates clearly a displacement to the right of the redox equilibrium between the carotenoids and their neighboring chlorophylls: $\text{Car}^+\text{Chl} \rightleftharpoons \text{CarChl}^+$. As described above, a carotenoid radical cation containing 9 conjugated double bonds has a higher reduction potential than β -carotene. Therefore, the larger amount of Chl^+ in the mutant PS II is due to the higher reduction potential of the carotenoid in the mutant, which makes it a better oxidant of Chl than β - Car^+ , shifting the equilibrium to the right (Fig. 10).

Temperature Dependence of Carotenoid Oxidation—Tracewell and Brudvig (11) showed that the Car^+ band in WT *Synechocystis* can be fit by two Gaussians, one centered at 982 nm (Car_A^+) and another at 1027 nm (Car_B^+). Although both Car_A^+ and Car_B^+ are β -carotenes, the difference in the absorbance maxima was attributed to differences in the local environments of the two carotenoids. Car_A^+ is dominant at 20 K and decreases in intensity at higher temperatures. Car_B^+ is more stable at higher temperatures and becomes equal in intensity to Car_A^+ at around 150 K. A similar phenomenon appears to be at work in the

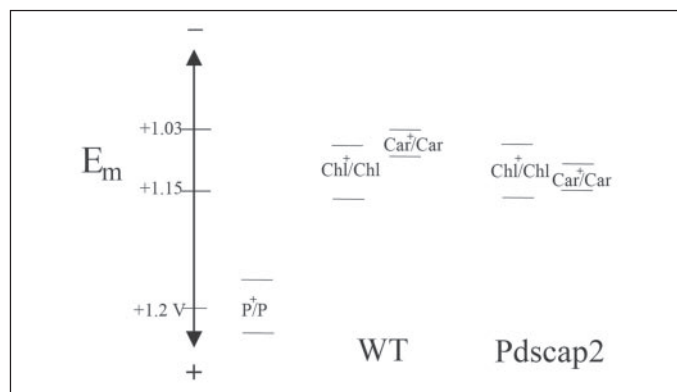


FIGURE 10. Relative positions of the reduction potentials for the PS II alternate pathway cofactors in WT and Pdsclap2 PS II core complexes.

Pdsclap2 PS II core complex with two or more carotenoid cation radicals contributing to the absorbance spectrum as the temperature increases. We observe a shift of the carotenoid radical absorbance maximum to longer wavelength and the peak broadens as the temperature of illumination is increased from 20 to 160 K. Gaussian deconvolution of the broad low energy tail on the Car^+ peak suggests that the shift in absorbance maximum is the result of the formation of two absorbance bands at ~ 940 and ~ 990 nm and a decrease in the 900 nm absorption band. These higher wavelength features cannot be due to more highly conjugated carotenoids as the Pdsclap2 core complexes do not contain carotenoids with more than 9 conjugated double bonds. It is likely that the 940 and 990 nm components correspond to cations derived from 9-conjugated double bond carotenoids in different environments. It is possible that at higher temperatures, electron transfer between the carotenoids in the D1/D2/Cyt b_{559} reaction center and those in CP43 and CP47 becomes energetically feasible resulting in the oxidation of a carotenoid bound to one of the more peripheral light harvesting proteins of the core complex. An increase in the width of the Car^+ absorbance peak was noted in WT PS II core complexes as the Car^+ species decayed and is likely an indication of a similar Car^+ heterogeneity (11).

There is evidence for carotenoid cation formation in PS II through a mechanism different from the one described here, and that is thought to occur both within and outside the PS II core complex. In higher plants, non-photochemical quenching of fluorescence occurs under conditions of illumination at high light intensity that produces an over-acidification of the thylakoid lumen. The major form of non-photochemical quenching occurs in the antenna and originates in the PsbS protein, where the quenching species is formed when violaxanthin (9 conjugated double bonds) is replaced by zeaxanthin (11 conjugated double bonds). The mechanism of non-photochemical quenching in PsbS involves the formation of a quenching species and a charge transfer complex involving a chlorophyll radical anion and carotenoid radical cation (53, 54). The formation of the radical pair in a chlorophyll/carotenoid heterodimer charge transfer complex requires that the reduction potential of the carotenoid be sufficiently low to be apt for oxidation, with the 11-double bond zeaxanthin easier to oxidize than the 9 double bond violaxanthin. Recently, a zeaxanthin-independent non-photochemical quenching mechanism localized in the PS II core complex was reported (55) and suggested to occur by a similar mechanism. It is likely that the sensitivity to light observed in the Pdsclap1 and Pdsclap2 mutant strains reflects a decreased capacity for the quenching of Chl triplet states, although an increased difficulty in generating a Chl^-Car^+ radical pair as the product of the quenching reaction upon substituting β -zeaxanthene for β -carotene could contribute as well.

Reduction of Car^+ by Q_A^- —As described above, Car^+ reduction may occur by charge recombination or Chl^+ formation. According to Ferreira *et al.* (14), Car 48 is within 7 Å of Chl_{ZD2} and at its closest point ~ 21 Å from P_A^+ (the principal location of the P^+ cation). Car 53 is about the same distance from P_A^+ but at 26 Å from its closest point to Chl_{ZD2} . Car 48 and 53 are within 6 Å of each other at their closest points. In light of these distances, it would appear likely that Chl_{ZD2} oxidation by Car^+ 48 is faster than its own oxidation by P^+ (via Car 53 or not) making it likely that Chl_{ZD2} oxidation occurs simultaneously with Car^+ formation. The timescale of minutes and hours that we observe for the decay of Car^+ (Figs. 8 and 9) is very likely the result of charge recombination with Q_A^- . The mechanism of charge recombination between the donor and acceptor sides of the PS II reaction center is thought to occur through $\text{P}_{680}^+\text{Q}_\text{A}^-$. Were this route to apply to Car^+ decay, then the recombination would be expected to proceed as shown in Reaction 1 at temperatures low enough to prevent electron donation by tyrosine Y_Z . Tyrosine Y_D is presumed to be already oxidized and to be too weak an oxidant to generate significant amounts of P_{680}^+ , and the secondary acceptor side quinone, Q_B , is missing from the core complexes.



Because β -zeacarotene has a higher reduction potential than β -carotene for cation formation, the foregoing equilibrium should shift to the right in the mutant, compared with WT, accelerating the rate of charge recombination. The decay kinetics of Car^+ in the WT and Pdsclap2 mutant PS II core complexes are directly compared at 20 K and 160 K in Fig. 9. This figure shows that, contrary to the prediction of the above reaction, Car^+ decays more slowly in the Pdsclap2 mutant than in WT. This observation indicates that Car^+ either does not equilibrate with P_{680}^+ or that it does not generate a sufficient equilibrated concentration of P_{680}^+ for $\text{P}_{680}^+\text{Q}_\text{A}^-$ to contribute significantly to Car^+ decay. We conclude that charge recombination is instead direct between Car^+ and Q_A^- , although possibly involving Pheo/Pheo $^-$ equilibrated with $\text{Q}_\text{A}/\text{Q}_\text{A}^-$.

The multiphasicity of charge recombination kinetics reflects the inhomogeneous composition of the PS II protein complex. This was observed previously for $\text{Car}^+\text{Q}_\text{A}^-$ recombination in WT PS II and was attributed to freezing the sample in multiple conformations that have different recombination rates. This is also occurring here, but we also note that the distribution of fast and slow decaying components is different for Pdsclap2 and WT PS II (Fig. 9). For the Pdsclap2 PS II sample, in addition to having a higher reduction potential of the carotenoid radical cation, the conjugation length of the carotenoid is also decreased. This reduces the distance over which it can act as an electron wire to bridge two redox components. In the case of β -zeacarotene, another point to consider is how the molecule binds to the β -carotene binding site. β -Carotene has C_2 symmetry. However, β -zeacarotene, with only one β -ionylidene ring, may be able to bind to each carotenoid binding site in either of two orientations. The distance between Q_A^- and the end of the conjugated chain would then depend on the orientation. Such heterogeneity of orientation would further contribute to the multiphasic decay kinetics, observed in the Pdsclap2 PS II core complex.

We have demonstrated that β -zeacarotene can replace β -carotene in PS II allowing assembly of this pigment-protein complex in *Synechocystis* sp. PCC 6803. We also show that P_{680}^+ is capable of oxidizing a carotenoid containing 9 conjugated double bonds (β -zeacarotene), consistent with recent estimates of a higher reduction potential for $\text{P}_{680}^+/\text{P}_{680}$. A consequence of the higher reduction potential of the carotenoid

cation generated in the mutant is that the amount of chlorophyll cation radical produced at the expense of the carotenoid cation radical is enhanced relative to what is observed in the WT. In a subsequent report in preparation,⁵ we will examine the kinetics and flash yield of oxidation of the carotenoid in the Pdscap2 mutant relative to WT, providing additional insights into the mechanism of carotenoid and chlorophyll oxidation.

Acknowledgments—We thank Dexter Chisholm for his technical advice on the construction of the mutants. We are also grateful to Dr. Harry Frank for his help in understanding carotenoid spectroscopy.

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-Conjugation than β -Carotene

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J. Biol. Chem. 2005, 280:38839-38850.

doi: 10.1074/jbc.M504953200 originally published online September 12, 2005

Access the most updated version of this article at doi: [10.1074/jbc.M504953200](https://doi.org/10.1074/jbc.M504953200)

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