Targeted Deletion Mutagenesis of the β Subunit of Cytochrome b_{559} Protein Destabilizes the Reaction Center of Photosystem II

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Oligonucleotide-directed mutagenesis techniques were used to delete the *psb*F gene, encoding the β subunit of the cytochrome b_{559} protein of the photosystem II complex in the cyanobacterium, *Synechocystis* 6803. Cyt b_{559} is an integral component of PS II complex. However, its precise functional role in PS II remains to be determined. Previously, we created a mutant in which the *psb*F gene as well as three of its neighbouring genes, *psb*E, *psb*L and *psb*J were simultaneously deleted from the chromosome of *Synechocystis* 6803 (Pakrasi, Williams and Arntzen, EMBO J. **7,** 325–332, 1988). This mutant had no PS II activity. However, the role of any one of the four individual gene products could not be determined by studying this mutant. The newly generated mutant, T256, had only one gene, *psb*F, deleted from the genome. This mutant was also impaired in its PS II activities. In addition, it had barely detectable levels of two other protein components, D1 (herbicide binding protein) and D2, of the reaction center of PS II, in its thylakoid membranes. In contrast, two other proteins of PS II, CP47 and CP43 were present in appreciable amounts. Fluorescence spectra (77 K) of the mutant showed the absence of a peak at 695 nm that was previously believed to originate from CP47. In addition, phycobilisomes, the light-harvesting antenna system of PS II, were found to be assembled normally in this mutant. We conclude that the presence of the β subunit of Cyt b_{559} in the thylakoid membranes is critically important for the assembly of PS II reaction center.

Introduction

Photosystem II – a protein complex in the thylakoid membranes of chloroplasts of vascular plants as well as of cyanobacteria – is the target site for a large number of commercially used herbicides. The structural details of this protein complex as well as its functional characteristics have been areas of intense research activities in recent times. At least eighteen different polypeptide components have now been identified in this pigment-protein complex [1]. In addition, research done over the past three years has indicated that the reaction center of PS II is localized on two ~32 kDa proteins – D1 and D2 [2, 3]. Among all of these subunit proteins, the role of cytochrome b_{559} has remained enigmatic for the longest period of time. Nucleotide sequence analysis of the genes encoding this protein has indicated that this cytochrome is composed of two subunits – α (~9 kDa) and β (~4.5 kDa) [4]. Spectroscopic studies on isolated Cyt b_{559} protein have suggested that its heme cofactor is coordinated between two histidine residues – most probably one in each of the α and β subunits [5].

We have used a directed mutagenesis approach to study the relationship between the structure and function of Cyt b_{559} in a unicellular cyanobacterium, *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis* 6803). Two neighbouring genes, *psb*E and *psb*F, encode the α and β subunits of Cyt b_{559} in this organism [6]. In addition, two other putative genes, *psb*L (previously called *psb*I, see [1]) and *psb*J are also present in close proximity to *psb*E and *psb*F [7]. The organization *psb*E – *psb*F – *psb*L – *psb*J of these genes in *Synechocystis* 6803 is very similar to that found in a number of chloroplast genomes [8]. Previously, we deleted the entire *psb*EFLJ region from the genome of *Synechocystis* 6803 to obtain a mutant, T1297, that was completely devoid of PS II activity [6, 9]. In this mutant, however, four genes were deleted simultaneously. Thus, it was not possible to determine whether the structural as well as functional defects in this mutant were due to the deletion of any one

Abbreviations: Chl, chlorophyll; Cyt, cytochrome; LiDS-PAGE, lithium dodecyl sulfate polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PS II, photosystem II.

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or more of these genes. To resolve this problem, we have recently used a synthetic oligonucleotide-directed deletion mutagenesis approach to systematically delete each individual gene in the *psb*EFLJ gene cluster and examine the phenotype of the resulting mutants. In this communication, we describe the creation of a deletion mutation in the *psb*F gene, encoding the β subunit of Cyt b_{559}, as well as the effect of such a mutation on the stability of the PS II complex.

Materials and Methods

Growth of cultures

Both wild type *Synechocystis* 6803 and strain T 256 were grown in BG 11 liquid medium supplemented with 5 mM glucose [6]. In addition, spectinomycin (10 μg/ml) was added to T 256 cultures. Cultures were propagated at 30 °C under a light intensity of 60 $\mu E/m^2/sec$, with continuous bubbling with water-saturated air.

Construction of deletion strain

Construction of the donor plasmid that contains the precise deletion of 41 out of 44 codons of the β subunit of Cyt b_{559} in *Synechocystis* 6803 has been described [10]. In addition to the desired deletion mutation in the *psb*F gene, this plasmid contained a spectinomycin resistance gene cartridge at an Nhe I site, immediately downstream of the *psb*J gene. This donor plasmid was used to introduce the deletion mutation into the T 1297 strain that lacks the entire *psb*EFLJ region [6]. Selection was made for cultures that could grow in the presence of spectinomycin.

PCR analysis

Chromosomal DNA was isolated from liquid cultures of wild type and T 256 strains, using a procedure described in [11]. Polymerase chain reaction (PCR) was carried out on ~50 ng chromosomal DNA, using two oligonucleotide primers (25 pmol each). One of these primers hybridizes to a sequence 5′ of *psb*E gene, and the other one hybridizes to the complementary strand of DNA on the 3′ edge of *psb*J gene. The reaction mixture included Taq salts (50 mM KCl, 10 mM Tris-Cl, pH 8.4, 2.5 mM $MgCl_2$, and 0.1% gelatin), 0.2 mM each of dATP, dCTP, dGTP and dTTP, and 2.5 units Taq polymerase (Cetus-Perkin Elmer). The reaction was carried out for 35 cycles of incubations at 93 °C for 30 sec, 46 °C for 1 min, and 72 °C for 1 min. Products were subsequently analyzed on 1% agarose gels.

Protein analysis and immunoblotting

Thylakoid membranes were isolated from 1 l cultures of mid-log phase wild type and T 256 cells. Cells were harvested by centrifugation at 8000 × *g* for 10 min at 4 °C, washed once in breakage buffer (0.2 M sucrose, 2 mM Na-EDTA, 50 mM MES, pH 6.2), and resuspended in 5 ml of the same buffer. Glass beads (75–150 μ, Sigma) were added to this suspension and the mixture was vortexed three times for 2 min each with 1 min intervals on ice. The broken cell mixture was filtered through Miracloth (Calbiochem), and spun at 2000 × *g* for 4 min to remove unbroken cells. The supernatant was then centrifuged at 100,000 × *g* for 45 min in a T 865 rotor (Sorvall). The resulting membrane pellet was resuspended in 1 ml breakage buffer. Chl *a* concentration was measured in 80% acetone [12].

Membrane proteins from wild type and T 256 cells were fractionated on a lithium dodecyl sulfate polyacrylamide gel system (LiDS-PAGE), essentially as described in [9]. Protein bands were visualized by staining with Coomassie blue. Western immunoblotting was performed according to the procedure in [9]. Primary antibodies were kindly provided by N.-H. Chua (*anti*-CP47 and *anti*-CP43) and Y. Inoue (*anti*-D1 and *anti*-D2). Alkaline phosphatase conjugated goat *anti*-rabbit IgG (Sigma) was used as the secondary antibody, and color development was carried out with BCIP (3-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt; Sigma) and NBT (nitroblue tetrazolium).

Fluorescence analysis

Fluorescence emission spectra of whole cells (frozen at 77 K) were obtained on a SPEX Fluorolog instrument. Excitation of chlorophyll was done at 440 nm whereas phycobilins were excited at 580 nm. The spectra are presented without correction for instrument response.

Results

In *Synechocystis* 6803, the gene cluster *psb*EFLJ is localized on a 2.2 kbp *Eco*RI-*Hind*III fragment

of chromosomal DNA [6]. Fig. 1 shows the nucleotide sequence of an 850 bp long stretch from this fragment that includes all of the four open reading frames as well as some untranslated sequences in the 5′-flanking, 3′-flanking and intergenic regions. In this diagram, the coding regions of *psb*E, *psb*F, *psb*L and *psb*J extend from nucleotides 121 to 366, 403 to 537, 547 to 666 and 699 to 818, respectively.

In order to confirm that the T256 mutant contains a deletion of the *psb*F ORF, we performed PCR amplification of chromosomal DNA from the mutant and the wild type cells, using two oligonucleotides (corresponding to the underlined sequences in Fig. 1) that hybridize to opposite strands of chromosomal DNA. If T256 contained the proper directed deletion mutation, we expected a change in the size of the major double-stranded PCR product from 784 bp to 660 bp. As shown in Fig. 2, the major PCR product from the wild type DNA is ~800 bp (lane 1) and that from the T256 DNA is ~650 bp (lane 2). In addition, DNA sequence analysis of the single-stranded PCR products corresponding to the same DNA region also showed that the deletion of 124 base pair region has indeed occurred [10]. Thus, we conclude that the T256 mutant contains a precise deletion of the *psb*F gene.

Analysis of proteins

Fig. 3A shows the profile of membrane proteins from wild type (WT) and T256 mutant (M) cells fractionated on a 10–20% gradient LiDS-PAGE system. A large number of polypeptide bands (>100) were detected in each lane. Thus, clear

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  1  GCTACCCCAG CACCGAGGTG GCAATGGTTC CCTAGGCGGC TCACAAAATA
 51  GTAGACTAGA CTCTACTTGC TTTGCATTTG TCAGTCAATG TTGTTTTGAA
101  AAATTGAAGG AGAACACAAA ATGTCAGGGA CTACCGGCGA GCGTCCATTT
151  TCCGATATTG TCACCAGCAT TCGCTACTGG GTGATCCACA GCATCACCAT
201  CCCGATGTTG TTTATTGCTG GTTGGTTGTT TGTCAGCACG GGCTTAGCCT
251  ACGATGCTTT TGGCACTCCC CGCCCCGATG AATATTTCAC CCAGACCCGT
301  CAAGAGTTGC CCATTCTCCA GGAACGCTAC GACATTAATC AGGAAATTCA
351  AGAGTTTAAT CAATAAAACA TTTAATTGTT CTTTTTTAGT TGGTAATTAA
401  CAATGGCAAC CCAAAATCCT AATCAACCGG TTACTTATCC CATTTTTACG
451  GTGCGCTGGC TGGCGGTTCA CACCCTGGCG GTGCCCTCTG TCTTCTTTGT
501  CGGGGCGATC GCCGCGATGC AATTTATTCA ACGCTAGGAG TTTTTCATGG
551  ACAGAAATTC AAACCCAAAC CGCCAACCGG TGGAATTGAA CCGCACTTCT
601  TTATACCTGG GTCTATTGTT GGTGGCTGTG TTGGGGATTT TGTTCTCCAG
651  CTATTTCTTT AACTAAACTT TTTTAATACG CAATTTAGGA GGCATGGTAT
701  GTTCGCAGAA GGCAGAATCC CTTTGTGGGT GGTGGGTGTA GTGGCCGGTA
751  TTGGCGCCAT TGGTGTTCTA GGATTATTTT TCTACGGAGC CTATGCTGGT
801  TTAGGTTCTT CCATGTAATC GAGGGCTAGC CGCCACACAA TATCATGGTT
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Fig. 1. Nucleotide sequence of an 850 bp long region of *Synechocystis* 6803 genome that contains four open reading frames, *psb*E, *psb*F, *psb*L and *psb*J (see text). The region between nucleotides 403 and 526 (boxed), corresponding to the first 40 codons of the *psb*F gene, was deleted by site-directed mutagenesis [10]. Two synthetic oligonucleotides, the first one (20 mer) having the exact same sequence as the underlined region between nucleotides 33 and 52, and the second one (18 mer) having a sequence complementary to the underlined region between nucleotides 801 and 818, were used for PCR amplification of the *psb*EFLJ region in the chromosomes of wild type and T256 cells. The Nhe I site at which the spectinomycin-resistance gene cartridge was introduced lies between nucleotides 825 and 830.

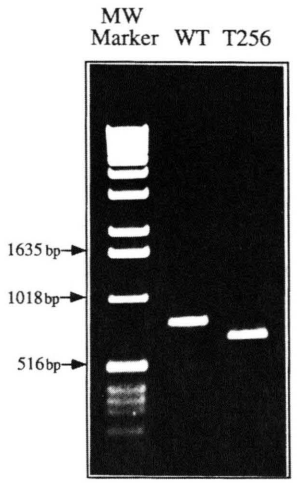

Fig. 2. Fractionation of double-stranded DNA molecules that are amplified products of polymerase chain reactions (PCR) on chromosomal DNA from wild type cells (WT lane) or the T256 mutant cells (T256 lane). The first lane shows molecular weight markers (1 kbp ladder, BRL Co.) that have been fractionated on the same agarose gel.

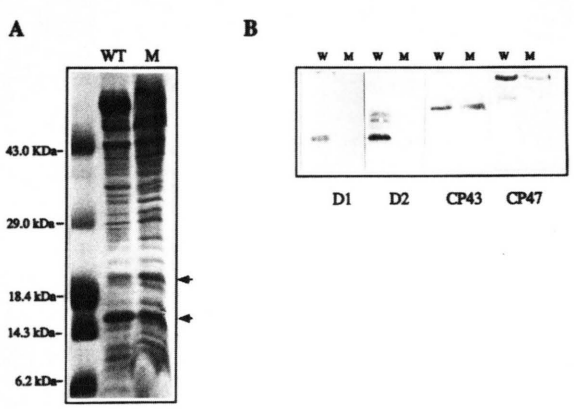

Fig. 3. Panel A: Electrophoretic separation of membrane proteins from wild type (WT) and T256 (M) cells on a 10–20% LiDS-PAGE system and subsequently stained with Coomassie blue. The first lane shows the migration of molecular weight marker proteins (BioRad Co.). Each arrow on right indicates a doublet of major phycobiliprotein bands. Panel B: Immunodecoration of proteins (Western blot) with antibodies against D1, D2, CP43 and CP47 proteins, respectively. For these experiments, membrane proteins from wild type (W) and T256 (M) cells were initially fractionated on LiDS-PAGE systems identical to the one shown in panel A.

identification of any PS II protein was essentially impossible just by examination of these Coomassie-stained protein profiles. However, four protein bands (indicated by two arrows) that were blue-colored even prior to staining with Coomassie blue and which corresponded to major phycobiliproteins, were present at nearly equivalent amounts in wild type and T256 cells. Thus, mutation in the *psb*F gene did not seem to affect the synthesis as well as pigment-binding properties of these proteins of phycobilisomes, membrane extrinsic pigment protein complexes that serve as an antenna system for PS II in cyanobacteria.

To detect the presence of various protein components of PS II, we used monospecific polyclonal antibodies raised against homologous proteins in PS II complexes in chloroplasts. As shown in Fig. 3B, antibodies raised against spinach D1 protein recognized a single protein band in wild type membrane fractions. However, the presence of this protein was barely (<5%) detectable in the membrane fraction from the T256 mutant. Antibodies raised against the D2 protein from spinach recognized a major protein band in membranes from wild type cells. Only a small amount of this protein (~7%) could be detected in T256 membranes. In comparison to these two proteins, apoproteins of CP47 and CP43 were present in significant amounts in the mutant cells in comparison to those in wild type cells. The amount of CP43 protein was >80% in the mutant as compared to that in wild type membranes, whereas that of CP47 protein was between 50 and 60%.

Fluorescence emission spectra

Fig. 4 shows the fluorescence emission spectra of intact wild type and T256 cells frozen at 77 K. As shown previously [9], preferential excitation of Chl molecules at 440 nm resulted in three emission peaks at 685 nm (F685), 695 nm (F695) and 720 nm (F720) (Fig. 4A). Among these, the first two are attributable to Chl molecules in PS II, whereas F720 originates from PS I Chl. Dzelzkalns and Bogorad [13] have recently shown that the origin of F685 (687 nm in their system) is most probably CP43, since a CP43-less mutant of *Synechocystis* 6803 lacked in fluorescence in this wavelength region. In addition, isolated CP47 proteins from cyanobacteria emit F695 [14]. Interestingly,

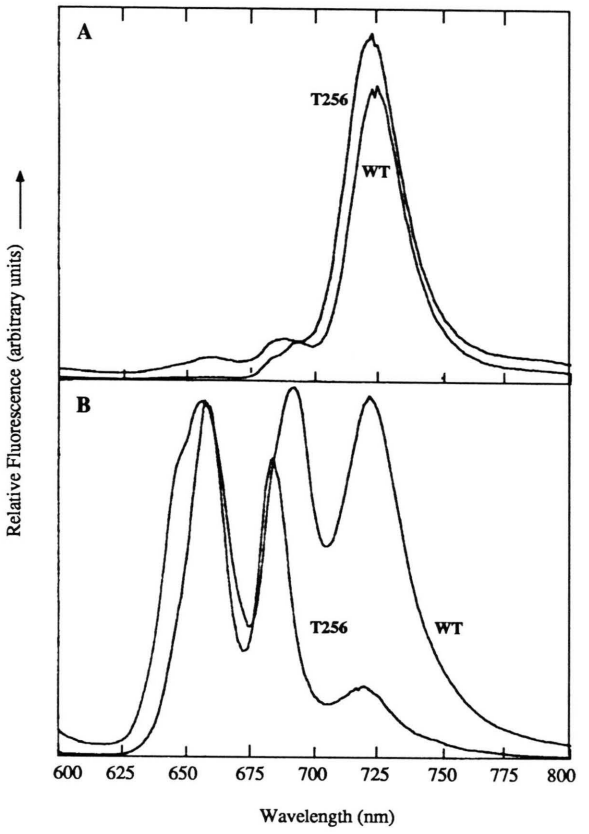

Fig. 4. Fluorescence emission spectra from intact wild type (WT) or T256 cells, frozen in liquid N_2 (77 K). Excitation was at (A) 440 nm or (B) 580 nm with 5 nm bandpass. Emission bandpass was 3 nm.

the T256 mutant lacked in F695, although F685 was present. Thus, this mutant showed the presence of appreciable amount of CP47 apoprotein but no F695. F720 was unaltered, indicating no change in PS I fluorescence emission from this mutant. This is very similar to the situation in the mutant T1297 that has the entire *psb*EFLJ region deleted from its chromosome [9].

Fig. 4B shows the fluorescence emission spectra at 77 K when phycobilin pigments were preferentially excited at 580 nm. In properly assembled phycobilisomes, light quanta are absorbed primarily by phycocyanins and then transferred sequentially through allophycocyanin and allophycocyanin B to Chl molecules that act as membrane internal light-harvesting antenna for PS II reaction center. Inhibition of energy transfer within and from phycobilisomes is manifested by characteristic fluorescence emission from individual pigments at 77 K. As shown in this figure, both wild type and T256 cells showed a fluorescence peak at 657 nm that originates from allophycocyanin, whereas an additional small shoulder at 648 nm, arising from phycocyanins, was present in the T256 sample. Thus energy transfer from phycocyanins to allophycocyanins was nearly normal in the T256 sample, indicating a normal assembly of phycobilisomes in this mutant strain.

This figure also demonstrates that the energy absorbed by phycobilins gave rise to F695 and F720 in wild type cells. Thus, light quanta absorbed in phycobilisomes were transferred to PS I *via* PS II. In the mutant T256, some energy was still transferred to PS I, although the quantum yield of PS I fluorescence was relatively much smaller. Thus, in this mutant, energy absorbed by phycobilins was transferred to the Chl antenna of PS I *via* the PS II complex that lacked in any properly assembled reaction center.

Discussion

The presence of Cyt b_{559} as a two subunit protein component of PS II complex in its most purified form suggests that this protein has some important structural and/or functional role in PS II [3]. Studies described here were aimed at performing a fine structure analysis of the role of the β subunit (*psb*F gene product) of Cyt b_{559} in PS II of *Synechocystis* 6803. As shown in Fig. 1, four open reading frames, *psb*E, *psb*F, *psb*L and *psb*J are tightly clustered in a 850 bp long region of the chromosome of this cyanobacterium. Moreover, transcript analysis of these genes indicate that all four of them are cotranscribed into a single ~900 base long message (data not shown). The same kind of tight clustering of these genes has also been observed in the chloroplast genome of a number of vascular plants as well as of *Euglena* [8]. A result of such clustering is that the potential ribosome binding site for *psb*L gene overlaps with the translational stop codon of *psb*F [10]. Thus, we used an oligonucleotide-directed deletion mutagenesis approach to delete the DNA region that includes codons 1 through 40 of the 44 codon *psb*F ORF

and thus minimized potential polar effects of such a deletion mutation on downstream genes. As shown in Fig. 2, as well as during nucleotide sequence determination of this region of chromosomal DNA [10], T256 had the desired deletion mutation. Interestingly, such a deletion mutation still had pleiotropic effects on the stability of the reaction center of PS II in a way very similar to that in T1297, a deletion mutant that had the entire *psb*EFLJ operon deleted from the chromosome [6, 9]. As shown in Fig. 3B, the steady-state levels of two reaction center proteins, D1 and D2, were very low in this mutant in comparison to those in wild type cells. Unfortunately, we did not have specific antibodies that recognized the gene product of each individual ORF in the *psb*EFLJ operon. T256 is expected to have an absence of the *psb*F gene product. However, the synthesis as well as stable assembly of the products of the *psb*E, *psb*L and *psb*J genes in the thylakoid membranes of this mutant need to be determined in the future. It is conceivable that in the absence of the β subunit, the heme (s) in Cyt b_{559} cannot be coordinated properly. This may in turn affect the stability of the reaction center of PS II. With respect to D1 and D2, the possibility remains that both of them are translated at normal levels and integrated into the membrane of the mutant cells. However, in the absence of Cyt b_{559}, a protein postulated to have a protective role against photoinhibition [15], the assembled D1-D2 complex may turn over rapidly. Thus, only a trace amount of each protein may be detected under steady-state conditions. Short-term radioactive pulse-labeling and subsequent chase experiments may elucidate the fates of these proteins, in future.

An alternative possibility is that the deletion mutation may actually affect the stability of the transcript from the *psb*EFLJ gene cluster. In this case, significant amount of *psb*E, *psb*L and *psb*J gene products would not be synthesized. Northern blot analysis of this transcript in T256 cells is currently in progress to examine this possibility.

Fig. 3B also indicates that significant amounts of CP47 and CP43 apoproteins – two antenna proteins in PS II core complex – accumulated in the membranes of T256 mutant. A similar phenomenon has been observed with the *psb*EFLJ deletion strain, T1297 [9]. These results are quite significant for the elucidation of the stepwise assembly process of the membrane protein complex, PS II. Analysis of other PS II-deficient mutants of *Synechocystis* 6803 have shown that null mutations in any one of the genes or gene families encoding CP47, D1 or D2 proteins affect the steady-state levels of the other two proteins [16, 17]. Among all such mutants, only *psb*A (encoding D1 protein) triple deletion mutation has been examined for the presence of the α subunit of Cyt b_{559}. Interestingly, this mutant contains near normal levels of this subunit protein [17]. Deletion studies on the *psb*C gene (encoding the CP43 protein) have shown that absence of CP43 still allows the synthesis of a CP47-D1-D2 complex that retains reaction center activities [16]. In addition, Simpson and colleagues [18] have recently described a barley mutant that has CP47, CP43, D1 and D2 missing from its thylakoids, whereas the α subunit of Cyt b_{559} is still present. All of these data indicate that Cyt b_{559} is one of the centrally critical protein subunits for the assembly of PS II. To date, our studies have been the only ones where defined mutations have been created in this protein to examine its role in PS II.

Fluorescence spectra at 77 K (Fig. 4A) indicated that F695, one of the two peaks originating from PS II was absent from the mutant, whereas the other one, F 685 was present. The origin of F 695 has previously been identified as some Chl molecule on CP47. However, T256 contained significant amounts of the CP47 apoprotein in its membrane. A possibility exists that this membrane-integrated protein might not have any Chl associated with it. However, de Vitry *et al.* [19] have recently demonstrated that in various PS II-deficient mutants of *Chlamydomonas,* CP47 and CP43 have Chl bound to them, although the PS II complexes in these mutants were not properly assembled. Thus, they concluded that binding of Chl to Chl-binding proteins precedes the assembly of such proteins into the multiprotein complex, PS II. We believe that the same events may very well occur in *Synechocystis* 6803. Thus, the absence of F695 may be the result of an absence of some specific interaction between CP47 and the reaction center of PS II. F685 in the mutant may originate from CP43 as found in wild type cyanobacterial cells [14].

Fluorescence analysis as well as examination of the protein profile on one-dimensional LiDS-

PAGE systems (Fig. 3 A) indicated that in T256, a mutant missing its PS II reaction center, the synthesis, pigment binding and assembly of phycobilisomes have not been affected appreciably. Similar observations have also been reported by Dzelzkalns and Bogorad [13] when they examined randomly generated PS II-deficient mutants of *Synechocystis* 6803, whose primary lesions are in genes encoding CP47 and CP43, respectively. Thus, the assembly of phycobilisomes seem to be independent of that of the PS II complex, even though the former serves as an antenna structure for the latter one.

In conclusion, the presence of the β subunit of Cyt b_{559} is critically important for the proper assembly of functional PS II complex. Experiments are currently in progress to assess the role of various domains as well as specific residues of this protein in PS II function.

Acknowledgements

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