Mutational Analysis of the PsbL Protein of Photosystem II in the Cyanobacterium *Synechocystis* sp. PCC 6803

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Z. Naturforsch. **48c**, 267–274 (1993); received December 2, 1992

Photosynthesis, Photosystem II, Cyanobacterium, Directed Mutagenesis, *psbL* Gene

The *psbL* gene is a member of the *psbEFLJ* gene cluster in the cyanobacterium *Synechocystis* sp. PCC 6803 and higher plants. *psbL*, a 4.5 kDa protein encoded by this gene, is a component of the photosystem II complex. The amino acid sequence of this protein indicates that it has a single membrane-spanning α-helical domain. We have used a targeted mutagenesis technique to delete the coding region of the *psbL* gene in *Synechocystis* 6803. The resultant mutant strain T345 did not show any PSII-mediated oxygen evolution activity and, as a result, could not grow under photoautotrophic conditions. However, it had normal PSI activity. The chlorophyll to phycobilin ratio in the T345 cells was significantly lower than that in the wild type cells. Fluorescence emission spectra (77 K) of the mutant cells showed the absence of a 695 nm band that usually originates from the PSII complex. Binding assays with radioactive diuron demonstrated that the mutant cells did not have any herbicide binding activity. However, immunostaining experiments showed that both the D1 (the herbicide binding protein) and the D2 proteins of the PSII reaction center were present at >25% of their normal levels in the thylakoid membranes of the T345 mutant cells. Our data indicate that the PsbL protein is essential for the normal functioning of PSII.

Introduction

Photosystem II, one of the two pigment-protein complexes in the thylakoid membranes of cyanobacteria, higher plants and eukaryotic algae, mediates light-induced electron transfer from water to plastoquinone molecules. Two polypeptides, D1 and D2, are known to form the binding environments for the reaction center chlorophyll(s) P680, pheophytin, quinone acceptors Q_A and Q_B as well as a four-manganese cluster involved in water oxidation (see [1] for a recent review). Cytochrome *b* 559, a heme-containing protein, is closely associated with D1 and D2, although its exact functional role is yet to be determined. PSII also contains two antenna chlorophyll-binding proteins, CP47 and CP43. In recent years, newly developed polyacrylamide gel systems and protein sequencing techniques have been used to reveal the presence of a number of small (< 10 kDa) proteins in the PSII complex [2]. However, the functional roles of most of these small proteins are currently unknown.

One of these small polypeptides is PsbL, a protein of approximate molecular mass of 4.5 kDa. The presence of this protein was discovered in purified PSII core complex preparations from wheat and spinach [3, 4] as well as in a PSII reaction center preparation from the cyanobacterium *Synechococcus vulcanus* [5]. The N-terminal sequences of these PsbL proteins matched with the deduced amino acid sequence of the protein encoded by *psbL*, an open reading frame in the chloroplast genome of tobacco that has been completely sequenced [6]. The exact function of the PsbL protein is unknown. Recent studies by Nagatsuka and co-workers [7] on the resolution and reconstitution of the PSII core complex have indicated that PsbL and a 4.1 kDa protein may play a regulatory role in the coordination of the primary electron acceptor, Q_A.

In chloroplasts of green plants [4], cyanelles of *Cyanophora paradoxa* [8] and cyanobacteria [9], the *psbL* gene is a member of a *psbEFLJ* operon. Among these, the *psbE* and the *psbF* genes encode the α (9 kDa) and the β (5 kDa) subunit proteins of cytochrome *b* 559 respectively, whereas the *psbJ* gene encodes a 4 kDa protein of PSII [10]. Interestingly, the translational initiation codon of the *psbL* gene in tobacco and spinach is ACG which is edited at the mRNA level to AUG, the normal initiation codon [11]. In most organisms these four genes are usually cotranscribed. However, in the green alga, *Chlamydomonas reinhardtii*, the *psbE*

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Verlag der Zeitschrift für Naturforschung,
D-W-7400 Tübingen
0939–5075/93/0300–0267 $ 01.30/0

gene is not located in the physical proximity of the *psbF* and the *psbL* genes [12].

We are using targeted mutagenesis techniques to understand the functional roles of the products of each of the four genes in the *psbEFLJ* gene cluster in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter called *Synechocystis* 6803) [13, 14]. In previous studies we have established that the products of the *psbE*, the *psbF* and the *psbJ* genes have structural as well as functional roles in the PSII complex [10, 13, 14]. The goal of the present study is to understand the role of the PsbL protein in PSII. For this purpose we have created a *psbL* deletion mutant, T345, which is unable to form functional PSII centers. However, D1 and D2, the two reaction center proteins accumulate in significant amounts in the thylakoid membranes of this mutant strain.

Materials and Methods

Growth of cultures

The wild type strain of *Synechocystis* 6803 and the *psbL* mutant T345 were grown in BG11 medium [15] which was supplemented with 5 mM glucose, when indicated. The medium used for T345 was also supplemented with 5 μg/ml spectinomycin (Sp). Cultures were grown under 60 μE m^{-2} s^{-1} of fluorescent light, with vigorous bubbling with room air. Growth of cyanobacterial strains was monitored by the measurement of scattering at 730 nm on a DW2000 spectrophotometer (SLM-Aminco Instruments, Urbana, IL).

Construction of T345, the psbL deletion strain

Site directed mutagenesis of the *psbL* gene was performed according to a previously published procedure [13]. An oligonucleotide primer 5′-CGCTAGGAGTTTTTCTTTTAATACGCAATT-3′ (30-mer) was synthesized to span the regions immediately 5′ and 3′ to the coding region of the gene (Fig. 1). This primer was used to create a deletion of the *psbL* coding region. A spectinomycin-resistance (Sp^r) cartridge was inserted into the Nhe I site downstream of the *psbEFLJ* gene cluster as described previously [14]. The resulting plasmid, pSL345, was transformed into *Synechocystis* 6803 strain T1297, which lacks the entire *psbEFLJ* region [16]. The resultant Sp^r transformant was designated T345. The presence of the *psbL* deletion mutation in these mutant cells was verified by polymerase chain reaction (PCR) mediated amplification as described elsewhere [13, 14]. The PCR reaction was carried out for 35 cycles of incubation at 95 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min on a Perkin-Elmer/Cetus DNA thermal cycler instrument.

Absorption and fluorescence spectral analysis

Absorption spectra of intact cyanobacterial cells were recorded at room temperature in the split-beam mode on a DW2000 spectrophotometer with a 2 nm slit width. Fluorescence emission spectra at 77 K were obtained on a homebuilt spectrofluorometer consisting of an INSTASPEC I diode-array (1024 detector elements) detection system (Oriel Corpn.) with a 1 nm slit width. Excitation of the samples was with a broad beam light filtered through a Corning CS 4-96 filter. The spectra are presented with correction for the instrument response. The software in the INSTASPEC package was used to obtain the derivative spectra shown in Fig. 6B and 6C. We used the Kaleidagraph program (Abelbeck Software) on a MacIntosh computer to normalize the spectral data.

The ratios of chlorophyll to phycobilin pigments in intact wild type and T345 cells were determined according to [17].

Protein analysis and immunoblotting

Thylakoid membranes from wild type and various mutant strains were isolated as described elsewhere [18]. Concentrations of chlorophyll were measured in methanolic solutions [19]. For each lane, 2.5 μg chlorophyll-containing sample was prepared and subjected to SDS-PAGE on 16% acrylamide gels as described elsewhere [18]. Fractionated proteins were transferred to 0.2 μm nitrocellulose filters (Schleicher and Schuell) using a semi-dry blotting apparatus (BIO-RAD) for 2 h at 10 V. Rabbit polyclonal antibodies raised against the D1 and the D2 proteins of PSII were kind gifts from Drs. M. Ikeuchi and Y. Inoue. Immunodetection was carried out according to a procedure described previously [14]. The secondary antibodies were alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Immunochemicals).

Measurements of electron transfer and herbicide binding activities

A Clark type oxygen electrode was used to measure the rates of light-induced oxygen evolution from intact cyanobacterial cells as described in [10]. Whole chain electron transport was measured in the BG 11 medium. Rates specific for PSII were determined using 0.25 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) and 1 mM $K_3Fe(CN)_6$ (FeCN) as electron acceptors. For PSI-mediated oxygen uptake rates, 1 mM 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD) and 1 mM sodium ascorbate (Asc) were added as electron donors and 1 mM methyl viologen (MV) was added as an electron acceptor.

To estimate the concentration of PSII (on a chlorophyll basis) in whole cells, we measured the binding of [^{14}C]diuron as described elsewhere [10].

Results

psbL gene in Synechocystis 6803

In *Synechocystis* 6803 as well as in green plants and cyanelles, *psbL* is the third gene in the *psbEFLJ* gene cluster. After the discovery of its presence downstream of the *psbF* gene, this gene was called *psbI* [8, 20] and later renamed as *psbL* [3, 4]. In Fig. 1, we present the sequence of an 180 nucleotides region of this gene cluster from *Synechocystis* 6803. This sequence corresponds to nucleotides 521 to 700 of that in Fig. 1 of [13, Genebank accession M 33897]. The translational start codon of the coding region of the *psbL* gene is preceded by a pentanucleotide polypurine sequence (AGGAG) that may function as a ribosome binding site according to Shine and Dalgarno [21]. Also shown in this sequence is the translational stop codon (nucleotides −12 to −10) of the *psbF* gene and the beginning of the translational start codon (nucleotide 153) of the *psbJ* gene. The PsbL protein is predicted to have 39 amino acids and a molecular mass of 4,473 Da. Analysis of the N-terminal amino acid sequence of this protein from *Synechocystis* 6803 indicated that the initiating Met residue is retained in the mature protein (Ikeuchi, Pakrasi and Inoue, unpublished). A similar finding has also been reported for *Synechococcus vulcanus,* another cyanobacterium [5]. In contrast, the initiating Met residue is cleaved off from the mature PsbL protein in wheat and spinach [4]. Fig. 2 shows a comparison of the predicted sequences of the PsbL protein from *Synechocystis* 6803, two higher plants and *Chlamydomonas,* an eukaryotic green alga. The high degree of similarity between these sequences indicate that this protein has been evolutionarily conserved. Hydropathy analysis

```
   -20                          1                          20
AATTTATTCA ACGCTAGGAG TTTTTCATGG ACAGAAATTC AAACCCAAAC CGCCAACCGG
                           M  D   R  N  S   N  P  N   R  Q  P  V
                           1                              10

   40                          60                         80
TGGAATTGAA CCGCACTTCT TTATACCTGG GTCTATTGTT GGTGGCTGTG TTGGGGATTT
  E  L  N   R  T  S   L  Y  L  G   L  L  L   V  A  V   L  G  I  L
                      20                                 30

  100                         120                        140
TGTTCTCCAG CTATTTCTTT AACTAAACTT TTTTAATACG CAATTTAGGA GGCATGGTAT
  F  S  S   Y  F  F   N  STOP
                     39
```

Fig. 1. Nucleotide sequence of the *psbL* gene. The deduced amino acid sequence of the PsbL protein is shown below the nucleotide sequence. The top line indicates the nucleotide number and the fourth line shows the amino acid residue number. A possible ribosome binding site is underlined. In the mutant T345, nucleotides 1 through 124 were deleted (see text for more details).

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Synechocystis 6803   ------MDRNSNPNRQ PVELNRTSLY LGLLLVAVLG ILFSSYFFN   (39)
Tobacco              ------ TQ-    E  N          W    IF  A V   N      (38)
Rice                 ------ TQ-    E  N          W    IF  A V   N      (38)
Chlamydomonas        MFKNFF A -P   K  V          W    IF  A V     I    (44)
```

Fig. 2. Comparison of the amino acid sequence of the PsbL protein from *Synechocystis* 6803 (this study), tobacco [6], rice [32], and *Chlamydomonas reinhardtii* [12]. Dashes indicate gaps for optimal alignment of the protein sequences. The underlined sequence corresponds to a hydrophobic domain and represents a putative membrane spanning α-helical region. The numbers within parenthesis indicate the number of amino acid residues in the PsbL proteins.

[22] indicated that the underlined sequence in the C-terminal half of this polypeptide represents a putative membrane spanning α-helical domain. In *Synechocystis* 6803, the N-terminal hydrophilic domain (residues 1 to 16) of the PsbL protein contains three positively charged Arg residues. According to the "positive-inside" rule of von Heijne [23], the N-terminus of this protein is thus expected to be exposed in the stroma. The N-terminal Thr residue in the PsbL protein in the wheat PSII complex has been postulated to be phosphorylated [3]. This Thr residue is missing from both the *Synechocystis* and *Chlamydomonas* sequences, implying that the PsbL proteins in these organisms are not phosphorylated.

Photoautotrophic competence of T345, the psbL deletion mutant

As described in the Materials and Methods section, the coding region of the *psbL* gene was deleted from the chromosome of *Synechocystis* 6803 to create a mutant T345. In order to confirm that this mutant strain had the desired deletion mutation, we performed PCR amplification of the chromosomal DNA from wild type and mutant cells. We expected a reduction in the size of the PCR product from 786 bp in the wild type cells to 662 bp in the mutant cells. As shown in Fig. 3, the PCR product from the wild type DNA was ~800 bp whereas that from the T345 DNA was ~650 bp. These results indicated that the *psbL* gene has been deleted from the chromosome of the T345 cells. As shown in Fig. 4, the mutant cells were unable to grow in BG11 medium without any added glucose. Interestingly, the T345 cells grew significantly slower than the wild type cells even in the presence of glucose in the growth media. Since these cells were grown in the presence of light (photoheterotrophic growth), these data may indicate that the growth light may have a damaging photoinhibitory effect on the T345 cells. The absence of photoautotrophic growth suggested that the T345 mutant cells did not have any functional PSII complex.

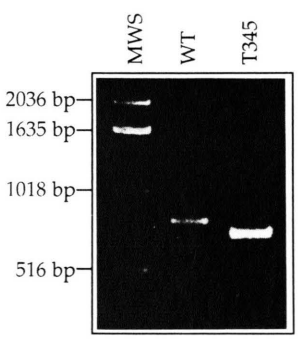

Fig. 3. Fractionation of double stranded DNA molecules that are amplified products of polymerase chain reactions on chromosomal DNA from wild type (WT lane) and T345 (T345 lane) cells, on an 1% agarose gel. MWS – molecular weight standards (Bethesda Research Labs.).

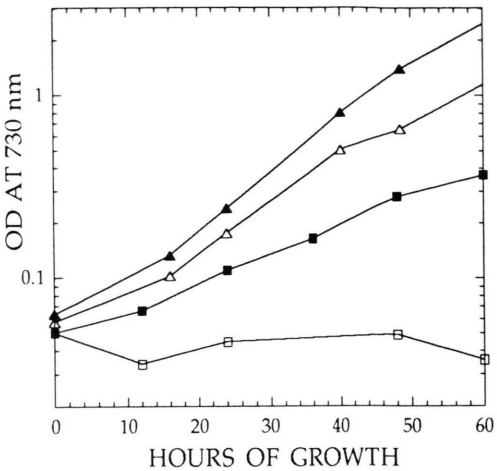

Fig. 4. Growth of wild type and T345 cells in liquid BG11 medium. Growth rates were estimated by measuring OD at 730 nm and plotting on a semilogarithmic scale. The growth medium for the T345 mutant was supplemented with Sp at 5 μg/ml. The different growth curves correspond to: (open triangle) wild type cells in BG11; (solid triangle) wild type cells in BG11 + 5 mM glucose; (open square) T345 cells in BG11; and (solid square) T345 cells in BG11 + 5 mM glucose.

Pigment composition and photosynthetic electron transfer activities

Fig. 5 shows the room temperature absorption spectra of wild type and mutant cells. The relative amplitude of the 620 nm peak from phycobilins with respect to the 680 nm peak from chlorophylls was significantly higher in the T345 cells compared to that in the wild type cells. On an equal cell basis, the mutant strain had 20% less phycobilins and 48% less chlorophylls as compared to the wild type strain. Consequently, the ratio of chlorophyll to phycobilins was decreased by 33% in the mutant cells (Table I).

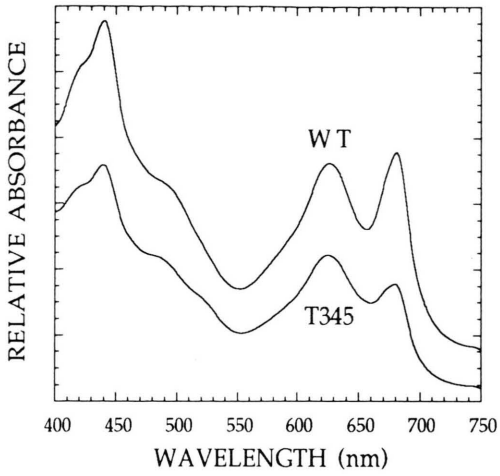

Fig. 5. Room temperature absorption spectra of intact wild type (WT) and T345 cells. The samples were adjusted for equal scattering at 730 nm and then offset for the ease of viewing.

Table I. Pigment composition, photosynthetic electron transfer rates and herbicide-binding activity of wild type and T345 strains.

	Wild type	T345
Chlorophyll/phycobilin (w/w)	0.15	0.10
Electron transport rates (μmol O_2 mg Chl^{-1} h^{-1})*		
H_2O to DCBQ/FeCN (PSII)	402	0
DAD/Asc to MV (PSI)	−328	−342
H_2O to CO_2 (whole chain)	182	0
Herbicide binding		
Chl/binding site	922	n.b.+
K_D (nM)	19.8	n.b.+

* Polarographically determined. Negative numbers indicate O_2 uptake.
Asc, sodium ascorbate; Chl, chlorophyll; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DCBQ, 2,6-dichloro-*p*-benzoquinone; FeCN, $K_3Fe(CN)_6$; and MV, methyl viologen.
+ n.b. – no detectable [^{14}C]diuron binding.

To examine the effect of the deletion mutation on photosynthetic activities, we used artificial electron donors and acceptors to measure PSII- as well as PSI-mediated electron transfer rates in intact wild type and T345 cells. As shown in Table I, there was no detectable PSII-mediated as well as whole chain electron transport activity in the mutant cells. In contrast, the PSI activity in the T345 cells was normal. These results demonstrate that the *psbL* deletion mutation resulted in the specific inhibition of PSII activity. In addition, the mutant cells were unable to bind [^{14}C]diuron (Table I), again indicating that functional PSII centers were absent in the T345 cells.

Fluorescence emission spectra

Fig. 6A shows the fluorescence emission spectra of wild type and T345 cells frozen at 77K. Upon excitation with a broad band light absorbed by both phycobilins and chlorophylls, four peaks, F665, F685, F695 and F720 were observed in the fluorescence emission spectrum of wild type cells (Fig. 6A) as well as in its first order derivative spectrum (Fig. 6B). Among these, F685 and F695 originate from PSII, F665 originate from phycobilisomes and F720 originate from PSI [24]. As shown in Fig. 6A and 6C, the T345 mutant cells lacked in the F695 peak. The origin of F695 has previously been identified as the CP47 protein [25]. However, a number of mutants of *Synechocystis* 6803 that do not exhibit the F695 emission have been found to have significant amounts of the CP47 apoprotein [13, 26]. The exact reason for the loss of this fluorescence peak from these mutants is currently unknown.

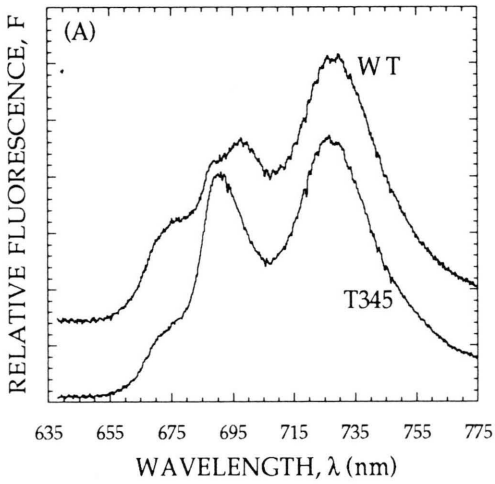

Fig. 6A. 77K fluorescence emission spectra of intact wild type (WT) and T345 cells at 5 µg chlorophyll/ml. The spectra were offset for the ease of viewing. Further details are given in the text.

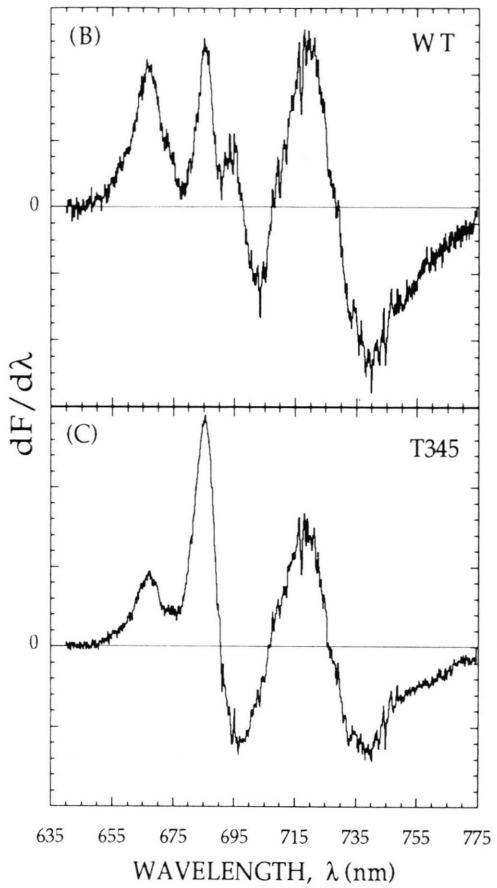

Fig. 6 B and C. Derivative spectra obtained upon differentiation of the data presented in Fig. 6 A. The differentiating intervals were 15 nm.

Immunostaining of thylakoid proteins

Previous studies have indicated that various targeted mutations in the *psbE* and the *psbF* genes affect the steady-state levels of other PSII proteins [9, 18]. In particular, T1297, a *psbEFLJ* deletion mutant, had significant amounts of D 1 but no detectable steady state amount of the D 2 protein [18]. As shown in Fig. 7, the T345 mutant cells had significant amounts of the D 1 and the D 2 proteins in its thylakoid membrane. However, on an equal chlorophyll basis, the levels of both of these proteins were significantly reduced in the mutant cells as compared to the wild type cells.

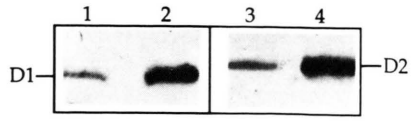

Fig. 7. Immunoblot analysis of membrane proteins. Duplicate samples of thylakoid proteins from T345 (lanes 1 and 3) and wild type (lanes 2 and 4) cells were fractionated on denaturing SDS-PAGE, transferred to a nitrocellulose filter and immunostained with rabbit antibodies raised against the D 1 (lanes 1 and 2) and the D 2 (lanes 3 and 4) proteins, respectively. Further details are given in the text.

Discussion

The PSII complex in the thylakoid membranes of oxygenic photosynthetic organisms contains at least ten different small (< 10 kDa) polypeptide components [2]. Four of these small proteins are encoded by the members of the *psbEFLJ* gene cluster. Previously we have shown that the α and the β subunits of the cyt *b*559 protein, encoded by the *psbE* and the *psbF* genes respectively, are essential for the stable assembly of the PSII reaction center [13, 18]. Recently we have also demonstrated that the product of the *psbJ* gene, a 4 kDa polypeptide, regulates the steady-state level of functionally competent PSII centers [10]. Other studies using site-directed mutagenesis have shown that PSII complexes formed in the absence of the *psbH* protein (10 kDa) show increased vulnerability to high light [27] whereas in the absence of the 3.9 kDa PsbK protein, PSII centers with near normal activity are formed [28]. In the present study, we have used a targeted mutagenesis approach to examine the role of the 4.5 kDa PsbL protein in *Synechocystis* 6803.

Our analysis of the *psbL* deletion mutant T345 shows that the PsbL protein is necessary for the formation of catalytically active PSII centers. The mutant T345 cells were incapable of PSII-mediated oxygen evolution (Table I) and as a consequence could not grow under photoautotrophic conditions (Fig. 4). In addition, the mutation led to significant alterations in the pigment composition of the thylakoid membranes. Fluorescence emission spectra (77 K) showed that F 695, a specific fluorescence emission peak originating from the PSII complex was missing from the mutant cells. These data can be easily explained if the reaction center of PSII could not form in the T345 mu-

tant cells; since we have earlier shown that D2, a member of the heterodimeric reaction center of PSII, is missing from T1297, a deletion mutant from which the entire *psbEFLJ* gene cluster has been deleted [18]. Contrary to this possibility, we found that both the D1 and the D2 proteins accumulate to >25% of their wild type levels in the *psbL* deletion mutant strain (Fig. 7). At present, we do not know whether these two proteins form a heterodimer in the membranes of T345 cells and coordinate the P680 reaction center. Interestingly, inactivation of the *psbC* gene, encoding the CP43 apoprotein in *Synechocystis* 6803, results in the formation of PSII reaction centers at a level 10% of that in wild type cells [29, 30]. The phenotypes of the T345 mutant and CP43-less mutants are very similar. The CP43-less mutant cells lack in their abilities to mediate light-induced water oxidation. Moreover, like the T345 mutant cells, they do not bind radioactive herbicides [31]. However, isolated reaction centers from CP43-less mutants have a normal complement of P680 as well as the acceptors pheophytin and Q_A [30].

Recently it has been shown that PSII core preparations from spinach thylakoids depleted of several small proteins are unable to retain photoreducible Q_A [7]. In addition, the presence of PsbL and another 4.1 kDa protein has been shown to be essential to reconstitute Q_A activity in these PSII preparations. Although these studies did not demonstrate that the PsbL protein alone is sufficient for the proper coordination of Q_A in the D1-D2 heterodimer, they are consistent with the findings in the present communication. Thus, it is conceivable that the D1-D2 reaction center complex is formed in the absence of the PsbL protein in the T345 cells without any photoreducible Q_A. In this case, the Q_B binding site would also be sufficiently perturbed so that diuron, a Q_B antagonist, can not bind to the assembled PSII centers (Table I). In the absence of any functional Q_A molecule, stable charge separation can not occur and as a consequence, water oxidation can not take place. Experiments are currently under progress to examine the presence of Q_A in the *psbL* mutant cells.

Acknowledgements

This research was supported by a grant from the National Institutes of Health (GM45797) to H. B. P. The authors wish to thank Karin Nyhus, Lili Zhang and Rosalind Wu for the construction of the pSL345 deletion plasmid; Drs. M. Ikeuchi and Y. Inoue for the gift of the antibodies used in this study and Dr. W. Vermaas for the gift of labeled diuron.

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