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Transcript Levels and Synthesis of Photosystem II Components in Cyanobacterial Mutants with Inactivated Photosystem II Genes

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After interruption or deletion of the photosystem II genes *psbB*, *psbC*, and *psbD* in the cyanobacterium *Synechocystis* sp. PCC 6803, thylakoids from such mutants were found to be depleted in a number of photosystem II proteins in addition to those for which the gene(s) had been inactivated. Transcript levels of photosystem II genes were measured and protein pulse-labeling was carried out to determine the reason for this effect. Transcripts of all photosystem II genes except the inactivated one(s) were found to be present in the various mutants. In certain cases, inactivation of one photosystem II gene led to overexpression of another. Protein pulse-labeling experiments using ^{35}S-methionine, in which not only the rapidly turning over D1 protein but also D2, CP43, and CP47 appear to be preferentially labeled, showed that the mutants studied synthesize the D1 protein as well as other photosystem II proteins whose genes were not inactivated. The fact that, in the various mutants, photosystem II proteins for which the gene is not inactivated are synthesized but do not accumulate in the thylakoid indicates that the *psbB*, *psbC*, and *psbD* gene products are all required for a stable assembly of the photosystem II complex.

INTRODUCTION

The cyanobacterial photosynthetic apparatus is very similar to that found in plant chloroplasts (Arntzen and Pakrasi, 1986; Bryant, 1986). Some of the initial processes in photosynthesis take place in photosystem II (PS II), one of the major pigment-protein complexes in the thylakoid membranes, which catalyzes the light-induced reduction of plastoquinone by water via a chain of redox reactions in higher plants, algae, and cyanobacteria (Barber, 1987; Andreasson and Vänngård, 1988; Vermaas, 1988). The PS II complex is composed of a number of proteins including D1, CP47, CP43, D2, apoproteins of cytochrome b_{559}, and the extrinsic manganese-stabilizing protein, along with a number of small proteins (Arntzen and Pakrasi, 1986; Gray et al., 1989; Vermaas, 1989).

In several instances, in both eukaryotes and prokaryotes, mutation in a single PS II gene has been documented to result in the disappearance of multiple components of the PS II complex from the thylakoid. Thylakoids from a *Chlamydomonas reinhardtii* mutant lacking *psbA* (Bennoun et al., 1986), as well as those from *Chlamydomonas* or *Synechocystis* sp. PCC 6803 mutants impaired in *psbD* (Erickson et al., 1986; Vermaas et al., 1987b, 1988), have been shown to be depleted severely in the other PS II core proteins (D2 or D1, CP47, CP43), in addition to lacking the protein (D1 or D2) encoded by the mutated gene. After deletion or interruption of the *psbB* gene, D1 and D2 cannot be detected in the thylakoid, whereas the amount of CP43 is significantly reduced (Vermaas et al., 1986, 1988). Upon deletion or interruption of the *psbC* gene, the level of other PS II core proteins has been drastically decreased (Dzelzkalns and Bogorad, 1988; Vermaas et al., 1988; Rochaix et al., 1989). Thus, generally speaking, in the absence of D1, D2, or CP47, the other large PS II proteins do not accumulate in the thylakoid membrane (Bennoun et al., 1986; Erickson et al., 1986; Jensen et al., 1986; Vermaas et al., 1988, 1989), whereas absence of CP43 leads to a decreased amount of other proteins of the PS II core complex in the thylakoid. The mechanism by which these proteins influence the presence or absence of other proteins in the PS II core complex could involve regulation at the transcriptional, translational, and/or post-translational level. In *Chlamydomonas*, post-translational regulation appears to be prevalent, although not exclusive. However, for cyanobacteria, of which a number of well-defined PS II mutants are available, the level of regulation has not yet been established. To determine the level at which inactivation of one PS II gene modifies the expression of other genes, we present here the results of PS II transcript level measurements and protein pulse-labeling experiments using a number of specific PS II mutants of the cyanobacterium *Synechocystis* sp. PCC 6803.

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Table 1. Summary of Photosystem II Genes in Wild Type and Mutants[a]

Strain	*psbAI/AII/AIII*	*psbB*	*psbC*	*psbDI*	*psbDII*	Reference
Wild type	+	+	+	+	+	
psbDI/C$^-$	+	+	−, Cm	−, Cm	+	This work
psbDII$^-$	+	+	+	+	−[b], Sm	This work
psbDI/C/DII$^-$	+	+	−, Cm	−, Cm	−[b], Sm	Vermaas et al. (1990)
psbB$^-$	+	−, Sm	+	+	+	This work
psbB-int	+	/, Km	+	+	+	Vermaas et al. (1988)
psbC-int	+	+	/, Km	+	+	Vermaas et al. (1988)

[a] Symbols are as follows: +, gene present; −, gene deleted and replaced by antibiotic resistance marker; /, gene interrupted by antibiotic resistance marker; Cm, gene interrupted or replaced by a chloramphenicol-resistance cartridge; Sm, gene interrupted or replaced by a spectinomycin-resistance cartridge; Km, gene interrupted or replaced by a kanamycin-resistance cartridge.
[b] One hundred sixty-one base pairs on the 5′ end and 17 bp on the 3′ end of *psbDII* are still present in this mutant.

RESULTS

Mutants Used in This Study

A number of mutants with inactivated *psbB*, *psbC*, and/or one or both copies of *psbD* have been generated by directed mutagenesis, of which six have been used for this study. The properties of some of these mutants have been described before (Vermaas, 1988; Vermaas et al., 1990) and are summarized in Table 1. Mutants used here that have not been reported before are *psbDI/C*$^-$, *psbDII*$^-$, and *psbB*$^-$. The mutant *psbDI/C*$^-$ contains a deletion of the entire *psbDI/C* operon with a chloramphenicol-resistance marker (from pBR325) replacing the EcoRV/DraI fragment covering *psbDI/C* (Vermaas et al., 1990). The *psbDII*$^-$ mutant has all but the first 161 bp and last 17 bp of *psbDII* deleted and replaced by a spectinomycin-resistance cartridge (Vermaas et al., 1990) from pHP 450Ω (Prentki and Kirsch, 1984). This mutant is photoautotrophic and is indistinguishable from wild type in its PS II composition and growth rates; thus, *psbDII* appears functionally dispensable under the conditions used. Note that in *Synechocystis* sp. PCC 6803, as well as in all other cyanobacteria studied thus far, *psbDI* overlaps with *psbC* (as is also found in higher plants), whereas *psbDII* does not appear to be co-transcribed with other genes. The *psbB*$^-$ mutant has the region between the *psbB* translation start site and a NcoI site 0.5 kb downstream from the end of the gene deleted and replaced by a spectinomycin-resistance marker. Note that the mutants *psbB*-int and *psbC*-int have been referred to as *psbB-I1* and *psbC-I1*, respectively, in Vermaas et al. (1988).

Transcript Detection

Blots of total RNA isolated from wild-type *Synechocystis* sp. PCC 6803 and the various mutants were probed with gene-internal regions of cloned *psbA*, *psbB*, *psbC*, and *psbD* from *Synechocystis*. The results are shown in Figure 1. As a control to verify equal loading of the various lanes, a gene-internal DNA fragment from *Synechocystis* sp. PCC 6803 *rbcL* (the gene coding for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) was also used as a probe; *rbcL* expression is assumed to be independent of expression of PS II genes, and the level of *rbcL* transcript indeed was observed to be very similar in the various mutants (Figure 1E).

In Figure 1A, the 1.2-kb bands represent transcripts of one or more *psbA* genes. Under stringent hybridization conditions, *psbAI* hybridization to RNA yielded significantly less intense bands (not shown) than when using a *psbAII* fragment of approximately equal length and radiolabeled at equal specific activity. This suggests that *Synechocystis psbAI*, which is relatively different from *psbAII* (Osiewacz and McIntosh, 1987; Ravnikar et al., 1989), is not highly expressed under the conditions used. The overall transcript level of *psbA* in the mutants is similar to that in wild type, although those in the *psbDI/C*$^-$ and *psbC*-int mutants are a little increased, whereas that in the *psbDI/C/DII*$^-$ mutant is somewhat decreased. Thus, the observations imply that the overall *psbA* transcript levels are not drastically affected by inactivation of other PS II genes.

In Figure 1B, the 2.0-kb band represents the *psbB* gene transcript. As expected, deletion of *psbB* in the *psbB*$^-$ mutant leads to a loss of *psbB* transcript. Because in *psbB*-int interruption of *psbB* occurred near the 3′ end of *psbB* (Vermaas et al., 1987a), the absence of a *psbB* transcript in the *psbB*-int mutant suggests that a *psbB*/ marker transcript formed is unstable and does not accumulate. Note that the *psbB* transcript level is increased if one of the two *psbD* copies is deleted but not if both (and *psbC*) are inactivated.

In Figure 1C, there are several bands representing *psbC* gene transcripts. The upper band (3.0 kb) represents transcripts containing both *psbDI* and *psbC*. This *psbDI/C* transcript apparently can be processed to a slightly smaller transcript containing both *psbDI* and *psbC* and to a 1.5-kb transcript containing mostly *psbC*. *psbC* transcripts were detected in wild type and in all mutants except in the *psbC* gene deletion mutants (*psbDI/C*$^-$ and *psbDI/C/DII*$^-$). It was

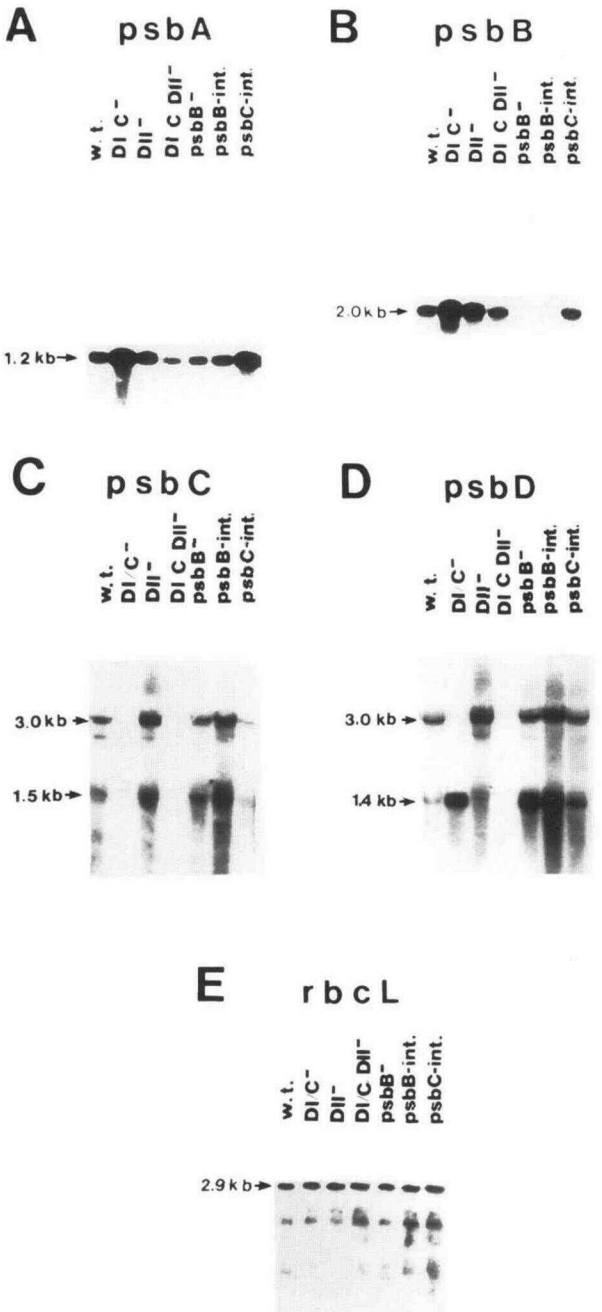

Figure 1. Detection of Transcripts for Major Components of the PS II Complex.

Blots of total RNA from wild type and mutant *Synechocystis* sp. PCC 6803 were probed with fragments of the following genes:
(A) *psbAII*.
(B) *psbB*.
(C) *psbC*.
(D) *psbDI*.
(E) As a control for equal loading of RNA in the various lanes, a blot was probed with an internal *rbcL* fragment from *Synechocystis* sp. PCC 6803.
The approximate transcript sizes have been indicated.

observed that, in the mutant in which *psbC* has been interrupted by a kanamycin-resistance marker at the SmaI site near the 3′ end of the gene (Vermaas et al., 1988), a *psbC* gene transcript of a size similar to that of wild type still was detected, although in decreased amount. Presumably, in the *psbC* interruption mutant transcription termination can occur in the kanamycin-resistance marker relatively close to the interruption site and yield a relatively stable transcript containing most of *psbDI/C* and some of the marker.

In Figure 1D, the 3.0-kb band represents the *psbDI/C* transcript that is identical to the band that is recognized by the *psbC* probe. The 1.4-kb band represents the *psbDII* gene transcript. In wild type, *psbDII* appears to be expressed at a relatively low but significant level. Expression is dramatically increased when *psbDI* has been deleted, whereas *psbB* interruption or deletion leads to increased *psbDII* expression. Upon deletion of *psbDII*, *psbDI/C* is more highly expressed than usual, and 1.5-kb to 1.6-kb bands, presumably processing products containing at least part of *psbDI*, become visible.

Thus, in each of the mutants, significant amounts of PS II transcripts were detected except for the genes that were inactivated. This indicates that the inability to synthesize one of the PS II proteins does not lead to a suppression of transcription of genes coding for other PS II core components such that transcript levels of these genes are dramatically decreased.

Steady-State Levels of Photosystem II Thylakoid Proteins and Protein Pulse-Labeling

The left panel of Figure 2 represents an immunoblot of thylakoid proteins from wild type and mutants probed with a mixture of D1, D2, CP43, and CP47 antisera. The location of the four major PS II components on the blot has been indicated. The antiserum mixture also recognized a protein of approximately 70 kD. Both the D1 and D2 antisera, but not the CP43 and CP47 antisera, recognize a band in this area of the blot (not shown). It is possible that the 70-kD band represents a D1/D2 (or D1/D1 and D2/D2) dimer, but it is striking that also in the *psbDI/C/DII*$^-$ mutant, which lacks a measurable steady-state amount of both D1 and D2 in its thylakoids, this band occurs.

The *psbDI/C*$^-$ mutant contains decreased, but significant, levels of CP47, D2, and D1 in its thylakoids and lacks CP43. This indicates that *psbDII* can be expressed, and, as noted before (Dzelzkalns and Bogorad, 1988; Vermaas et al., 1988), that CP43 is not absolutely necessary for a relatively stable assembly of other major PS II components. The *psbDII*$^-$ mutant contains a full complement of PS II proteins and is photoautotrophic, indicating that *psbDII* is dispensable with respect to PS II structure and function under these conditions. The *psbDI/C/DII*$^-$ mutant lacks a measurable amount of D1, D2, and CP43 but, under the

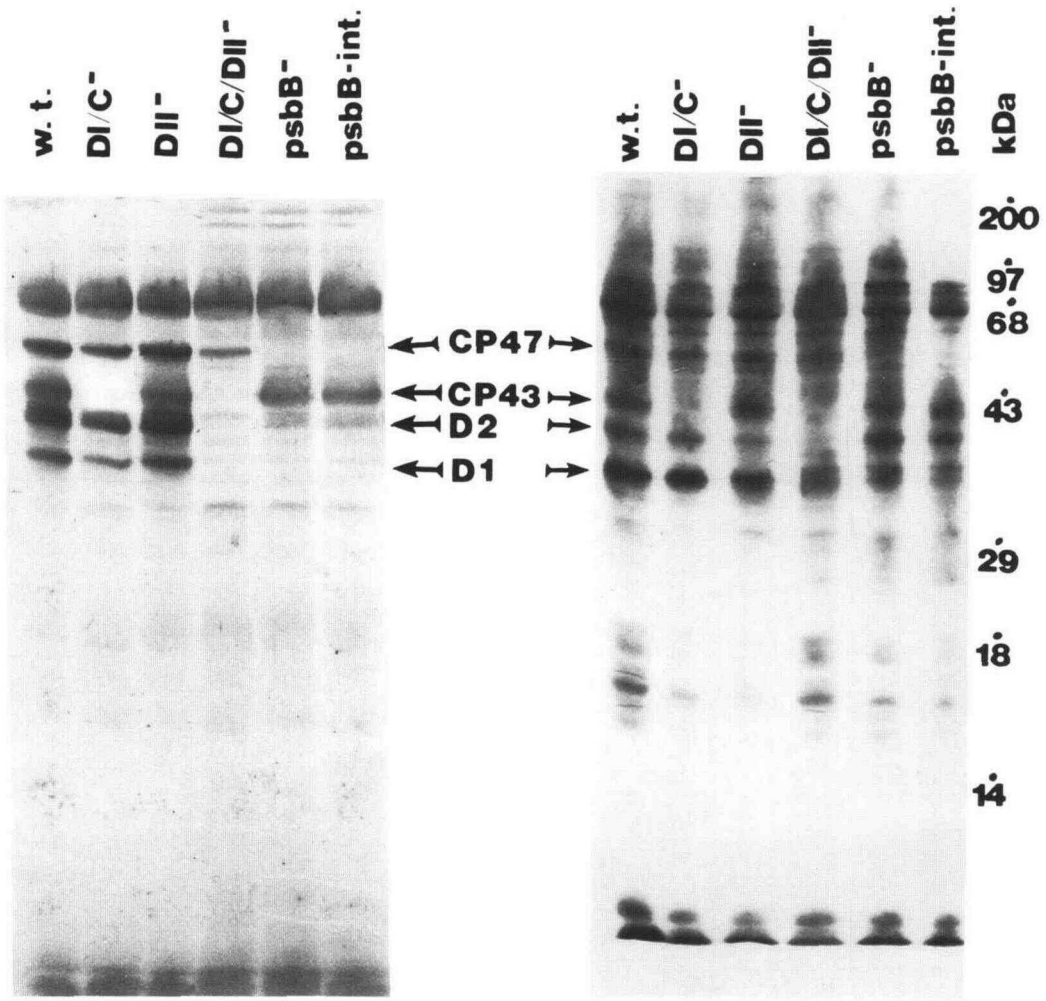

Figure 2. Steady-State Photosystem II Protein Composition (Left) and Thylakoid Protein Pulse-Labeling (Right) of Wild Type and Mutants.

(Left panel) Thylakoid proteins from wild type and five PS II mutants of *Synechocystis* sp. PCC 6803 were separated by SDS-PAGE, blotted onto nitrocellulose, and incubated with a mix of D1, D2, CP43, and CP47 antisera.

(Right panel) Wild type and five PS II mutants of *Synechocystis* sp. PCC 6803 were labeled in the light with radioactive ^{35}S-methionine in vivo for 30 min. Thylakoid membranes were isolated, and thylakoid proteins were separated on SDS-PAGE and detected by autoradiography. The positions of the D1, D2, CP43, and CP47 proteins have been indicated.

conditions used, a small amount of CP47 can be observed in this mutant; CP47 was not found in a similar mutant according to an earlier report (Vermaas et al., 1988). This small difference in results may originate from a larger sensitivity of the present experiments or from the fact that somewhat different *psbDI/C/DII*$^-$ mutants were used for the two studies. Also, a difference in the growth conditions of the cells can have an effect on the apparent protein composition of the thylakoid. The *psbB*$^-$ as well as the *psbB*-int mutants lack CP47 and D1, contain a significant amount of CP43, and have a trace of D2 in the thylakoid. The PS II protein composition in the various mutants is summarized in Table 2.

The right panel of Figure 2 presents results of pulse-labeling experiments in mutants and wild type with ^{35}S-methionine. The "32-kD" D1 protein is characterized by a light-dependent rapid turnover, making it easily distinguishable upon pulse-labeling in the light. The identity of the heavily labeled band in the 34,000 M_r region in Figure 2 has been confirmed to be D1 by comparison of the relative migration of the band and of the protein recognized by D1 antisera (compare both panels of Figure 2).

The D1 protein appears to be synthesized in all mutants shown in Figure 2, even though, under steady-state conditions, D1 is not detectable by protein gel blotting in thylakoids from some of those mutants. This implies that

Table 2. Effect of PS II Gene Inactivation on Steady-State Levels of PS II Proteins in the Thylakoid[a]

Strain	*D1*	*CP47*	*CP43*	*D2*
Wild type	+	+	+	+
psbDI/C$^-$	+/−	+/−	−	+/−
psbDII$^-$	+	+	+	+
psbDI/C/DII$^-$	−	−/+	−	−
psbB$^-$	−	−	+/−	−/+
psbB-int	−	−	+/−	−/+
psbC-int	+/−	+/−	−	+/−

[a] Symbols are as follows: +, gene product present at normal level in thylakoid; +/−, level of gene product significantly decreased in thylakoid; −/+, gene product detectable in thylakoid in trace quantities; −, gene product cannot be detected by protein gel blotting and immunodetection.

the *psbA* transcript is translated and that the stability of synthesized D1 is dramatically decreased in certain mutants, indicative of post-translational regulation.

In wild-type *Synechocystis* sp. PCC 6803, there is no evidence for a "33.5-kD" D1 precursor as observed in plants (Reisfeld et al., 1982; Marder et al., 1984; Minami and Watanabe, 1985; Inagaki et al., 1989). However, this may be due to a decreased life time of the precursor compared with the mature protein in cyanobacteria and does not imply that there would be a fundamental difference between D1s from plants and cyanobacteria in this respect. It should be noted that, in mutants with rapid turnover of D1 (most notably in the *psbB*-int and *psbB*$^-$ mutants), there is a radiolabeled band just above D1 in a position comparable with that of D1 precursor in plants. We postulate that this band indeed may be the D1 precursor and that it is visible because of a higher rate of D1 synthesis in these mutants (D1 is very rapidly degraded here) so that an appreciable amount of precursor can accumulate. Unfortunately, confirmation of this postulate by protein gel blotting is not possible because the steady-state concentration of the putative D1 precursor and of D1 is below the minimum level of detection by protein gel blotting (Figure 2). In wild type, bands are visible on the autoradiogram that correspond to the location of the D2, CP43, and CP47 proteins on the protein gel blot, and we attribute these to D2, CP43, and CP47 on the basis of their apparent molecular weight and, more importantly, on the basis of their disappearance in the appropriate mutants. This is discussed in more detail in Discussion.

The band attributed to D1 was shown to be degraded rapidly in the light: from the pulse/chase experiment shown in Figure 3, the half-life of D1 is approximately 2 hr to 3 hr (which is similar to that found for *Synechococcus* sp. PCC 7942; Goloubinoff et al., 1988), whereas some of the other labeled protein bands (but not including the putative CP47, CP43, and D2 protein bands) do not decrease much in intensity within 24 hr. It is estimated that the bands on the autoradiogram that we presume to originate from CP47, CP43, and D2 proteins in *Synechocystis* sp. PCC 6803 have a half-life that is only a few times larger than that of D1.

DISCUSSION

Although inactivation of one gene did not lead to dramatic suppression of transcription of other genes in any of the mutants, there are interesting variations in the gene transcript levels between wild type and the various mutants. This indicates that, upon deletion or interruption of a specific gene, expression of other genes has been modified.

Under normal conditions, the *psbDI* gene rather than *psbDII* is preferentially expressed in *Synechocystis*. A similar phenomenon is observed in *Synechococcus* sp. PCC

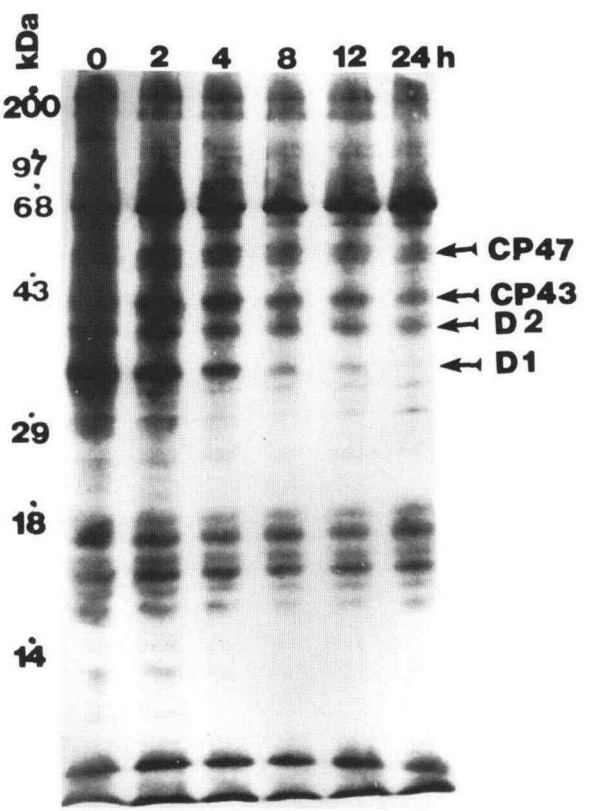

Figure 3. Pulse-Chase Labeling of Thylakoid Proteins from Wild-Type *Synechocystis* sp. PCC 6803.

Synechocystis sp. PCC 6803 (wild type) was labeled in the light with radioactive ^{35}S-methionine in vivo for 30 min. The radioactivity was subsequently chased for 0 hr, 2 hr, 4 hr, 8 hr, 12 hr, and 24 hr.

7942, where the *psbDII* transcript is virtually undetectable (Golden and Stearns, 1988). In wild-type *Synechocystis*, the 3.0-kb *psbDI/C* transcript is about 4 to 5 times more abundant than the 1.4-kb *psbDII* one. [Because *psbDI* and *psbDII* sequences are virtually identical in *Synechocystis* (Williams and Chisholm, 1987), direct comparison of the intensity of bands in Figure 1D provides a reasonable approximation of the relative amount of transcript of the two genes.] If one of the two copies of the *psbD* genes has been inactivated, the other copy of the gene is expressed more, presumably to compensate the inactivation. In the *psbDI/C*$^-$ mutant, no 3.0-kb *psbDI/C* transcript was detected, but the 1.4-kb *psbDII* band was an order of magnitude more intense than that of wild type. Upon deletion of most of *psbDII*, the *psbDI/C* transcript band was about 5 times to 8 times more intense than that of the wild type, and the faint 1.5-kb to 1.6-kb bands presumably reflect processed *psbDI* transcripts from the *psbDI/C* precursor, which are not detectable in wild type or in other mutants. The molecular mechanism underlying the compensation effects is as yet unresolved.

Modulation of *psbD* expression also altered expression of *psbA* and *psbB*. When one of the two copies of *psbD* has been deleted, the transcript level of *psbB* has been significantly increased. However, deletion of both *psbD* gene copies (along with *psbC*) inhibits the increase in *psbB* transcript level. There is no known linkage between the *psbD* genes and *psbB* on the *Synechocystis* genome. Thus, it seems likely that either the *psbD* transcript or gene product or a *psbD*/D2-regulated factor influences transcription or transcript stability of *psbB*. Also, inactivation of *psbB* influences *psbD* transcript levels, particularly *psbDII*. It should be noted that, in *Chlamydomonas*, no effect of inactivation of *psbB* on transcript levels of *psbA*, *psbC*, and *psbD* has been observed (Jensen et al., 1986). As will be discussed later, *psbC* appears to affect *psbA* transcript levels. Thus, the increase in *psbA* transcript levels in *psbDI/C*$^-$ may be due to the deletion of *psbC*. However, the fact that the *psbA* transcript level is decreased in *psbDI/C/DII*$^-$ argues for at least some (direct or indirect) interaction between *psbD* and *psbA* expression.

The data presented in Figure 1 indicate that *psbC* or its product influences the *psbA* but not *psbB* transcript level. Upon interruption of *psbC*, the *psbA* transcript level is significantly increased to an extent comparable with that present in the *psbDI/C*$^-$ mutant.

It appears that, in *Synechocystis* sp. PCC 6803, there is appreciably more effect of various PS II gene inactivations on the transcript level of other PS II genes than has been observed in *Chlamydomonas* (Erickson et al., 1986; Jensen et al., 1986), even though the reasons for the effects observed in the cyanobacterium are still unclear.

Comparing the pulse-labeling pattern of wild type and various mutants with the position of the large photosystem II core components on a protein blot (Figure 2), in wild-type thylakoids there is a distinct pulse-labeled band not only at the position of D1, but also where D2, CP43, and CP47 usually run. This we interpret to indicate that in *Synechocystis*, in contrast to the situation in plants, the turnover of not only D1 but also of D2, CP43, and CP47 in the light is rapid enough to yield a significant incorporation of radiolabeled methionine into these proteins. In strong support of this hypothesis, the *psbDI/C/DII*$^-$ mutant lacks radiolabeled bands at 37 kD and 42 kD (corresponding to D2 and CP43, respectively, and present in wild type), whereas the *psbDI/C*$^-$ mutant is depleted only in the 42-kD component (CP43; *psbDII* is still active so that D2 can be synthesized in this mutant). The *psbB* deletion and interruption mutants are depleted in a 52-kD component that has a similar electrophoretic mobility as compared with CP47. Thus, we postulate that, in *Synechocystis*, the synthesis rate of D2, CP43, and CP47 in the light is sufficiently high to visualize these proteins with pulse-labeling. Therefore, from the results shown in Figure 2, it can be concluded that, when one or two genes coding for PS II core complex components have been inactivated, the translation of other major PS II components is not dramatically affected.

It is interesting to note that, in the *psbDII*$^-$ mutant, the relative intensity of the pulse-labeled band presumed to represent D2 is lower than that in the *psbDI/C*$^-$ mutant, whereas the intensity of this band in the *psbDI/C*$^-$ mutant is similar to that in wild type. Thus, even though the *psbDII* transcript is less abundant than that of *psbDI*, it is possible that the *psbDI* transcript is translated less than that of *psbDII*.

In conclusion, upon inactivation of the gene(s) coding for one or more of the components of the PS II core complex, the other components are still synthesized at relatively normal rates, but the PS II complex (if formed at all) is not stable in the absence of one or more of its major core components and turns over rapidly.

METHODS

Growth Conditions

Synechocystis sp. PCC 6803 wild type and mutants were grown in BG-11 medium (Rippka et al., 1979) plus 5 mM glucose at 30°C. Solid medium was supplemented with 1.5% (w/v) agar, 0.3% sodium thiosulfate, and 10 mM Tes/KOH buffer, pH 8.2 (Vermaas et al., 1987a).

Isolation of Total RNA

Total RNA was isolated from wild-type and mutant *Synechocystis* sp. PCC 6803 according to a procedure developed for *Anacystis nidulans* R2 and *Anabaena* sp. PCC 7120 (Golden et al., 1987). We harvested 0.5 L to 1 L of cyanobacterial liquid culture at an early logarithmic growth stage (OD_{730} = 0.3 to 0.5). RNA prepa-

rations from denser cultures often contain a smear of transcripts that are smaller than usual, probably reflecting breakdown products. For improved RNA yield, the cell pellet was frozen in −70°C for 30 min and thawed at room temperature before starting the RNA isolation.

RNA Denaturation and Electrophoresis

Denaturation of RNA by glyoxal was done according to Ogden and Adams (1987). The gel electrophoresis unit was soaked with 0.1 M NaOH overnight to inactivate RNase. Ten micrograms of total RNA was loaded per lane (16-μL volume) onto a 1% agarose gel (20 cm long) in 10 mM sodium phosphate, pH 7.0. The gel was run at 80 V for 6 hr with constant circulation of buffer.

RNA Gel Blotting and Reversal of Glyoxalation

RNA was transferred to GeneScreen-*Plus* (Du Pont) by capillary blotting. Subsequently, glyoxal was removed by treatment in a 50 mM NaOH solution for 15 sec, followed by incubation in 1 × SSC/0.2 M Tris-HCl, pH 7.5, solution for 30 sec. The blots were dried under a heating lamp.

Hybridization of RNA and Washing Procedures

The blots were probed with 20 ng of ^{32}P-dATP nick-translated gene-internal *psbA*, *psbB*, *psbC*, *psbD*, and *rbcL* probes from *Synechocystis* at 42°C for 24 hr with constant agitation in 30 mL of hybridization solution containing 10% dextran sulfate, 50% deionized formamide, 1 M sodium chloride, and 1% SDS. The hybridized membrane was washed twice with 2 × SSC at room temperature for 5 min, then twice for 30 min in 2 × SSC plus 1% SDS at 60°C, and, finally, twice with 0.1 × SSC at room temperature for 30 min with constant agitation. After drying, the membrane was exposed to x-ray film in a cassette with intensifying screens at −70°C and developed after 1 day to 3 days.

Protein Pulse-Labeling with ^{35}S-Methionine

Pulse-labeling with ^{35}S-methionine was used to detect the rapidly turning over D1 protein in wild type and mutants. A 400-mL culture of cyanobacteria was harvested at logarithmic growth stage (OD_{730} = 0.3 to 0.5) by centrifugation at 1100*g* (2500 rpm) for 10 min at room temperature. The cell pellet was resuspended in liquid BG-11 in which the sulfate-containing constituents $MgSO_4$, $ZnSO_4$, and $CuSO_4$ had been replaced by an equal concentration of $MgCl_2$, $ZnCl_2$, and $Cu(NO_3)_2$, respectively. ^{35}S-methionine was added to the medium to a final concentration of 1 μCi/mL, corresponding to 0.6 nM. The culture was incubated in the light for 30 min. For experiments incorporating a chase of the radiolabeled material, cells labeled with radioactive ^{35}S-methionine were transferred to fresh BG-11 without sulfate but with 1.7 mM unlabeled methionine. The cells were grown for different times (0 hr, 2 hr, 4 hr, 8 hr, 12 hr, or 24 hr) under normal culture conditions and harvested, and thylakoids were isolated.

Thylakoid Isolation and Protein Gel Electrophoresis

For thylakoid preparation, cyanobacterial cells were pelleted (1100*g* for 10 min) and resuspended into 1 mL of "inhibitor buffer" containing 50 mM Mes/NaOH, pH 6.5, 50 mM $CaCl_2$, 0.3 M sucrose, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, and 2 mM ϵ-aminocaproic acid). Subsequently, one-third volume of 0.1-mm to 0.5-mm glass beads was added after transfer of the cyanobacterial cells to a microcentrifuge tube. The cells were broken in a mini-beadbeater (Biospec Products, Bartlesville, OK) by two high-speed bursts of 30-sec duration. Samples were put on ice between the two bursts to cool down. After centrifugation at 1100*g* (2500 rpm) for 10 min to remove cell debris, the supernatant was centrifuged again at 15,000*g* (14,000 rpm) for 15 min to 20 min in a Beckman Microfuge to pellet the thylakoids. The pellet was resuspended in the above inhibitor buffer and brought to a final concentration of 0.33 mg of chlorophyll/mL. SDS-PAGE was done using 12.5% acrylamide gels in the presence of 6.5 M urea at 4°C at 100 V to 200 V. The gel was stained with Coomassie Brilliant Blue for 40 min and destained overnight with constant agitation. After destaining, the gel was treated with Fluoro-Enhance (Research Products International Corp., Mount Prospect, IL) for 30 min before drying and x-ray autoradiography.

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