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Analysis of chimeric spinach/cyanobacterial CP43 mutants of *Synechocystis* sp. PCC 6803: The chlorophyll-protein CP43 affects the water-splitting system of Photosystem II

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Mutants of the cyanobacterium *Synechocystis* sp. PCC 6803 have been generated in which parts of *psb*C (the gene encoding the Photosystem II chlorophyll-protein CP43) have been replaced with the homologous gene fragment from spinach. Upon the replacement of all but the 3′ 84 bp of the cyanobacterial *psb*C gene with the homologous fragment from spinach, an obligate photoheterotrophic mutant was generated. Two photoautotrophic derivatives of this mutant were made reincorporating 3′ cyanobacterial sequences back into the spinach *psb*C gene of the mutant. These two mutants are similar to each other, carrying a chimeric CP43 with the N-terminal half from spinach. These mutants are photosynthetically active at a rate of about half that of wild type, which correlates with a decreased Photosystem II/chlorophyll ratio in these mutants. Thylakoids from the chimeric mutants contain a CP43 protein which migrates slightly more slowly on SDS-polyacrylamide gels than the native *Synechocystis* CP43. Interestingly, these mutants show significant shifts in thermoluminescence peaks, reflecting altered thermodynamic properties of the back reaction between the acceptor side and the water-splitting system. On the basis of the oscillations of these shifts with number of flashes, we conclude that S_2 is stabilized and S_3 is destabilized in these mutants. This represents evidence for an involvement of CP43 in events associated with water splitting.

Introduction

In Photosystem II (PS II), the chlorophyll-binding proteins CP47 and CP43 function primarily as the interior chlorophyll-*a*-containing antenna polypeptides in higher plants, algae, and cyanobacteria (reviewed in Ref. 1). Aside from their role in light harvesting, CP43 and CP47 are needed for stable PS II assembly [2] and domains in a large hydrophilic loop in the C-terminal half of CP47 appear to have a close structural association with the PS II donor side [1].

From hydropathy analysis it has been suggested that both CP47 and CP43 contain six transmembrane regions [3,4], five of which are in the N-terminal half of the CP47 and CP43 proteins. These putative membrane-spanning regions are highly conserved between plants and cyanobacteria. Both proteins also have a large hydrophilic region between the fifth and sixth postulated membrane-spanning regions. For CP47 this region is thought to span residues 260 to 450 [1], and for CP43 the corresponding region seems to extend approximately from residues 245 to 410. At least for CP47 this region is thought to be shielded by the manganese-stabilizing protein (reviewed in Ref. 1).

Little is known about the functional contribution of the CP43 protein to PS II; interruption of *psb*C, encoding CP43, leads to a loss of water-splitting activity while the PS II reaction center appears to retain its function [2,5]. This suggests that CP43 is involved, directly or indirectly, in creation of the proper environment for the water-splitting complex. Also, loss of *psb*C expression results in a destabilization of the PS II reaction center complex [2].

To map potentially important regions of CP43, we chose to introduce a large number of potentially functional mutations at once rather than to do site-directed

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Abbreviations used: PS II, Photosystem II; CP, chlorophyll-binding protein; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-hydroxypropanesulfonic acid); PCC, Pasteur Culture Collection.

mutagenesis. To introduce a large number of mutations, we replaced the native *psb*C gene of the transformable, facultative photoheterotrophic cyanobacterium *Synechocystis* sp. PCC 6803 with the corresponding region from spinach. Simple specific mutagenesis procedures for *Synechocystis* sp. PCC 6803 have been established, and have been reviewed in Refs. 6, 7.

Our results demonstrate that replacement of the native *Synechocystis psb*C gene with that from spinach yields unstable and inactive PS II complexes in the cyanobacterium. This is in contrast to *psb*A, for which a higher-plant copy of the gene functionally replaces the cyanobacterial one [8]. However, chimeric *psb*C mutants, carrying the 5′ half of the spinach *psb*C gene (coding for the N-terminal half of CP43), show photoautotrophic growth, and properties of the water-splitting complex have been modified in such mutants.

Materials and Methods

Cyanobacterial cultures. Synechocystis sp. PCC (Pasteur Culture Collection) 6803 was grown at 30°C in BG-11 medium [9] under continuous illumination as described in Ref. 7. Mutants defective in PS II were propagated on media containing 5 mM glucose. Culture doubling times were determined as in Ref. 3.

Cloning of psbDI/C carrying cyanobacterial and spinach sequences. A pUC-derived plasmid was constructed containing 94% of *psb*C from spinach and flanked by complementing cyanobacterial sequences by utilizing two restriction sites, recognized by *Sau*I and *Sph*I, which are conserved in the *psb*DI/C operon from spinach and *Synechocystis*. The plasmid carries up- and downstream flanking regions from *Synechocystis*, along with a selectable kanamycin-resistance marker downstream of the operon. This plasmid, pKdelDIC + spi, is shown in Fig. 1.

Cyanobacterial transformations. Synechocystis transformation protocols have been described in Ref. 3. A *Synechocystis* mutant in which the *psb*DI/C operon had been deleted and replaced by a chloramphenicol-resistance cartridge [10] was transformed with pKdelDIC + spi to generate a mutant carrying *psb*DI/C at its native position, but with 94% of the *psb*C gene sequence coming from spinach. The *psb*DI/C-deletion mutant still carried *psb*DII, a functional and unlinked second copy of *psb*D. The *Synechocystis* mutant carrying most of the spinach *psb*C gene was an obligate photoheterotroph. This mutant was subsequently transformed with a plasmid, named pX3, which carried the 3′ half of *psb*C (coding for the C-terminal half of CP43) from *Synechocystis*, as well as sequences downstream of this gene (Fig. 1). Photoautotrophic transformants were selected. Cyanobacterial DNA isolation procedures were essentially identical to those described in Ref. 11.

*DNA amplification and sequencing. psb*C from chimeric *Synechocystis* mutants was amplified by PCR (polymerase chain reaction) for subsequent cloning and sequencing. PCR-amplified DNA fragments to be sequenced were cloned into M13mp18 and M13mp19 [12]. Single-stranded M13 DNA was obtained [13] and

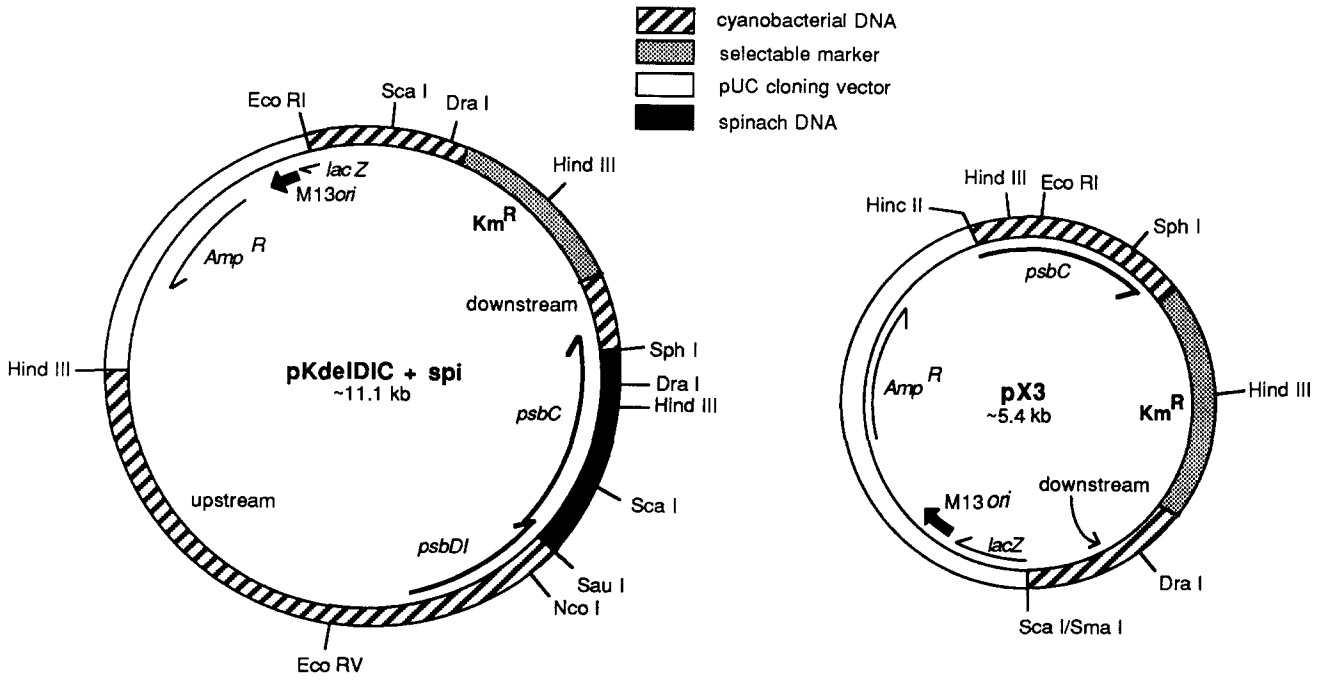

Fig. 1. Constructs used for the generation of *Synechocystis* mutants carrying parts of spinach *psb*C. The genes *psb*C and *psb*DI are indicated by arrows. The M13 origin of replication is indicated by a thick arrow. All relevant restriction enzyme sites are marked. pKdelDI/C+spi was generated in pUC119.

sequenced by the dideoxynucleotide chain termination method [14].

Southern blotting. For Southern hybridization analysis, DNA from agarose gels was transferred to GeneScreen Plus hybridization transfer membranes (NEN-Du Pont, Boston MA) and hybridized to ^{32}P-nick translated probes and washed essentially as recommended by the GeneScreen Plus manufacturer.

Thylakoid preparation. Cyanobacterial cultures in logarithmic phase were harvested, and thylakoids were prepared essentially as described in Ref. 15. The chlorophyll concentration was determined according to Ref. 16.

Immunodetection. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of thylakoid proteins, protein blotting onto nitrocellulose, and immunoreaction with polyclonal antibodies raised against spinach CP43, CP47, D2, and D1 was carried out as described in Ref. 2. For immunodetection, secondary antibodies conjugated with alkaline phosphatase (Bio-Rad, Richmond CA) were used, and alkaline phosphatase activity was detected with BCIP (5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt)/NBT (*p*-nitro blue tetrazolium chloride) staining.

Herbicide binding. To determine the diuron affinity and the number of binding sites for this herbicide on a chlorophyll basis (thus estimating the number of functional PS II reaction centers) in wild-type and mutant cells, binding of [^{14}C]diuron (Amersham; 243 μCi/mg) to intact cyanobacterial cells was measured according to a procedure similar to that in Ref. 17.

Fluorescence emission. Fluorescence emission spectra were recorded with a SPEX Fluorolog 2 scanning spectrometer.

Oxygen evolution. To measure oxygen evolution rates of intact cells, cyanobacterial cultures in logarithmic phase were harvested and resuspended in 25 mM Hepes (pH 7.0) to a concentration of 10 μg/ml chlorophyll. Oxygen evolution was measured upon illumination with a saturating light intensity (7000 μE m^{-2} s^{-1}; $\lambda \geq 560$ nm) in the presence of 0.25 mM 2,6-dimethyl-*p*-benzoquinone.

Thermoluminescence. The home-made apparatus used for thermoluminescence was constructed based on those operating at the Solar Energy Research Group at RIKEN [18] and at the Academy of Sciences in Szeged [19]. The apparatus consists of a fixed teflon-insulated copper stage (2 cm in diameter); before each measurement, a disk of Kodak lens-cleaning paper of the same diameter was placed on the stage to facilitate even spreading of the sample, and the sample (up to 400 μl of cell suspension) was applied. The sample compartment was then closed using a transparent plastic window 2 mm above the sample to prevent water vapors to reach the photomultiplier.

Wild-type and mutant cells to be used for thermoluminescence measurements were grown in BG-11 medium supplemented with 5 mM glucose and were harvested in their late-logarithmic growth phase. Cells were washed once and resuspended in 25 mM Hepes/NaOH (pH 7.0). Cells were dark-adapted at room temperature for approximately 2 h, and then aliquoted in dim light into tubes on ice containing glycerol, yielding a final concentration of 15% (v/v) glycerol and 75 μg/ml chlorophyll per 400 μl volume. After an additional incubation on ice of 30 min or more in complete darkness, 400 μl aliquots were transferred to the thermoluminescence stage in very dim light, and were dark-adapted for 3 min at 20–30°C before cooling of the stage with liquid nitrogen. Saturating light flashes (from an EG&G type FX-124 Xenon arc bulb powered by a PS-350 power supply at 1000 V using a 1 μF capacitor; 0.7 J/flash; 3–5 μs duration) were given at 1–2 Hz when the stage had reached −5°C. At the end of the flash train, the stage temperature was between −5 and −10°C (depending on the length of the flash train). After further cooling of the stage, a photomultiplier tube (EMI 9558) was brought over the sample replacing the flash lamp. The PM tube was protected by a Compur shutter. The distance between the phototube and sample was 12 mm. The apparatus was mounted in a closed metal box, and all operations after the sample was placed were carried out by external levers.

The stage was cooled by passing liquid N_2 through its base, and linear heating was obtained by a computer-controlled heating body in the base. The signal from the phototube as well as the stage temperature were processed by an IBM-compatible computer, and were stored for further analysis.

Results

Chimeric psbC mutants

By introducing altered *psb*C gene sequences into *psb*C deletion mutants, modified *psb*C can be directly incorporated into the *Synechocystis* genome by homologous recombination in the regions around the gene. Using the gene replacement scheme described in Fig. 2, and utilizing the facts that a *Sau* I site just upstream from the *psb*C start codon and a *Sph* I site about 80 bp before the end of the *psb*C gene have been conserved between spinach and *Synechocystis* sp. PCC 6803, we have generated a cyanobacterial mutant (KdelDI/C + spi) containing a hybrid *psb*C gene with all but 84 nucleotides at the 3′ end (94% of the gene) from spinach. In addition, the 3′ 30 nucleotides of *psb*DI (coding for the C-terminal end of D2) also originate from spinach (the 3′ end of *psb*DI overlaps with the 5′ end of *psb*C). Even though this region of 30 nucleotides in *psb*DI from spinach is different at three bases compared to that from *Synechocystis*, the *psb*DI

sequences from the two organisms encode identical amino acids in this region. A Southern blot of genomic DNA from wild type and the KdelDIC + spi mutant probed with a 3′ fragment of *psb*C is shown in Fig. 3 to illustrate that the plasmid construction was properly incorporated into the genome of the deletion mutant.

The KdelDIC + spi mutant, with most of its *psb*C sequence of spinach origin, was found to be an obligate photoheterotroph. Photoautotrophic growth of this mutant could be restored by transformation with pX3, a plasmid containing the 3′ 50% of *psb*C from *Synechocystis* along with downstream sequences (Fig. 1).

After transformation with pX3, photoautotrophic transformants were selected. Two of the mutants thus created, X3M and X3S, were used for further analysis. These mutants showed a more than 2-fold decrease in photoautotrophic growth rate as compared to wild type (Table I). From herbicide-binding experiments it appears that these mutants have a decreased number of binding sites (and thus functional PS II centers) on a chlorophyll basis as compared to wild type, while the diuron affinity remains unchanged (Table I); this suggests that the remaining PS II centers are structurally normal. The decreased number of PS II reaction centers on a chlorophyll basis contributes to or may be the major reason for the decreased photoautotrophic growth rate in the mutants; this decrease of the relative amount of PS II in X3M and X3S leads to a corresponding decrease in the rate of oxygen evolution at saturating light intensity (Table I), but does not lead to a significant change in the amount of light needed to half-saturate oxygen evolution (not shown). This strongly suggests that the remaining PS II centers in X3M and X3S functionally are quite normal. The decreased PS II content of the thylakoids is in agreement with the data obtained from Western blots of thylakoid proteins from wild type and mutants (Fig. 4): a somewhat decreased intensity of cross-reactivity with antisera raised against spinach CP43 and CP47 was obtained in the X3M and X3S mutants as compared to in wild type. The obligate photoheterotrophic mutant KdelDI/C + spi (carrying spinach *psb*C) does not contain detectable amounts of CP43 in its thylakoids, and resembles mutant S2–1, which is a mutant in which *psb*C has been interrupted by a kanamycin-resistance marker. The CP43 protein from X3S and X3M thylakoids exhibits a slightly different migration rate and runs as a much sharper band on the gel as compared to

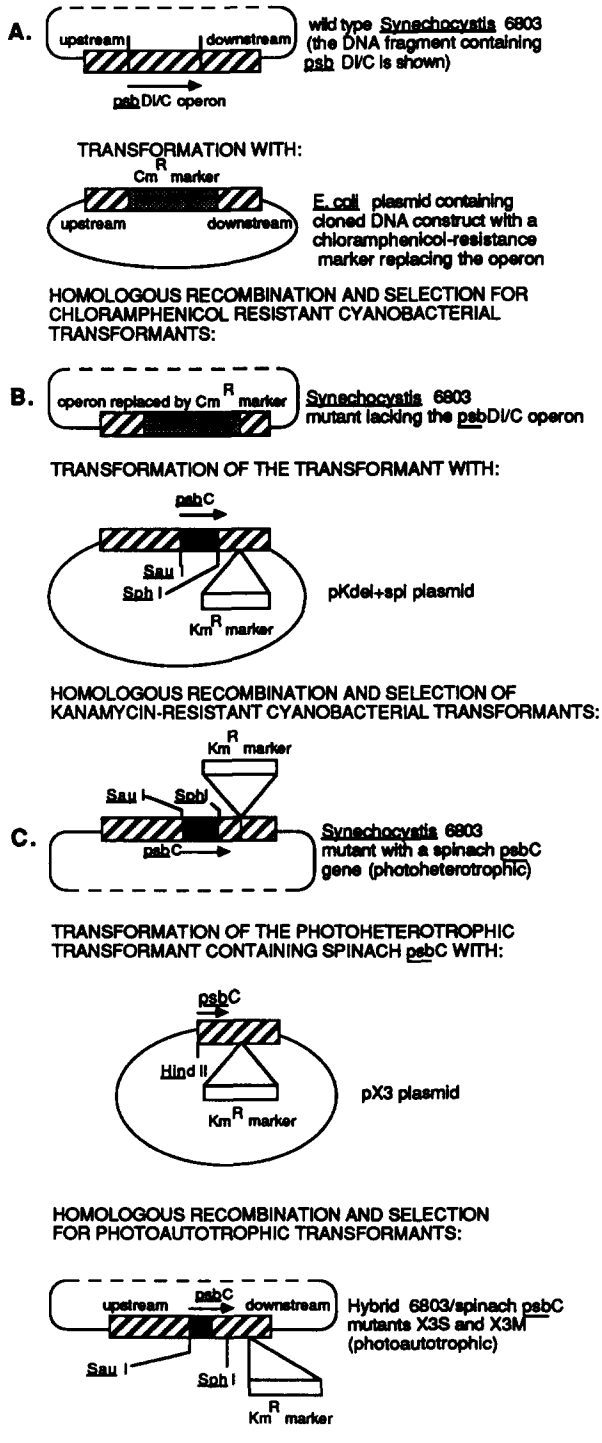

Fig. 2. Scheme showing the generation of the mutants X3S and X3M. The cross-hatched pattern indicates cyanobacterial sequences, and the dark shaded regions indicate a spinach origin. (A) The construction of the deletion mutant lacking *psb*DI/C. (B) Transformation of the deletion mutant with the spinach construct yielding obligate photoheterotrophic *Synechocystis* mutants which contain spinach *psb*C sequences between the conserved restriction sites, *Sau*I and *Sph*I (for more detailed maps see Figs. 1 and 3). The replacement of *psb*C between the *Sau*I and *Sph*I sites results in three mutations in *psb*DI as well, but does not lead to amino acid substitutions in D2. (C) The spinach mutant obtained in (B) can regain photoautotrophic potential by transformation with a plasmid, pX3, which contains the 3′ cyanobacterial *psb*C sequence starting at an internal *Hin*cII site (about 650 bases upstream of the *Sph*I site) and an extensive downstream region, which includes a kanamycin resistance marker at a position not interfering with *psb*C expression.

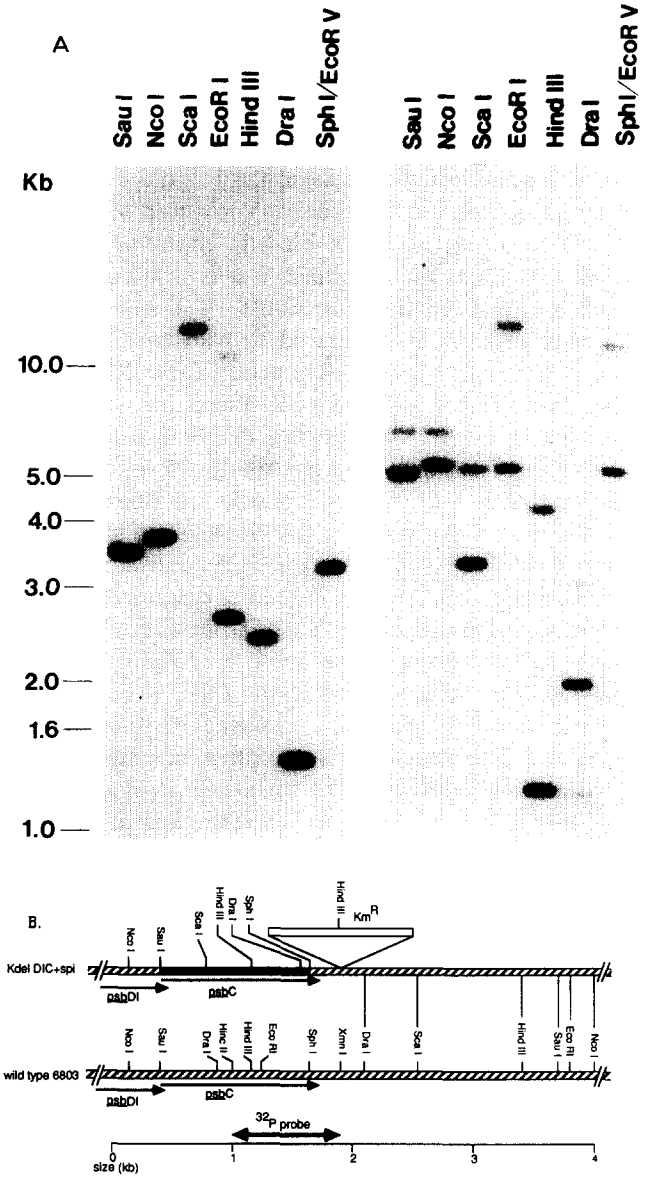

Fig. 3. (A) Southern blot analysis of genomic DNA of *Synechocystis* 6803 wild type (Wt) and the *psb*C spinach replacement mutant KdelDI/C+spi cut with the designated restriction enzymes and probed with a 0.9 kb internal portion of the 3′ end of *psb*C. (B) Relevant restriction enzyme sites in the *psb*DI/C gene region.

wild-type *Synechocystis* CP43; this may be due to the fact that a significant portion of this protein is of spinach origin. The apparent molecular mass of the spinach CP43 protein varies from 40 to 45 kDa [1]. Western blots probed with D1 and D2 antisera showed slightly decreased amounts of these PS II proteins in X3M and X3S as compared to wild type, whereas the amounts were significantly decreased in KdelDIC + spi and S2–1 (not shown).

psbC sequence of the chimaeric mutants

To analyze the location of the crossover events in *psb*C upon transformation of the spinach *psb*C mutant

TABLE I

Growth rates (doubling times) under photoautotrophic conditions (in BG-11 medium without glucose), oxygen evolution capacity at saturating light intensity, herbicide ([^{14}C]diuron) binding affinity, and binding site quantitation on a chlorophyll basis of wild-type Synechocystis and hybrid mutants

Strain	Doubling time (hours)	Oxygen evolution rate (μmol O_2 (mg Chl h)$^{-1}$)	Diuron dissociation constant (nM)	Chlorophylls per herbicide binding site
Wild type	11	310	13	800
X3M	23	160	13	1600
X3S	31	140	13	1400

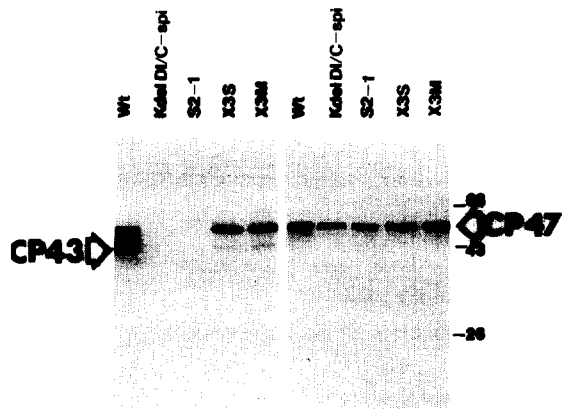

Fig. 4. Immunoblots of thylakoid proteins from wild type (Wt), the spinach replacement mutant (KdelDI/C + spi), cyanobacterial/spinach CP43 hybrids (X3S and X3M), and the *psb*C gene interruption mutant (S2–1). The blots were probed with antibodies against CP43 (left 5 lanes) and CP47 (right 5 lanes).

with pX3 giving rise to X3S and X3M, *psb*C from X3M and X3S was amplified and cloned into M13. Sequence analysis on these mutants indicates that the crossover point is in the region of the glycine codon 235 in *Synechocystis* sp. PCC 6803 (which is codon 236 in spinach) for both mutants (Fig. 5). Gly-236 in spinach is close to the end of putative helix IV of CP43; a topological model of CP43 and the location of introduced mutations in X3M and X3S is shown in Fig. 6. The X3M and X3S mutants differ from wild type in 41 positions beyond residue 11 (before this residue the

```
          Leu Ile Cys Ile Ser Gly Gly Ile Trp His Ile Leu
                                  700
6803 ... TTG ATC TGT ATT TCC GGT GGT ATC TGG CAC ATT CTG ...
X3S  ... GTC ATT TGT ATA CTT GGT GGT ATC TGG CAC ATT CTG ...
X3M  ... GTC ATT TGT ATA CTT GGT GGA ATC TGG CAC ATT CTG ...
spi  ... GTC ATT TGT ATA CTT GGT GGA ATC TGG CAT ATT TTA ...
                                  700
          Val Ile Cys Ile Leu Gly Gly Ile Trp His Ile Leu
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Fig. 5. Comparison of the *psb*C sequence in *Synechocystis* (regular type), spinach (italicized), and the mutants X3M and X3S in the region of the recombination in the hybrid mutants. The precise region of crossover in X3S and X3M has been underlined. The numbers represent the nucleotide positions in the *psb*C gene from the two organisms.

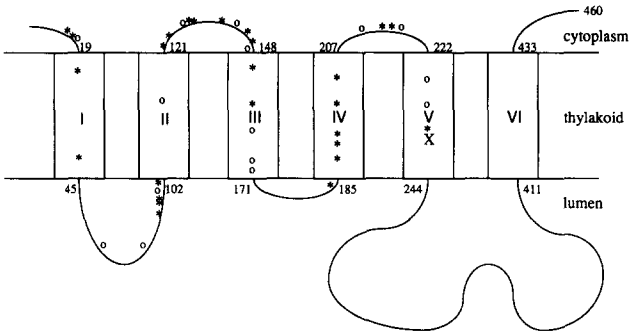

Fig. 6. Topological model of CP43 as it is thought to fold through the thylakoid membrane. The location of non-conservative amino acid changes in X3M and X3S as compared to wild type is indicated by an asterisk; the location of conservative changes is indicated by a circle. In Table II, the differences between the CP43 sequences in wild type and in X3S/X3M have been summarized.

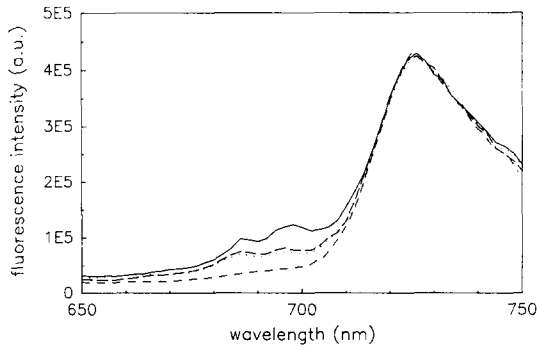

Fig. 7. 77 K fluorescence emission spectra of wild type (———), and hybrid mutants, X3M (- - - - -), X3S (· · · · · ·), and the *psb*C-interruption mutant S2-1 (— — —) in the presence of 60% glycerol. Wild type has the normal PS II peaks at 685 nm and 695 nm in addition to the PS I peak at 725 nm. X3S and X3M show a decrease in both the 685 nm and 695 nm peaks indicating a lower number of PS II centers. The excitation wavelength was 440 nm. Band width for excitation was 18 nm; the emission band width was 3.6 nm.

alignment between spinach and *Synechocystis* CP43 is equivocal); of these differences, 14 mutations are conservative. A listing of the mutations is provided in Table II. The sequences of *Synechocystis* 6803 and spinach *psb*C have been published [4,20].

X3S and X3M were the result of two independent recombination events, but both occurred at virtually identical locations. This might imply either that recombination occurs preferentially at certain 'hot spots', or that only recombination in this area leads to a photoautotrophic phenotype. For this reason, we investigated whether this region was more homologous between the two organisms than other areas of the *psb*C gene. It was found that in the *psb*DI/C operon the region of highest sequence identity between spinach and *Synechocystis* sp. PCC 6803 lies around the *psb*C translational start site, and that the sequence identity index in the region of crossover is not significantly higher than in many other regions of the gene (not shown). Therefore, the apparently high frequency of crossover events in the region around codon 235 in *psb*C is not related to an unusually high homology in this region, but could be the result of (1) a requirement of the cyanobacterial sequence close by to obtain a photoautotrophic phenotype, or (2) a particular secondary structure of the DNA, either hindering recombination elsewhere in the gene or facilitating homologous recombination in this region.

TABLE II

Mutations in CP43 of X3M and X3S as compared to wild type Synechocystis sp. PCC 6803

The approximate location of the mutations has been indicated in Fig. 6. CP43 residue numbering is according to the sequence in *Synechocystis*. Only mutations beyond residue 11 have been indicated, as the alignment is equivocal before this residue.

Residue number	*Synecho-cystis*	X3M/ X3S	Residue number	*Synecho-cystis*	X3M/ X3S
15	Leu	Gln	146	Asn	Thr
16	Pro	Glu	148	Ile	Leu
17	Ser	Thr	150	Tyr	Ile
24	Ser	Ala	156	Cys	Ile
42	Arg	Ala	160	Leu	Phe
63	Ile	Val	167	Met	Leu
79	Ile	Leu	168	Phe	Tyr
89	Ala	Gly	185	Val	Lys
93	Thr	Ile	189	Pro	Val
95	Ile	Thr	192	Asn	Ser
98	Phe	Tyr	194	Ala	Ser
101	Val	Ser	199	Tyr	Cys
114	Leu	Phe	203	Ala	Ser
122	Arg	Leu	212	Ile	Val
126	Val	Thr	215	Asn	Asp
130	Tyr	Ser	216	Asn	Asp
131	Ser	Phe	217	Met	Leu
132	Ser	Pro	225	Ile	Val
137	Asp	Val	229	Leu	Val
141	Lys	Arg	233	Ser	Leu
143	Gln	Lys			

Fluorescence properties

To investigate whether the presence of mutations in CP43 led to anomalies in energy transfer, fluorescence emission spectra were measured at 77 K upon 440 nm (chlorophyll) excitation (Fig. 7). These spectra revealed some decrease in the 685 nm and 695 nm peaks of the PS II emission in both mutants, consistent with a relative decrease in the number of active PS II centers. However, the position of the corresponding peaks was similar to that in wild type, which suggests that the presence of the chimeric CP43 protein does not lead to major structural or functional rearrangements of PS II-associated chlorophylls in these mutants. In addition, excitation and emission spectra were measured at room temperature. The results (not shown) are indicative of rather normal energy transfer from chlorophyll and phycobilins to the PS II reaction center.

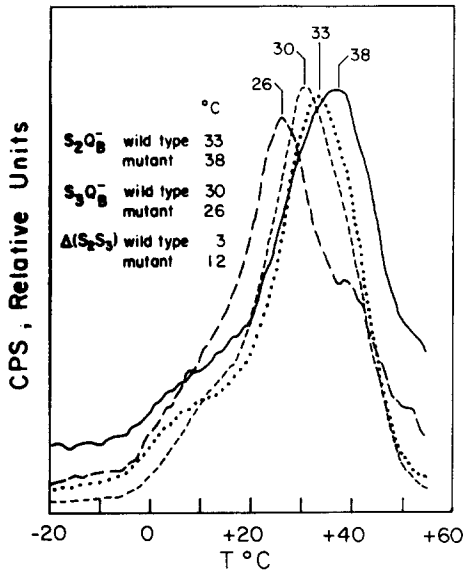

Fig. 8. Thermoluminescence intensity as a function of temperature in wild type and X3M cells. Samples had received one or two saturating flashes before freezing. ———: wild type, one flash; · · · · · · : X3M, one flash; - - - - - -: wild type, two flashes; — — —: X3M, two flashes.

Thermoluminescence

Thermoluminescence measurements provide a sensitive tool to monitor the activation energy needed for recombination of charges between the acceptor and donor sides of PS II. This provides simultaneous information regarding the acceptor and donor sides [18,21]. For thermoluminescence measurements, dark-adapted whole cells were frozen at a rate of about 2 C°/s, while the temperature was monitored continuously. One or more flashes were given at −5°C, at which temperature normal charge separation between the S states and Q_B occurs. The samples were frozen further to about −35°C, and were then heated up at a constant rate in darkness. At a certain temperature, charge recombination between acceptor and donor sides becomes possible when there is sufficient thermal energy to have some $P680^+$ and $Pheo^-$ formed from the oxidized S states and reduced Q_B or Q_A. This leads to a burst of chlorophyll luminescence at that temperature. The temperature of recombination depends on the height of the energy barrier for recombination, which is a function of the redox potentials both of the quinones Q_A and Q_B, and of the S states [21].

The thermoluminescence intensity as a function of temperature in wild-type and X3M cells is presented in Fig. 8. The thermoluminescence properties of X3S are very similar to those of X3M (not shown). In wild type, the peak of thermoluminescence after one flash is at 33–34°C, which decreases after the second flash to 30–31°C, signifiying a decreased energy needed to form $P680^+$ and $Pheo^-$. This 3°C decrease in glow temperature at the second flash is generally observed in PS II [22]. However, in X3M the thermoluminescence peak temperature after one flash (38°C) is about 12°C higher than that observed after two flashes (26°C). After two flashes, the thermoluminescence signal of X3M shows a shoulder at 38–40°C, probably reflecting centers in $S_2 \cdot Q_B^-$ state. This large temperature shift in the X3M mutant could originate from either donor-or acceptor-side events. To distinguish between these two possibilities, we measured the thermoluminescence peak temperature as a function of flash number. The glow temperature oscillates with a periodicity of four (originating from the water-splitting system), superimposed on a smaller oscillation with periodicity of two (originating from the acceptor side) [23]. In X3M and X3S, a much larger oscillation is seen in the peak temperature of thermoluminescence emission: the peak at the first flash is shifted to higher values as compared to wild type, while at the second flash an 11°C drop in peak temperature is observed. As indicated in Fig. 9, in X3M the glow temperature after five flashes is significantly higher than at the 2nd through 4th flashes, and there is a clear periodicity of four in the peak temperature, superimposed on an oscillation with a periodicity of two that is similar to that seen in wild type. This indicates that the alteration of the flash pattern in X3M and X3S originates from donor-side changes. The simplest explanation is that the S_2 state is thermodynamically somewhat stabilized (the redox potential gap between S_2 and P680 is somewhat larger than in wild type), while S_3 is destabilized. A destabilization of S_3

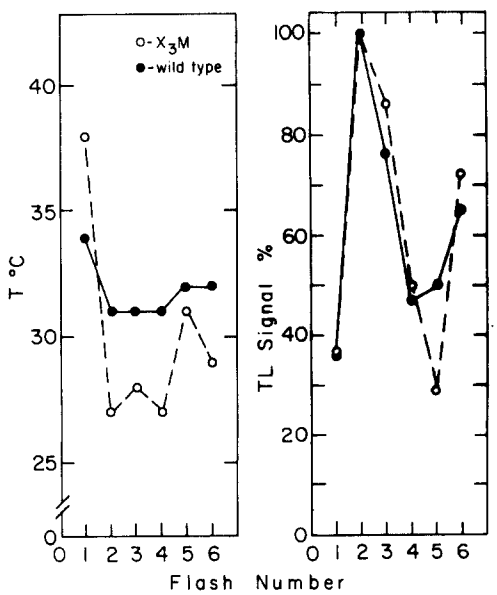

Fig. 9. Thermoluminescence peak temperature (left) and signal amplitude (right) in wild type (•, solid lines) and X3M (○, broken lines) as a function of flash number in intact cells. The TL signal amplitude was normalized to 100% at the second flash. Per measurement, a quantity of cells corresponding to 30 μg chlorophyll was used.

in the X3M and X3S mutants would indicate that the lifetime of this state likely is decreased. Preliminary results of the measurement of the S_3 lifetime of X3S and X3M indicate that this indeed is the case, and that the lifetime is decreased by about a factor of two as compared to wild type (not shown).

Discussion

Specific mutagenesis has been shown to be a powerful technique contributing new insight towards the understanding of the structure and function of PS II. With directed mutagenesis procedures, generation of specific PS II mutants in which a specific protein has been genetically altered or removed can be accomplished relatively easily. This provides a direct means of elucidating the function of a certain protein or residue therein within the PS II complex.

We have applied gene replacement techniques to the functional analysis of CP43 in *Synechocystis* sp. PCC 6803. Our chimeric gene studies show that, despite the high amino acid sequence identity (85%) between CP43 from spinach and *Synechocystis*, a hybrid protein consisting almost entirely of spinach sequences appears to be unable to be stably incorporated into PS II complexes; however, we cannot exclude the possibility that this hybrid protein is not found in thylakoids because it is not expressed well due to unsuitable codon usage or secondary structure of the spinach gene, or to instability of the mRNA. In any case, the impairment of PS II by the spinach *psb*C gene can be overcome by replacing the spinach sequence in the 3′ half of the gene with its cyanobacterial counterpart through homologous recombination.

The *psb*A gene from *Poa annua* can functionally replace cyanobacterial *psb*A in *Synechocystis* sp. PCC 6803 [8]. In contrast, spinach CP43 cannot substitute for the *Synechocystis* homolog. This may be related to the fact that *psb*A is a little more conserved between higher plants and cyanobacteria (the most highly expressed form of *Synechocystis* D1 is 86% identical with that from *Poa*) than *psb*C, but on the other hand interactions between D1 and other PS II components may be more extensive than in the case of CP43, because of the function of D1 as a central reaction center protein (however, see Ref. 24).

The chimeric mutants have about half of the number of PS II complexes on a chlorophyll basis as compared to wild type. This may be related to either a lower stability or a modified synthesis rate of PS II components in X3M and X3S. However, in many respects, the PS II complexes that remain in the X3S and X3M mutants appear functionally normal. The oxygen evolution rate per PS II is similar in mutants and wild type, the antenna function appears to be normal (as measured by room-temperature fluorescence and light-saturation characteristics of oxygen evolution), and the changes in fluorescence excitation and emission characteristics can be attributed to the decreased number of PS II reaction centers on a chlorophyll basis in the chimaeric mutants.

Perhaps the most surprising feature of the X3S and X3M mutants is the large change in thermoluminescence properties. Charge recombination in the $S_2 \cdot Q_B^-$ state occurs at about 4°C higher temperature, while recombination involving S_3 occurs at 4°C lower temperature than in wild type. This results in a separation of the two thermoluminescence peaks if both S_2 and S_3 are present in the sample (Fig. 8). We base our interpretation that the shifts in peak temperature are related to donor- rather than acceptor side effects on three pieces of evidence: (1) the major oscillation in thermoluminescence temperature has a periodicity of four; (2) the $S_2 \cdot Q_A^-$ thermoluminescence peak temperature in X3M (measured in the presence of diuron) was 3–4°C higher than the $S_2 \cdot Q_A^-$ temperature in wild type (data not shown); and (3) the Q_B/herbicide binding environment is similar in X3M and X3S as compared to wild type, judging from the diuron affinity in the mutants. To our knowledge, this is the first time mutants have been found that are phenotypically rather normal but that have modified S_2 and S_3 redox properties. The interesting implication of our observation is that the N-terminal half of CP43 influences, directly or indirectly, the water-splitting system. This indicates that this part of CP43 is close enough to the water-splitting system to have a significant effect on the S states. Thus, apart from effects of the long hydrophilic loop in the C-terminal half of chlorophyll-binding proteins in PS II (at least in CP47 this loop appears to affect the water-splitting complex [1]), also regions in the N-terminal half modify the properties of the water-splitting system. Most likely, this implies that lumenally exposed regions in this part of the protein directly or indirectly interact with the oxygen-evolving complex.

It is interesting to estimate the order of magnitude of the apparent changes in S_2 and S_3 midpoint redox potential that lead to a 4°C shift in thermoluminescence. As a rough calibration factor, one can use the effects of DCMU on the thermoluminescence peak position after one flash. In the absence of DCMU, thermoluminescence originates from $S_2 \cdot Q_B^-$ recombination, which is at 34°C in wild type, while in the presence of DCMU (when the $S_2 \cdot Q_A^-$ state is formed after one flash) the thermoluminescence peak temperature shifts to 9°C. Assuming the equilibrium constant of $Q_A^- \cdot Q_B \leftrightharpoons Q_A \cdot Q_B^-$ to be about 20 (reviewed in Ref. 25), the difference in operational midpoint potential between Q_A/Q_A^- and Q_B/Q_B^- is about 80 mV. As an 80 mV shift leads to a 25°C change in thermolumines-

cence temperature, it is clear that the shifts in E_m of S_2 and S_3 in X3M and X3S are much less than 80 mV. This illustrates the sensitivity of thermoluminescence measurements: such small changes in midpoint potential are difficult to measure directly, but cause a significant change in thermoluminescence properties.

At this moment, we cannot address the interesting question of mutation of exactly which residues leads to the shift in S_2 and S_3 properties. Also, the mechanistic nature of the change in S_2 and S_3 properties is unknown. However, it is interesting to note the parallel between thermoluminescence properties of X3M and X3S and those of wild-type systems at low pH [26]. According to the topological model shown in Fig. 6, and the summary of mutations in Table II, five non-conservative and three conservative changes have been introduced in regions that presumably are on the lumenal side of the thylakoid, while 10 non-conservative and 6 conservative mutations have been introduced in hydrophobic regions of the protein; the non-conservative changes in the hydrophobic region include the deletion of a positive charge (Arg42 to Ala), and the change of a Pro residue (189) to Val close to the lumenal side of the thylakoid. These mutations, and/or one or more mutations in the (presumably) lumenal region of CP43 may be responsible for the changes in S_2 and S_3 properties.

The results presented here clearly indicate that the role of CP43 is not solely that of an antenna protein, but that CP43 is also involved, directly or indirectly, in determining the properties of the water-splitting system. Relatively conserved modifications in CP43 lead to changes in the thermodynamic properties of PS II, which are best explained by an effect on the water-splitting apparatus. This highlights the intricate interactions that appear to exist between different functional and structural subunits of the PS II complex.

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