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Directed inactivation of the *psbI* gene does not affect Photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract PsbI is a small, integral membrane protein component of photosystem II (PSII), a pigment-protein complex in cyanobacteria, algae and higher plants. To understand the function of this protein, we have isolated the *psbI* gene from the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 and determined its nucleotide sequence. Using an antibiotic-resistance cartridge to disrupt and replace the *psbI* gene, we have created mutants of *Synechocystis* 6803 that lack the PsbI protein. Analysis of these mutants revealed that absence of the PsbI protein results in a 25–30% loss of PSII activity. However, other PSII polypeptides are present in near wild-type amounts, indicating that no significant destabilization of the PSII complex has occurred. These results contrast with recently reported data indicating that PsbI-deficient mutants of the eukaryotic alga *Chlamydomonas reinhardtii* are highly light-sensitive and have a significantly lower (80–90%) titer of the PSII complex. In *Synechocystis* 6803, PsbI-deficient cells appear to be slightly more photosensitive than wild-type cells, suggesting that this protein, while not essential for PSII biogenesis or function, plays a role in the optimization of PSII activity.

Key words Photosynthesis · Photosystem II · Oxygen evolution · Targeted mutagenesis · Light sensitivity

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Introduction

Photosystem II (PSII), a multimeric pigment-protein complex in the thylakoid membranes of cyanobacteria, green algae and higher plants, mediates light-driven transfer of electrons from water to plastoquinone, with concomitant release of oxygen. A number of polypeptide components of PSII are integral membrane proteins, while others are peripherally associated with the membrane (see Ikeuchi, 1992 for a review). The PSII complex also binds a number of inorganic cofactors involved in electron transfer, including chlorophyll, manganese, pheophytin, iron and plastoquinone.

The protein components of one of the most highly resolved photoactive preparations of PSII from higher plants (reaction center preparation) are D1, D2, cytochrome *b559*, PsbI and PsbW (Nanba and Satoh 1987; Ikeuchi and Inoue 1988a; Webber et al. 1989, Lorkovic et al. 1995). Together, the D1 and the D2 proteins form the site of primary photochemistry and are believed to coordinate all of the cofactors described above (see Debus, 1992 for a recent review). Additionally, the D1 protein provides a tyrosine residue that functions as the immediate electron donor to the reaction center (RC) chlorophylls. Cyt *b559* plays a role in stabilizing the PSII complex, and is believed to participate in protecting the RC from damage caused by photoinhibition (reviewed in Whitmarsh and Pakrasi, 1995). The function of PsbW, a recently identified nuclear-encoded protein, is unknown.

The role of the PsbI protein is also not known. This 4.8 kDa integral membrane protein is present in isolated RC preparations from both spinach and cyanobacteria (Ikeuchi et al. 1989a). The PsbI protein has between 36 and 39 amino acids, which are predicted to form a single membrane-spanning domain with short, exposed C- and N-terminal regions (Ikeuchi and Inoue 1988b; Webber et al. 1989). Cross-linking studies of isolated reaction center complexes from spinach

indicate that the N-terminal domain of PsbI is in close contact with D2 and the α-subunit of cyt *b559* on the stromal side of the membrane (Tomo et al. 1993).

In a recent study, a targeted mutagenesis approach was used to disrupt the *psbI* gene in the chloroplast genome of *Chlamydomonas reinhardtii* (Künstner et al. 1995). The resultant mutant strains accumulated only 10–20% wild-type levels of the PSII complex. With this significant decrease in the titer of PSII and its oxygen-evolving activity, the mutant cells were highly light-sensitive. However, under low light conditions, they were still able to grow autotrophically, implying that the PsbI protein plays no direct role in the photochemical activity of the PSII complex.

We have isolated the *psbI* gene from *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis* 6803). This unicellular, cyanobacterium is naturally transformable with exogenous DNA, and targeted modifications of various genes can be generated by using gene replacement techniques (Pakrasi 1995). During this study, we generated two PsbI-deficient mutant strains of *Synechocystis* 6803 and determined the effects of such mutations on the form and function of the PSII complex.

Materials and methods

Isolation of the *psbI* gene from *Synechocystis* 6803

PSII-enriched preparations from *Synechocystis* 6803 cells were purified according to Noren et al. (1991). These preparations were fractionated on SDS-PAGE and proteins in the 4 to 5 kDa size range were subjected to N-terminal sequencing as described in Ikeuchi and Inoue (1988b). Based on these amino acid sequence data, mixed oligonucleotide primers were designed and used for polymerase-chain reactions (PCR) with genomic DNA from *Synechocystis* 6803 cells. The amplification products were separated by agarose gel electrophoresis (Sambrook et al. 1989) and DNA bands in the expected size range of 75 bp were cloned into modified pUC118 plasmid vectors using the 'T-A' ligation system (Invitrogen). Double-stranded plasmid DNA from clones containing inserts of the expected size were subjected to dideoxynucleotide sequencing (Sanger et al. 1977).

A recombinant plasmid containing an insert corresponding to the *psbI* gene was used as a hybridization probe for the screening of a plasmid library of *Synechocystis* 6803 genomic DNA, as described in Ikeuchi et al. (1991).

Interruption and deletion of the *psbI* gene in *Synechocystis* 6803

To create mutant strains with inactivated *psbI*, a plasmid (pI41) was engineered to contain a spectinomycin/streptomycin-resistance cartridge (Ω fragment, see Pakrasi et al. 1991) as described in the Results section. The recombinant plasmids were used to transform wild-type *Synechocystis* 6803 cells using methods described by Williams (1988). Spectinomycin-resistant colonies were selected and grown in the BG11 medium supplemented with 10 μg/ml spectinomycin, 5 mM glucose and 5 μM atrazine (when necessary) under a light intensity of 50 $\mu E/m^2$ per s.

Analysis of the composition and activity of the PSII complex

Growth curve analysis and determination of oxygen evolution rates were carried out as described by Ikeuchi et al. (1991). Immunoblot analysis was performed as described in Ikeuchi et al. (1989b).

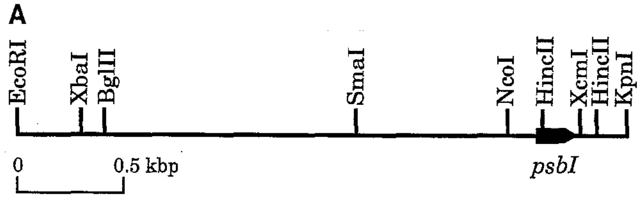
Results

Isolation and characterization of the *psbI* gene from *Synechocystis* 6803

Analysis of the subunit composition of the PSII complex has been greatly facilitated by the development of a modified SDS-PAGE system that provides high resolution in the low molecular weight range (Ikeuchi and Inoue 1988a). Under such conditions, several small protein bands are observed in isolated PSII complexes from various organisms (Ikeuchi et al. 1989a). N-terminal amino acid sequencing of such small proteins has identified at least three PSII subunits which comigrate in the 4.4 to 4.8 kDa region: PsbI, PsbL and the β subunit of cyt *b559* (Ikeuchi et al. 1989b). We have used this system to analyze PSII preparations isolated from *Synechocystis* 6803 using a method described by Noren and coworkers (1991). Determination of the N-terminal sequence of the polypeptides migrating in the 4.5 to 5 kDa range showed that these three proteins are present in the PSII preparations from *Synechocystis* 6803 (data not shown). Based on the N-terminal sequence of the PsbI protein, we designed two mixed oligonucleotide primers: (i) 5′-ATG(T/C)T(T/A/G/C)AC(G/C/T)(C/T)T(T/A/G/C)AA(A/G)AT(C/T)CG-3′, and (ii) 5′-T(T/C)(T/C)TT(C/A/T)GG(C/A)TT(C/T)TA(T/C)TT(A/T/C/G)T-3′. These oligonucleotides were used for PCR amplification of genomic DNA from *Synechocystis* 6803. Amplification products of the expected size (74 bp) were cloned into pUC vectors, and their nucleotide sequences were determined. Two of the clones contained sequences corresponding to that of the *psbI* gene.

Screening of a *Synechocystis* 6803 genomic library showed that these cloned fragments of the *psbI* gene hybridized to a 2.6 kb *Eco*RI-*Kpn*I fragment, which was isolated and ligated into pUC118 vector to yield the recombinant plasmid pI41, shown in Fig. 1A. Restriction analysis with various enzymes revealed several convenient recognition sites in this fragment, including a *Hin*cII site in the 5′ region of the *psbI* coding region, as well as additional *Hin*cII and *Xcm*I sites downstream of it. The nucleotide sequence of the *psbI* gene and its flanking regions is shown in Fig. 1B. Based on its deduced amino acid sequence, the PsbI protein appears to be highly conserved in various photosynthetic organisms, and contains a 21 residue segment which may form a transmembrane domain (Fig. 2). It is noteworthy that the PsbI protein in cyanobacteria has

A



B

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  1 TAGAGCTGGG GAGATAACCG CCACTGCCAG TACCTCCTGC TGGATACCTA
 51 GAGGTTTAGT ACAGTCATGG AAACGGGGAT CAATCACCTT CTGCCAAGCT
101 TCTTTGACCA ATTGGTAACG GCGCAGTTGT TCCCACCCTG GCGATCGCCC
151 CAGGCGGCCC AGTAATTGTT CGAGATTGTT TAAGCCCATG GTGTGCTCCC
201 TTGGCGGTGG AAATTCCCCG GCCATCTCCC CAAAGCCTAG ACCGAAATGG
251 GGTAAAGTAG GCAAGGTAGA ATGGTTTCTG CGCCCGGATT TTTACCCAAA
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351 CTTAAAATCG CCGTTTATAT CGTTGTTGGC TTGTTTATTT CCTTATTTAT
401 CTTCGGCTTT CTTTCCAGTG ACCCCACCCG GAACCCTGGC CGCAAAGACT
451 TTGAATAAGC TTTAGCCCAA AATTCTTCCT TCTCTCCCTA GACTAATTTT
501 GGTGCCAAGG GTAGATTGGA ACCTGATTAC TCTCCCCCAC CGGAGAGTTT
551 TTTGTCACTG GGGCCACAGA CGAAACGATT TCTGGCAATT GTTCGTCCCC
601 CTTGTTAACC AAGCCCGGTT AGCCTTAGCT TTATCTCAGT CCAAACAAAG
651 CAAATGTTAG CGTTGGCGTT TAGTTCGTCG ATTTAAATGC CCCCTATTGT
701 TTCCCCTCCC CCTCCTCC
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Fig. 1A A restriction map of the region of the *Synechocystis* 6803 genome containing the *psbI* gene. The coding region of *psbI* (*heavy arrow*) contains a *Hin*cII site in its 5′ region. This 2.6 kb insert was cloned in the plasmid vector pUC118 to generate the plasmid pI41. **B** Nucleotide sequence of the *psbI* gene from *Synechocystis* 6803. The coding region is *boxed* and a possible ribosome binding site is *doubly underlined*. The *arrows* indicate an inverted repeat which may function as a transcription terminator. This sequence has been deposited in the GenBank-EMBL database under the accession number U28040

39 amino acids, and is slightly larger than the PsbI protein in algae and higher plants.

Directed mutagenesis of the *psbI* gene in *Synechocystis* 6803

The plasmid pI41, containing a 2.6 kb fragment encompassing the *psbI* gene and its flanking regions, was used as a template for directed mutagenesis of the *psbI* gene (Fig. 3). Insertion of a spectinomycin-resistance cartridge (Ω fragment, see Pakrasi et al. 1991) at the *Hin*cII site in the *psbI* coding region resulted in a disruption of this gene (IS1). To delete the ORF entirely, a 263 bp *Hin*cII fragment was removed and replaced with the Ω cartridge (IS2). Additionally, two constructs that contained this Ω cartridge inserted at a *Hin*cII and an *Xcm*I site downstream of the *psbI* coding region were generated, thus effectively tagging the gene with a selectable marker (IS7, IS8). These constructs were used individually to transform *Synechocystis* 6803 wild-type cells. Following selection and segregation of spectinomycin-resistant colonies, interruption or deletion of the *psbI* gene in the chromosomes of these strains was confirmed by PCR analysis (data not shown).

PsbI is not essential for PSII activity

To confirm that interruption or deletion of the *psbI* gene resulted in the loss of the PsbI protein, thylakoid membranes isolated from IS1, IS2 and wild-type strains were subjected to immunoblot analysis using antibodies raised against individual PSII proteins. As shown in Fig. 4, CP47, D2, PsbH and PsbK proteins are present in both wild-type and *psbI*-inactivated strains. However, the PsbI protein is found only in the wild-type thylakoids, indicating that disruption or deletion of the *psbI* gene leads to a specific loss of the PsbI protein. As demonstrated by the presence of near-normal amounts of other PSII proteins in the *psbI* mutant strains, loss of the PsbI protein in *Synechocystis* 6803 does not result in any significant destabilization of the PSII complex. Despite the specific loss of PsbI from the PSII complex, mutant *Synechocystis* 6803 cells were able to grow photoautotrophically (in media lacking glucose) at rates comparable to those of control cells (Fig. 5). These results indicate that under our growth conditions, the PsbI protein is not necessary for photosynthetic growth of *Synechocystis* 6803 cells.

Fig. 2 Alignment of the sequences of the PsbI protein from several organisms. A 21-residue putative transmembrane domain is indicated. The sequences designated with asterisks at their C-termini are deduced from nucleotide sequence, while others were partially determined by N-terminal protein sequencing. Residues that are conserved in all organisms are indicated by the asterisks above the first line

		aa	MW	pI
	`*  **  **     *  ** **  * ** * *`			
Synechocystis 6803	MLTLKIAVYIVVGLFISLFIFGFLSSDPTRNPGRKDFEL*	39	4419	9.50
Synechococcus vulcanus	MEILKITVYILITIFVLLFVFGLLSGDPA/			
Synechococcus 6301	MLALKVTVYVLILIFVALFVFGLLSSDPARTPSRKDLED*	39	4352	6.89
Euglena gracilis	MLILKVFVYALILIFVSLFVFGLLSNDPGRNPYDDTGELMREVFYKDPYKGPL*	53	6129	4.76
Chlamydomonas reinhardtii	MLTLKIFVYTVVTFFVCLFIFGFLSNDPARNPGKNLD*	38	4614	9.28
Liverwort	MLTLKLFVYTVVIFFVSLFVFGFLSNDPGRNPGRKE*	36	4153	10.13
Tobacco	MLTLKLFVYTVVIFFVSLFIFGFLSNDPGRNPGREE*	36	4168	6.95
Spinach	MLTLKLFVYTVVIFFVSLFI/			
Sinapsis	MLTLKLFVYTVVIFFVSLFIFGFLSNDPGRNPGREE*	36	4168	6.95
Rice	MLTLKLFVYTVVIFFVSLFIFGFLSNDPGRNPGRDE*	36	4154	6.88
Wheat	MLTLKLFVYTVVIFFVSLFIFGFLSNDPGRNPGREE*	36	4168	6.95
Barley	MLTLKLFVYTVVIFFVSLFIFGFLSNDPGRNPGRQE*	36	4167	9.58
	+ |——21——| − +			

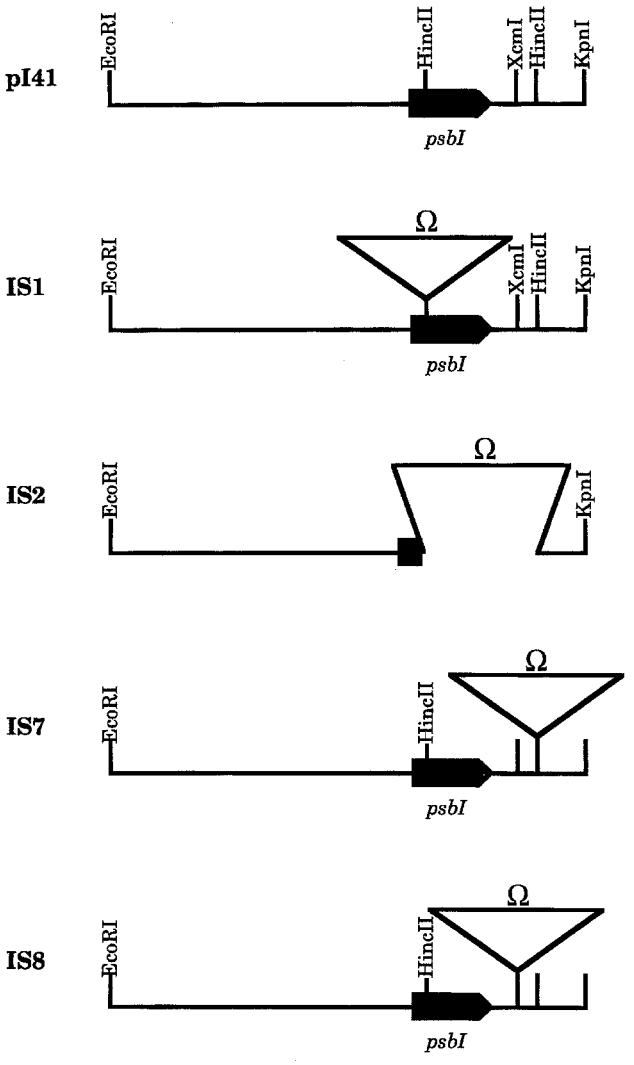

Fig. 3 Cartridge mutagenesis of the *psbI* gene. Plasmid pI41 was used as a template for insertion (IS1) and deletion (IS2) mutagenesis of *psbI*. Two mutants containing the spectinomycin-resistance marker (Ω) inserted downstream of the *psbI* ORF were also generated (IS7, IS8)

The rates of PSII-mediated O_2 evolution and doubling times of control and *psbI* deletion strains are shown in Table 1. These data indicate that a loss of the PsbI protein results in a moderate (~ 25%) loss of steady-state oxygen evolution activity. Moreover, under continuous illumination conditions, inactivation of steady-state oxygen evolution in the PsbI-deficient mutant cells occurred faster than in the wild-type cells, suggesting that the photosensitivity of these cells is somewhat enhanced. This photosensitivity was further confirmed by the analysis of the light dependence of oxygen evolution rates. As shown in Fig. 6, the IS1 insertion mutant (as well as the IS2 deletion strain) exhibited lower rates of light-dependent oxygen evolution than the control IS7 strain (as well as the IS8 mutant). These results, together with the observed increase in the doubling times of the mutant cells (Table 1), indicate that loss of the PsbI protein causes a small decrease in the efficiency of photosynthesis, probably due to an increased sensitivity to light.

It is known that the PSII complex is severely destabilized in mutant strains in which the *psbEFLJ* gene cluster (encoding cyt b559, PsbL and PsbJ), or the three copies of the *psbA* gene (encoding D1), or the *psbDC* gene cluster (encoding D2 and CP43) are deleted (reviewed in Pakrasi and Vermaas 1992). As expected, all of these strains lacked the PsbI protein (data not shown). However, PsbI was detected in thylakoid membranes of a mutant strain in which the *psbK* gene has been inactivated (data not shown). The PsbK-deficient mutant has a near-normal content of PSII centers, and a loss of PsbK does not inhibit photosynthetic growth or activity of *Synechocystis* 6803 cells (Ikeuchi et al. 1991). Our results indicate that PsbI, like PsbK, is not essential for PSII assembly or activity, but is needed for optimal PSII function.

Discussion

In this study, we have isolated and characterized the *psbI* gene from the unicellular cyanobacterium

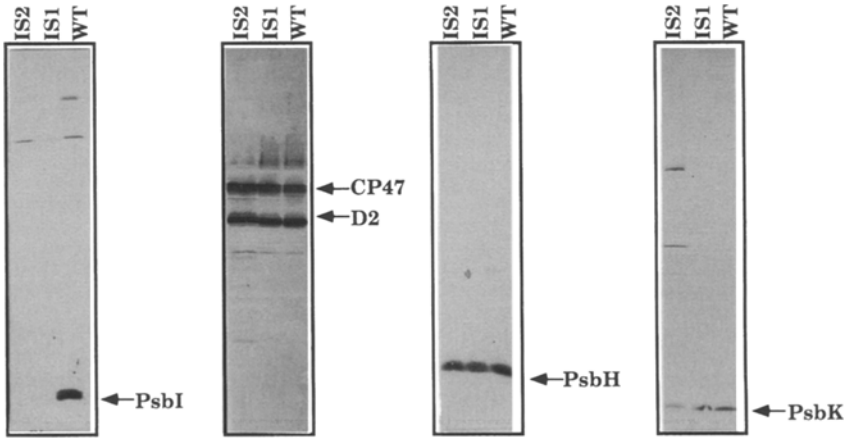

Fig. 4 Immunoblot analysis of PSII proteins in thylakoids of wild-type, IS1 and IS2 strains of *Synechocystis* 6803. Antibodies against PsbI, CP47, D2, PsbH and PsbK proteins were used to identify these polypeptides in thylakoid samples separated by SDS-PAGE and transferred to nitrocellulose filters

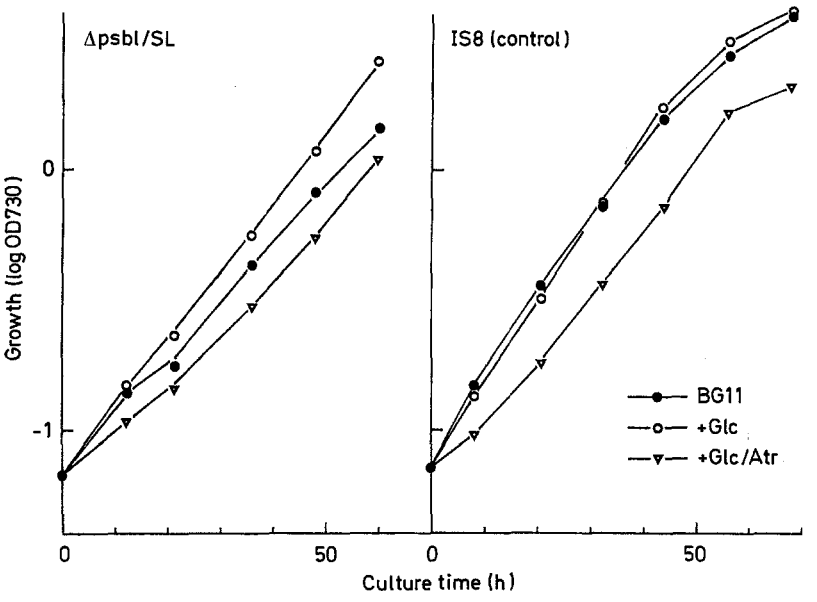

Fig. 5 Analysis of growth of *psbI* deletion (IS2) and control (IS8) strains of *Synechocystis* 6803. Cells were cultured in basal (BG11) and supplemented (+ Glc, + Glc/Atr) media and grown under 50 μE/m² per s white light. Glc, glucose; Atr, atrazine

Table 1 Growth properties and PSII activities of a PsbI-deficient mutant strain of *Synechocystis* 6803

Strain	Doubling time (h)			O_2 evolution[a] (μmol O_2/ mg Chl per h)	O_2 inactivation[b] (min)
	BG11	+ G	+ G/Atr		
IS8 (control)	10.1	9.5	13.5	422 (100%)	2.4
IS2 (Δ *psbI*)	14.0	11.5	16.0	310 (73%)	2.1

[a] PSII-mediated oxygen evolution rates measured from water to 2,6-dimethyl-*p*-benzoquinone

[b] Time at which steady-state oxygen evolution rates, under constant illumination, begin to decrease from the maximum rate observed. + G, plus 5 mM glucose; + G/Atr, plus 5 mM glucose and 5 μM atrazine

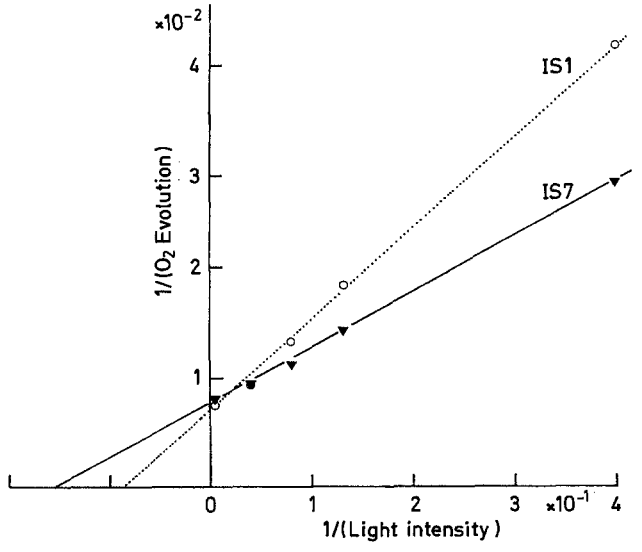

Fig. 6 Light dependence of the rates of PSII-mediated oxygen evolution from intact cells of control (IS7) and *psbI*-less (IS1) strains of *Synechocystis* 6803

Synechocystis 6803. As in other O_2-evolving organisms examined so far, the PsbI protein in this cyanobacterium is present in isolated photoactive PSII preparations. We have shown that, in *Synechocystis* 6803, loss of the PsbI protein does not severely affect PSII assembly or function. Cells which lack the PsbI protein appear to be slightly more photosensitive than the wild-type cells, and exhibit only a 25–30% loss of their PSII activity. Our results indicate that while the PsbI protein is not essential for the biogenesis or function of the PSII complex, it may play a role in the optimization of PSII activity.

These data contrast with those from a recent study in which mutants of the green alga *C. reinhardtii* that lack PsbI were analyzed for the assembly and function of the PSII complex (Künstner et al. 1995). In these mutant strains, a loss of the PsbI protein resulted in an 80–90% loss of PSII complexes. On the other hand, PsbI-less mutant strains of *Synechocystis* 6803 have a near-normal content of a number of PSII proteins (Fig. 4). Furthermore, PsbI-less mutants of *Synechocystis* 6803 do not display the extreme light sensitivity observed in the *Chlamydomonas* PsbI mutants. Although the *Chlamydomonas* mutants were able to grow photosynthetically under very low light, moderate to higher photon flux had a severe inhibitory effect on their growth (Künstner et al. 1995), a phenotype not observed in the *Synechocystis* mutants described here. Clearly, in the cyanobacterial system, the PsbI protein is not required for biogenesis, stabilization or functioning of the PSII complex.

These findings with PsbI parallel the data available for the PsbK protein of PSII. Inactivation of the *psbK* gene in *Chlamydomonas* results in the accumulation of PSII at < 10% of its normal level, and inability of the cells to grow photoautotrophically (Takahashi et al. 1994). In contrast, in the analogous cyanobacterial

mutants, the number of PSII centers was decreased by less than a factor of 2, leading to the conclusion that the PsbK protein is not an essential component of PSII (Ikeuchi et al. 1991). In higher plants, selective removal of this protein from isolated O_2-evolving PSII preparations has no significant effect on PSII activity, indicating that PsbK is not required for the stability of PSII in these preparations (Ikeuchi et al. 1989a). Based on these data, the stability of the PSII complex in cyanobacteria and higher plants seems to be substantially different from that of their *Chlamydomonas* counterpart.

One explanation for the distinct phenotypes of the PsbI-less mutants strains of the cyanobacterium and the green alga may lie in the unique evolutionary path taken by *Chlamydomonas*. The arrangement of PSII genes in the plastid genome of *Chlamydomonas* is significantly different from that in other photosynthetic organisms. For example, unlike the case in other organisms, the genes encoding the two subunits of cyt *b559* are not part of an operon in this eukaryotic alga (Mor et al. 1995). In contrast to higher plants, the *psbI* gene in *Chlamydomonas* is not cotranscribed with the *psbK* gene. It is possible that the PSII complex of Chlamydomonas contains as yet unidentified polypeptides, which are unique to this organism, closely associated with PsbI, and required for PSII stability. A closer examination and comparison of PSII components in cyanobacteria, algae and higher plants may discern such differences.

Such differences also suggest that details of the biogenesis of the PSII complex may be distinctly different between *Synechocystis* 6803 and *Chlamydomonas*. It has been shown that a number of nuclear gene products, not present in cyanobacteria, are necessary for the stabilization of PSII components in *Chlamydomonas* (reviewed in Erickson and Rochaix 1992). The requirements for such proteins may reflect steps in the PSII assembly process that are absent in cyanobacteria. It is also noteworthy that inactivation of the *psaC* gene, encoding an iron-sulfur protein in the PSI complex, destabilizes PSI in *Chlamydomonas*, whereas the PSI reaction center complex remains relatively unperturbed in *psaC*-less mutant strains of cyanobacteria (see Pakrasi, 1995 for a discussion).

In the PSII reaction center complex, the PsbI protein is present in close proximity to the D2 and the cyt *b559* proteins. Thus, the finding that PsbI is not essential for PSII assembly or function is surprising. It is usually assumed that the spatial relationships between proteins in the PSII complex, and the reaction center complex in particular, are closely regulated for reasons of efficiency in electron transport reactions. Absence of a polypeptide component, such as PsbI, is thus thought to be highly damaging for the form and function of such a protein complex. When other components of the isolated reaction center complex, such as D1, D2 or cyt *b559*, are deleted, the entire complex is destabilized, resulting in a loss of PSII function (reviewed in Pakrasi and Vermaas, 1992). However, analysis of some other small PSII proteins, not present in the isolated reaction center complex, has indicated that these polypeptides, while not essential, contribute to the overall efficiency of PSII assembly and function (Ikeuchi et al. 1991; Lind et al. 1993). In the case of the PsbH protein, a detailed functional analysis has demonstrated that this protein plays a role in optimizing the efficiency of electron flow from Q_A to Q_B on the acceptor side of PSII (Mayes et al. 1993). To delineate the specific functions of the PsbI protein, similar detailed mechanistic analysis of the PsbI-less mutants will be necessary in the future.

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