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Construction of an Obligate Photoheterotrophic Mutant of the Cyanobacterium *Synechocystis* 6803[1]

INACTIVATION OF THE *psbA* GENE FAMILY

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ABSTRACT

***psbA* in *Synechocystis* 6803 was found to belong to a small multigene family with three copies. The *psbA* gene family was inactivated *in vitro* by insertion of bacterial drug resistance markers. Inactivation of all three genes resulted in a transformant that is unable to grow photosynthetically but can be cultured photoheterotrophically. This mutant lacks oxygen evolving capacity but retains photosystem I activity. Room temperature measurements of chlorophyll *a* fluorescence induction demonstrated that the transformant exhibits a high fluorescence yield with little or no variable fluorescence. Immunoblot analyses showed complete loss of the *psbA* gene product (the DI polypeptide) from thylakoid membranes in the transformant. However, the extrinsic 33 kilodalton polypeptide of the water-splitting complex of photosystem II, is still present. The results indicate that assembly of a partial photosystem II complex may occur even in the absence of the intrinsic D1 polypeptide, a protein implicated as a crucial component of the photosystem II reaction center.**

Oxygenic photosynthesis in higher plants, algae, and cyanobacteria involves the cooperation of two photosystems, PSI and PSII, operating in series. The structure of the protein-Chl complex of PSII, its biogenesis, assembly, and turnover are currently the subject of intense research. The reaction center core of PSII consists of Chl *a* and carotenoids, and at least six intrinsic polypeptides with approximate mol wt of 47,000, 43,000, 32,000, 32,000, 10,000, and 4,000 (4, 12). The two larger polypeptides (CP-47 and CP-43) have been identified as Chl-binding polypeptides. One of the 32 kD polypeptides, D1, is likely to harbor the secondary quinone acceptor Q_B near the external face of the thylakoid membrane. Functions of the other 32 kD polypeptide, designated D2, and of the 10 and 4 kD polypeptides (the two subunits of cytochrome *b-559*), have yet to be established. Three extrinsic polypeptides with approximate mol wt of 33,000, 23,000, and 16,000 have been shown to be constituents of the water-splitting system of PSII (2, 4, 12). The 33 kD polypeptide is involved in stabilizing the manganese redox center, and the 23 and 16 kD polypeptides in increasing the affinity for Ca^{2+} and Cl^- at the water oxidizing site. In cyanobacteria, the 23 and 16 kD polypeptides seem to be missing. Associated with the PSII core is an outer pigment-protein bed that funnels excitation energy to the reaction center Chl *a* P680. In higher plants this light harvesting complex contains Chl *a* and *b*. Cyanobacteria, which lack Chl *b*, have the analogous complex organized in unique structures called phycobilisomes.

The location of the various electron carriers and other cofactors in the protein matrix of PSII is unclear. Different lines of evidence have given rise to conflicting hypotheses concerning the site of the primary charge separation. It has long been thought that P680 together with the intermediate acceptor pheophytin and the primary quinone acceptor Q_A is bound to CP-47 (for reviews see 4, 12). On the other hand, sequence homologies between the D1/D2 polypeptides of PSII and reaction center subunits of photosynthetic bacteria, together with the crystallographic structure of the reaction center from *Rhodopseudomonas viridis*, have recently led to the proposal that charge separation in PSII is functionally located in the D1/D2 unit (9, 19, 29). This latter proposal has gained recent support from studies on a photochemically active PSII complex consisting of D1, D2, and cytochrome *b-559* (20, 24).

In the present study we set out to examine the function of the D1 polypeptide and its interaction with other PSII polypeptides. Our approach has been to construct a well-defined mutant of the cyanobacterium *Synechocystis* 6803 lacking the D1 polypeptide. In addition to attributes such as small genome size (15) and short generation time (<24 h), *Synechocystis* 6803 has two main features important for our studies. First, the cells are naturally competent. They can take up exogenous DNA and integrate it into their chromosome by homologous recombination. This feature enables us to modify a gene *in vitro* and shuttle it back into the cells, where it will replace the wild type gene. Foreign genes can also be introduced by this method of gene replacement. The second important feature is that *Synechocystis* 6803 possesses the ability to grow photoheterotrophically, thus allowing rescue and functional analyses of mutants impaired in photosynthetic capacity. Such analyses permit specific aspects of photosynthetic function to be related to individual polypeptides or polypeptide domains.

MATERIALS AND METHODS

Growth Conditions. *Synechocystis* 6803 was grown in BG 11 medium with 5 mM TES (pH 8.0), at 33°C in constant light (33).

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For selection and propagation of the different transformants the medium was supplemented with 5 mM glucose, 10 μM DCMU (Sigma), and appropriate combinations of antibiotics (Sigma). The concentrations of antibiotics were 5 μg/ml kanamycin, 10 μg/ml streptomycin, 20 μg/ml spectinomycin, and 2.5 μg/ml chloramphenicol.

Cloning of *psbA* genes. *psbA*-1 and *psbA*-2 were cloned from a plasmid library in pUC18 (Fig. 1) using the *psbA* gene from *Amaranthus hybridus* as a heterologous probe (16, 33). The clones contained the entire *psbA*-1 with flanking sequences, and the 3′ half of *psbA*-2 with flanking sequence, respectively. A *psbA*-3 clone was isolated from a *lambda* EMBL3 library using *psbA*-2 as a probe. This clone contained the 3′ half of *psbA*-3 with flanking sequence. A *SalI-BamHI* portion of the phage clone, containing the *psbA*-3 gene fragment, was subcloned in pUC19.

Inactivation of *psbA* Genes. The protocol for inactivation is given in Figure 1. The kanamycin resistance gene was isolated as a *Hin*cII fragment from pUC4K (31). The linked streptomycin and spectinomycin resistance genes were isolated as a *Sma*I fragment from pHP45 (27). The chloramphenicol resistance gene from pKT210 (5) was isolated as a *Hin*cII fragment from pRL 171 (pRL 171 was a kind gift was Jeff Elhai and Peter Wolk). After transformation (33) the cells were spread on nitrocellulose filters (MSI, Fisher Scientific) overlaying nonselective plates. After 48 h the filters were transferred to plates containing appropriate selective media. Transformed colonies were visible after 5 to 8 d.

DNA Isolation and Hybridization. Genomic DNA from *Synechocystis* 6803 was isolated from CsCl gradients (33). Southern blot analyses were performed according to standard procedures.

Isolation of Thylakoid Membranes. All steps were carried out at 4°C. Cells were harvested and washed once in preparation buffer (50 mM Hepes [pH 6.5], 10 mM $Na_4P_2O_7$, 25% v/v glycerol). The washed cells were passed four times through a prechilled French pressure cell (Aminco) at 20,000 p.s.i. The broken cells were diluted 4-fold with preparation buffer and centrifuged for 10 min at 25,000*g* to pellet unbroken cells and cell debris. The thylakoid fragments were pelleted by centrifugation for 30 min at 200,000*g*, washed once in preparation buffer without $Na_4P_2O_7$, and finally suspended in the same buffer at a Chl *a* concentration of approximately 2 mg/ml.

Polypeptide Analyses. Thylakoid polypeptides were separated by SDS-PAGE as before (1). Protein transfer to nitrocellulose was carried out for 2 h at a current of 1 amp and a temperature of 4°C. Antibody probing was performed according to the protocol of Blake *et al.* (7) with minor modifications, using alkaline phosphatase conjugated to either protein A, anti-rabbit IgG, or anti-mouse IgG (Sigma). Total proteins were stained by incubating the nitrocellulose (Schleicher and Schuell) blot in India ink (14). Antibodies prepared against the D1 polypeptide from *Amaranthus hybridus* (23) and against the extrinsic 33 kD polypeptide from Spinach (1) were used as probes for the analogous polypeptides in *Synechocystis* 6803.

Measurements of Electron Transport and Fluorescence. All steps were carried out at room temperature. Cells were harvested and washed three times in assay medium (30 mM Hepes [pH 7.4], 20 mM NaCl) supplemented with 200 mM sorbitol. The washed cells were suspended in assay medium and kept at room temperature in the dark. Oxygen evolution was measured with a Rank Brothers oxygen electrode at room temperature and with saturating white light using DMQ[2] (Aldrich) and $K_3Fe(CN)_6$ as a combined electron acceptor. PSI activity was followed as O_2 uptake via the Mehler reaction, with duroquinonediimide (Aldrich) as electron donor, methylviologin (Sigma) as electron acceptor, and ascorbate as O_2^- scavenger. Chl *a* fluorescence induction was measured in a fluorimeter of local design. Further details are given in the legends to Figures 3 and 4.

Chl Determination. Chl *a* concentrations were determined, with some modifications, according to Mackinney (18).

RESULTS

Inactivation of the *psbA* Gene Family. Using a combination of homologous and heterologous probes we found that *psbA* in *Synechocystis* 6803 belongs to a small multigene family with three members. These genes are designated *psbA*-1, *psbA*-2, and *psbA*-3, in order of identification (Figs. 1 and 2). Furthermore, the three genes are not immediately adjacent on the *Synechocystis* chromosome. The complete sequence of *psbA*-1 has been determined and will be presented elsewhere (H Osiewacz, L McIntosh, unpublished data). Preliminary sequence data and Northern blot analyses (not presented) confirm the identity of *psbA*-2 and *psbA*-3 as full-length *psbA* genes. The strategy for inactivation of the *psbA* gene family is outlined in Figure 1. Constructions containing the interrupted *psbA* genes were cloned in the vector pUC19, which does not replicate in *Synechocystis*, and the recombinant plasmids were used for transformation. Disruption of the *psbA* genes within the chromosome was confirmed by Southern blot analyses (Fig. 2).

PSII Activities. We found that inactivation of *psbA*-1 alone, or of *psbA*-1 together with *psbA*-2, did not affect the photosynthetic growth of the cells. Inactivation of all three *psbA* copies, however, prevented photosynthetic growth, and the resulting triple-transformant ($T_{1,2,3}$) required a reduced carbon source in the presence of light. To learn more about the photosynthetic capacity of the mutant, electron transport activities were investigated. The oxygen evolution measurements in Figure 3 reflect electron transfer from water to the exogeneous PSII acceptor DMQ. DMQ intercepts electrons at a site between Q_B and the PQ pool. The DMQ-supported activity is sensitive to DCMU,

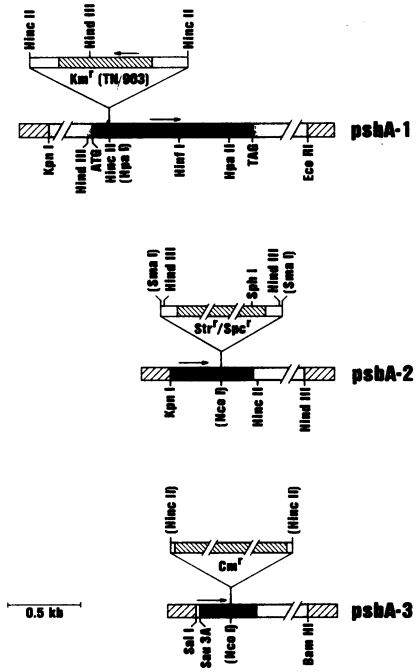

FIG. 1. The *psbA* genes from *Synechocystis* 6803 were inactivated *in vitro* by insertion of marker genes conferring antibiotic resistance. The kanamycin resistance gene (Km^r) was inserted as a *Hin*cII fragment, the streptomycin and spectinomycin resistance cartridge (Str^r/Spc^r) as a *Sma*I fragment, and the chloramphenicol resistance gene (Cm^r) as a *Hin*cII fragment.

[2] Abbreviation: DMQ, 2,5-dimethyl-*p*-benzoquinone.

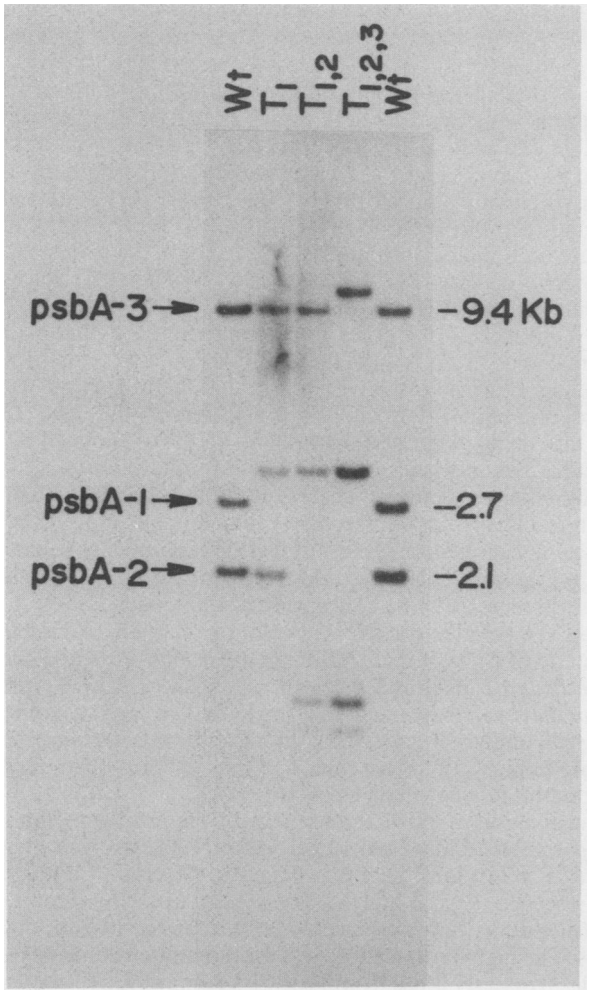

FIG. 2. Southern blot analyses of genomic DNA from *Synechocystis* 6803 digested with *Hin*dIII and probed with a mixture of ^{32}P-labeled *psbA*-1, *psbA*-2, and *psbA*-3. The lanes show hybridization to DNA from wild type (Wt) and from mutants obtained after transformation of Wt with the inactivated *psbA*-1 (T_1), of T_1 with the inactivated *psbA*-2 ($T_{1,2}$) and of $T_{1,2}$ with the inactivated *psbA*-3 ($T_{1,2,3}$). The inactivated *psbA*-1 and *psbA*-3 genes gave rise to larger *Hin*dIII fragments as compared to the native genes due to the Km^r and Cm^r inserts. The inactivated *psbA*-2 showed two smaller fragments because of the two *Hin*dIII sites in the Str^r/Spc^r cartridge (Fig. 1). Sizes in kilobase pairs (Kb) are indicated.

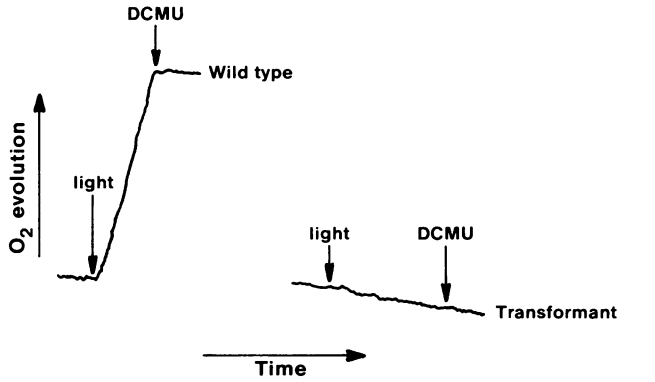

FIG. 3. Oxygen evolution measured at room temperature with saturating white light. The reaction mixture contained 30 mM Hepes-HCl (pH 7.4), 20 mM NaCl, 1 mM DMQ, 3 mM $K_3Fe(CN)_6$, and 15 μg Chl *a* ml^{-1}. DCMU (10 μM) was added as indicated. The rate for the wild type was 201 μmol O_2 mg^{-1}Chl *a* h^{-1}. No activity could be detected in the transformant.

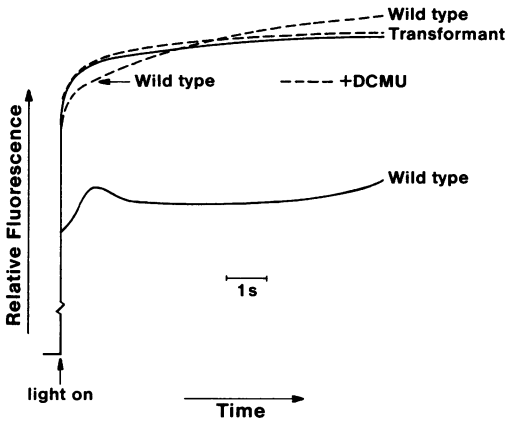

FIG. 4. Time course of room temperature Chl *a* fluorescence induction measured through a red (Corning 2-64) emission filter and with a blue (Corning 4-96) excitation filter. The reaction mixture contained 30 mM Hepes-HCl (pH 7.4), 20 mM NaCl, and 5 μg Chl *a* ml^{-1}. DCMU (10 μM) was added as indicated. Samples were dark adapted for 10 min. Note that the (+)DCMU and (−)DCMU traces for the transformant overlap.

which blocks electron transport presumably by binding to the Q_B binding site on the D1 polypeptide. The wild type showed high rates of O_2 evolution while no activity could be detected in the mutant, even with increased concentrations of electron acceptor. The PSI activity was not affected in the transformant (not shown).

To characterize further the photosynthetic activity of the transformant, room temperature measurements of Chl *a* fluorescence induction were performed (Fig. 4). The wild type cells exhibited an oscillation in variable fluorescence similar to that reported for other cyanobacteria. The peak yield represents the maximum accumulation of reducing equivalents in the electron transport chain between Q_A and PSI. Blocking PSII electron transfer with DCMU abolished the oscillation and caused a rapid rise in fluorescence yield up to a high semi-steady state level. The fluorescence for the transformant lacked the transient feature observed for the wild type and instead displayed kinetics resembling that of DCMU-poisoned wild type cells, with a rapid rise to a high steady state level. The addition of DCMU to the transformant did not affect the fluorescence. The results in Figure 4 were obtained using broadband emission and excitation filters. Similar results were obtained when most of the phycobilisome fluorescence was removed by a 690 nm (10 nm bandwidth) interference emission filter (not shown), suggesting that much of the high fluorescence in the transformant originates from PSII Chl *a*.

Polypeptide Composition. In order to confirm that inactivation of the *psbA* gene family prevents synthesis of D1, thylakoid membranes were isolated from wild type and mutant cells of *Synechocystis* 6803. The membranes were subjected to SDS-PAGE, followed by electrophoretic transfer of proteins to nitrocellulose (Fig. 5). Staining the nitrocellulose for total proteins did not reveal any differences between wild type and transformant. However, probing with an antibody against D1 showed no D1 polypeptide present in the thylakoids from the transformant. Furthermore, no aberrant molecular weight forms of D1 could be detected. We cannot exclude, however, that modified *psbA* products are synthesized in the mutant but are unable to be inserted into the thylakoid membranes. Consistent with the functional analyses, inactivation of *psbA*-1 alone, or together with *psbA*-2, did not decrease the amount of the D1 polypeptide (not shown).

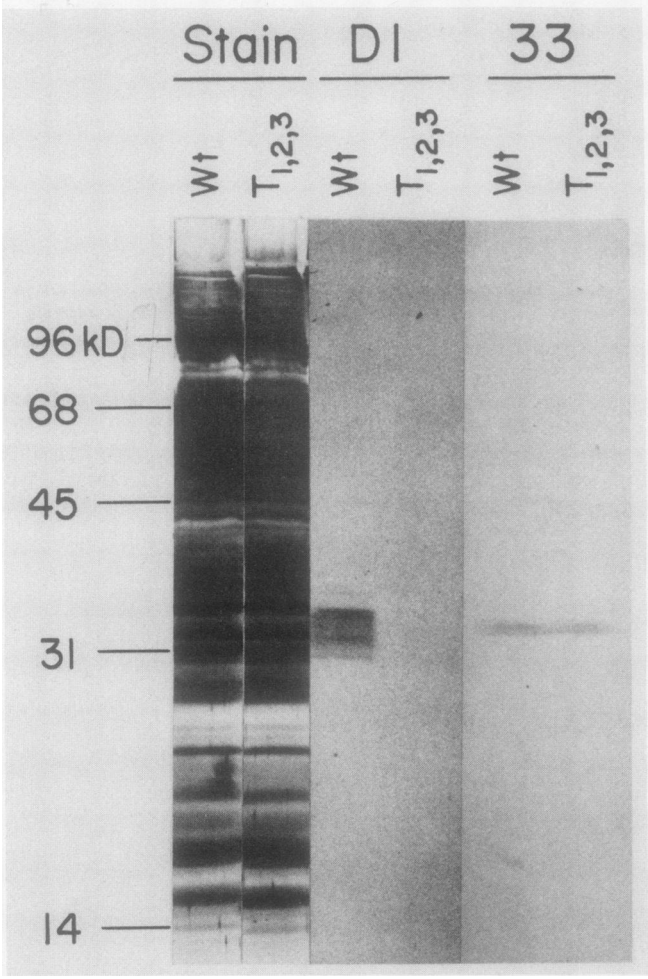

FIG. 5. Thylakoid polypeptides from wild type (Wt) and mutant ($T_{1,2,3}$) were separated by SDS-PAGE and subsequently transferred to nitrocellulose. After transfer, one part of the nitrocellulose was incubated in India ink to stain for total proteins. The other parts were incubated with antibodies against either the D1 polypeptide or the extrinsic 33 kD polypeptide. Each sample contained thylakoids corresponding to 5 μg Chl *a*. Sizes in kilodaltons (kD) are indicated.

Investigations with photosynthetic mutants of *Chlamydomonas* (6, 11) have shown that inability to synthesize either D1 or D2 results in a rapid loss of most PSII polypeptides, intrinsic as well as extrinsic, from thylakoid membranes. It seems reasonable to expect that the lack of the intrinsic D1 polypeptide in the *Synechocystis* mutant would prevent the remaining PSII polypeptides from being stably integrated into the thylakoid membrane. Therefore, it was of interest to probe the protein blots with an antibody against the extrinsic 33 kD polypeptide in the water-splitting complex of PSII. As is evident from Figure 5, this polypeptide is present in the thylakoids from the transformant.

DISCUSSION

The presence of a multigene family for *psbA* in *Synechocystis* is in agreement with studies on other cyanobacteria (13, and references therein). In *Anacystis nidulans* R2 two of the three *psbA* genes encode identical polypeptides (13). DNA:DNA hybridizations performed under stringent conditions (not shown) indicate that in *Synechocystis* 6803 *psbA*-2 and *psbA*-3 are very homologous, while *psbA*-1 is more divergent. It remains to be seen if the polypeptides encoded by *psbA*-2 and *psbA*-3 are identical. *psbD*, encoding the D2 polypeptide (11), exists as two copies in the *Synechocystis* genome (33). The utility of multigene families for *psbA* and *PsbD* in cyanobacteria is not understood. In higher plants and eukaryotic algae there is generally only one copy of these genes per plastid genome. *Chlamydomonas* is an exception with two identical copies of the *psbA* gene (10). A possible explanation for multiple *psbA* genes in cyanobacteria could be that different *psbA* polypeptides serve different functions. As already pointed out (13), that the D1 polypeptide might have functions in addition to its role in PSII is indicated through studies on tomatoe chromoplasts (26) and on the endosymbiont *Anabaena azollae* (21). In both these systems PSII activity is absent, yet the level of *psbA* transcript remains high. Alternatively, multiple *psbA* genes might be required to maintain an optimal steady state level of the D1 polypeptide in cyanobacteria. Labeling experiments have shown that in higher plants and eukaryotic algae D1 is rapidly turned over in the light (17). It has also been suggested that there are two pools of D1 in thylakoids membranes (3, 32). These results imply a need for highly efficient synthesis of D1. It should also be noted that whereas higher plant chloroplasts may contain more than 100 copies of their genome, the number of genome equivalents per cell in cyanobacteria only varies between 1 to 16 (13, 15). Thus, it can be argued that duplications of cyanobacterial *psbA* may have been selected for during evolution in order to maintain a high rate of D1 synthesis. Although the results presented here for *Synechocystis* 6803 and those for *A. nidulans* R2 (13) demonstrate that cyanobacteria containing only one copy of *psbA* grow normally under laboratory conditions, cyanobacteria growing in nature may require more than one 'species' of *psbA* to facilitate adaptation to a changing environment.

Inactivation of all three members of the *psbA* gene family in *Synechocystis* 6803 blocks PSII activity. The oxygen evolving capacity is lost and there is a dramatic increase in nonvariable fluorescence (Figs. 3 and 4). The results from the fluorescence measurements can most simply be interpreted in either of two ways. The high fluorescence yield for the mutant could be caused by decaying excitation from PSII Chl *a* and phycobilisomes that are no longer able to funnel the excitation to a reaction center. Alternatively, the high fluorescence may reflect inhibition in electron transfer on the reducing side of PSII between Q_A and the Plastoquinone pool. The latter possibility would imply that the water-splitting system of PSII is still able to feed electrons to P680. Thus, a more comprehensive understanding of the fluorescence data will require that the oxidizing side of PSII be examined. Preliminary results from measurements of Signal II, EPR signals arising from components on the oxidizing side of PSII (4), indicate that the transformant is blocked at this site. This supports the conclusion that the high fluorescence phenotype of the transformant results from PSII Chl *a* and phycobilisomes no longer functionally linked to a reaction center. Whether this is a consequence of a specific loss of the reaction center P680, or of a general disruption of the PSII complex, remains to be seen.

It is not clear to what extent a PSII complex can assemble in mutants lacking specific functional PSII genes. That the high fluorescence of the transformant (Fig. 4) is also observed at 690 nm, where primarily Chl *a* fouoresces, suggests that PSII Chl *a* is still present even in the absence of D1. The extrinsic 33 kD polypeptide in the water-splitting system of PSII could still be detected in thylakoid membranes from the mutant (Fig. 5). It cannot be ruled out that the 33 kD polypeptide is nonspecifically bound to the membranes. However, reconstitution experiments with thylakoids from higher plants indicate that there are no nonspecific binding sites for the 33 kD polypeptide on the thylakoid membranes (1). Apart from the presence of the 33 kD polypeptide, little is known about the polypeptide composition of the water-splitting system in cyanobacteria. It is not known if the 33 kD polypeptide has binding sites on any of the core

polypeptides in the PSII reaction center and/or on other intrinsic PSII polypeptides. In any case, the presence of both the 33 kD polypeptide, phycobilisomes and PSII Chl *a* in the thylakoids of the transformant suggests that part of PSII may still assemble in the absence of the D1 polypeptide. These conclusions are consistent with studies demonstrating that mutants of *Synechocystis* 6803 lacking either CP-47 (30) or Cyt *b-559* (25) still contain some PSII polypeptides in the thylakoid membrane. It appears that loss of an intrinsic PSII polypeptide does not necessarily result in degradation of the entire PSII complex.

The diffuse appearance of the band obtained after probing the Western blot with the antibody against the D1 polypeptide (Fig. 5) is a phenomenon observed also by others (28). It is known that the D1 polypeptide often gives rise to diffuse bands on polyacrylamide gels. Whether or not this is due to degradation of the D1 polypeptide is not clear. In our experiments, the presence of protease inhibitors during thylakoid preparation and subsequent steps did not improve the signal. The D1 polypeptide possesses significant sequence homologies to the D2 polypeptide (29). Thus, it could be argued that the diffuse band in Figure 5 may result from a cross-reaction between the D2 polypeptide and the antibody against the D1 polypeptide. We do not totally exclude that possibility; however, a recent study on higher plants (22) suggests that such a cross-reactivity does not exist.

A further characterization of the functional and structural defects of the *psbA* transformant is now in progress. Also, the presence of a multigene family for *psbA* in cyanobacteria poses the important question of how these different genes are regulated. Preliminary data for the expression of the *psbA* genes in *Synechocystis* 6803 indicate differential control of expression (C Jansson, L McIntosh, unpublished data). This is consistent with results for *A. nidulans* R2 (13) and *Anabaena* 7120 (8), and could possibly confer an adaptive advantage to these photosynthetic organisms. We believe that the construction of *psbA* mutants in *Synechocystis* 6803 will provide an essential basis for addressing questions concerning function and regulation of the different *psbA* genes.

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