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# Role of the PSII-H Subunit in Photoprotection

NOVEL ASPECTS OF D1 TURNOVER IN *SYNECHOCYSTIS* 6803*

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**Photosystem I-less *Synechocystis* 6803 mutants carrying modified PsbH proteins, derived from different combinations of wild-type cyanobacterial and maize genes, were constructed. The mutants were analyzed in order to determine the relative importance of the intra- and extramembrane domains of the PsbH subunit in the functioning of photosystem (PS) II, by a combination of biochemical, biophysical, and physiological approaches. The results confirmed and extended previously published data showing that, besides D1, the whole PsbH protein is necessary to determine the correct structure of a $Q_B$/herbicide-binding site. The different turnover of the D1 protein and chlorophyll photobleaching displayed by mutant cells in response to photoinhibitory treatment revealed for the first time the actual role of the PsbH subunit in photoprotection. A functional PsbH protein is necessary for (i) rapid degradation of photodamaged D1 molecules, which is essential to avoid further oxidative damage to the PSII core, and (ii) insertion of newly synthesized D1 molecules into the thylakoid membrane. PsbH is thus required for both initiation and completion of the repair cycle of the PSII complex in cyanobacteria.**

Photosystem (PS)[1] II is the pigment-protein complex, of both prokaryotic and eukaryotic thylakoid membranes, which is deputed to the splitting of water in oxygen and protons. Its functioning is understood in greater detail than its architecture, which is very highly structured in terms of protein number and interactions. Knowledge of the supramolecular organization of the system is rapidly increasing, parallel with the progressively better resolution obtained by crystallographic analysis (1). However, the topology and accessory functions of low molecular mass subunits, about half of the almost 30 different polypeptides implicated in PSII structure, are far from being established.

One of the reasons for this is the current limiting resolution of 3.8 Å of the crystal structure. Second, despite the strong homology in PSII among organisms that perform oxygenic photosynthesis, some small subunits such as PsbR, $PsbT_n$, and PsbW are present in the eukaryotic complex, but missing in cyanobacteria. Other subunits are present and highly conserved in the PSII complex of all organisms performing oxygenic photosynthesis. This is true for PsbH, a component of PSII originally detected as a 9-kDa phosphoprotein in pea thylakoid membranes (2). However, phosphorylation site(s) (3), located at the N-terminal, extramembrane 12 amino acid extension typical of eukaryotes, is (are) absent in the cyanobacterial polypeptide. The function of PsbH in PSII has been associated, through analysis of a *Synechocystis* mutant lacking the coding gene, with control of the electron flow from $Q_A$ to $Q_B$ (4), protection from photoinhibition (5), contribution of important structural features to the $Q_B$/herbicide-binding site (6), and stabilization of the PSII complex and bicarbonate binding on its acceptor site (7). The required presence of PsbH in the assembly and/or stability of PSII in the eukaryotic green alga *Chlamydomonas reinhardtii* has been clearly demonstrated, also by the construction of deletion mutants (8, 9). Other aspects of the role of PsbH remain to be clarified: precise location (1, 10), significance of phosphorylation in chloroplasts (9), and possible participation in signal transduction (11). So, while PsbH in cyanobacteria appears to be partially dispensable and accessory, its fundamental role in eukaryotic PSII cannot definitely be investigated by reverse genetics in higher plants, since they are compulsory phototrophs.

In a previous work (6), we showed that the maize PsbH subunit could functionally replace the endogenous one in the PSII of *Synechocystis* 6803 (hereafter *Synechocystis*). The heterologous protein brought about modifications of the $Q_B$ site, which were hypothetically ascribed to its distinctive N-terminal extension. Here, we describe the analysis of four mutants of *Synechocystis* carrying artificial PsbH subunits, derived from different combinations of wild-type cyanobacterial and maize genes. For their construction, we took advantage of a PSI-less strain (12), which is highly appropriate for the study of both PSII structure (13) and function (14, 15). We initially addressed the question of which domain of the PsbH polypeptide is important for the structure-function of the $Q_B$/herbicide-binding niche and the $Q_A$ to $Q_B$ electron transfer. The behavior of mutants with respect to the turnover of the D1 protein in response to treatments with high light was then compared and revealed significant differences. In particular, it showed that the correct structure of PsbH is fundamental in the final steps of the repair cycle of PSII, *i.e.* prompt removal of damaged D1 polypeptides and insertion of new ones into the thylakoid membrane.

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[1] The abbreviations used are: PS, photosystem; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MALDI-MS, matrix-assisted laser desorption/ionization-mass spectrometry.

## EXPERIMENTAL PROCEDURES

*Strains and Culture Conditions*—*Synechocystis* 6803 PSI-less strain (*psaAB*$^-$) (12) and PSI-less/*psbH* double mutant strains were grown at 30 °C and at 5 $\mu$E m$^{-2}$ s$^{-1}$ light intensity, in BG11 medium supplemented with 10 mM glucose. When optical density at 730 nm was used to evaluate cell numbers in liquid cultures, a value of 0.25 was considered to correspond to $10^8$ cells ml$^{-1}$. Lincomycin was used at a final concentration of 1 mM.

*Construction and Genomic Analysis of Mutants*—Construction of plasmids pSH233k and pMH264k has been described elsewhere (6). Their parent plasmid, containing a 1319-bp fragment of the *Synechocystis psbN-psbH-petC-petA* cluster centered around the *psbH* gene, at the 5′ and 3′ of which *Hin*dIII and *Bam*HI sites had been respectively introduced, was used for the construction of three other plasmids. (i) pCH269 was obtained by cutting the parent vector with *Hin*dIII and *Eco*RV, and legating the double-strand DNA formed by pairing of oligonucleotides syn13 (5′-AG CTT ATG GCT ACT CAG ACC GTT GAA GAC TCG AGC AGA CCT AAG CCT AAG CGC ACT CGG TTA GGA GAT-3′) and syn 14 (5′-ATC TCC TAA CCG AGT GCG CTT AGG CTT AGG TCT GCT CGA GTC TTC AAC GGT CTG AGT AGC CAT A-3′) into the obtained ends. (ii) pΔH228 was constructed by substituting the *Hin*dIII-(*psbH*)-*Bam*HI fragment of the parent vector with a fragment obtained by PCR, using primers sr-delta (5′-AAG CTT ATG GCT AAA CGG ACT GGC GCA GG-3′) and cp2 (5′- TTG GAT CCA AAA ACT ATG AAG TC-3′) on template pMH264k (Chiaramonte *et al.*, Ref. 6), cut by the same enzymes, and (iii) pH-less, obtained by substituting the *Hin*dIII-(*psbH*)-*Bam*HI fragment of the parent plasmid with a cohesive-end adaptor duplex formed by annealing undecamers $H_3P_1$ (5′-AGCTTCT-GCAG-3′) and $P_1B_1$ (5′-GATCCTGCAGA-3′). The kanamycin cassette derived from pUC4K (Amersham Biosciences) was cloned into the single *Bam*HI sites of (i), (ii), and (iii), giving the final constructs pCH269k, pΔH228k, and pH-lessk, respectively. All constructs were completely sequenced to ensure that no undesirable mutation had occurred during the cloning procedure.

The PSI-less *Synechocystis* strain was transformed by electroporation, as already described (Chiaramonte *et al.*, Ref. 6). Recombinant colonies were subcloned 7–9 times in BG11 containing both 5 mM glucose and 100 $\mu$g ml$^{-1}$ kanamycin. Genomic DNA from selected clones was extracted and analyzed by PCR with primers syn7 (5′-TTACCAAGGAGCTCTTTGGCC-3′) and syn8 (5′-CAAGGAGATCTT-TACTGGCA-3′). Genomic DNA was otherwise subjected to Southern blotting after digestion with *Nco*I; *Synechocystis* or maize-specific *psbH* probes were synthesized by PCR with primers syn2 and syn4 (Chiaramonte *et al.*, Ref. 6) or sr-delta and cp2 respectively. Labeling, hybridization and detection were performed using a chemiluminescent detection system (DIG DNA labeling and detection kit, Roche Applied Science).

*Preparation of Thylakoid Membranes and MALDI Mass Spectrometry*—For SDS-PAGE and Western blotting, thylakoids were prepared from 5 ml of cell culture (~2 $\mu$g of chlorophyll) following the procedure of Komenda and Barber (5). Final pellets were resuspended in 50–100 $\mu$l of 50 mM Tris, pH 7.5, 1 M sucrose and quantified by both chlorophyll and protein dosage.

For MALDI mass spectrometry, thylakoids from 4 liters of cell culture ($OD_{730}$ = 0.8) were prepared following the procedure described in Szabò *et al.* (13). MALDI measurements were performed as described therein, on a REFLEX time-of-flight instrument (Bruker-Franzen Analytik) equipped with a SCOUT ion source operating in positive linear mode.

*Analysis of Chlorophyll, Protein, and Cell Concentrations*—To measure chlorophyll, cells were sedimented by centrifugation at 10,000 × *g* for 3 min and pigments were extracted with 100% methanol. Extracts were centrifuged, and the spectra of the clear supernatant were recorded from 300 to 750 nm. Chlorophyll concentrations were calculated from absorbance at 666 and 750 nm, according to Lichtenthaler (16).

Protein concentrations in thylakoid extracts were determined by the BCA (bicinchoninic acid) Protein Assay Reagent (Pierce), according to the manufacturer's standard procedure, with reading of the absorbance at 562 nm. Cell numbers were determined by flow cytometry using a Becton Dickinson FACScan instrument and CellQuest software (BD Biosciences, San Jose, CA). Cell suspensions were analyzed at a flux of 12 $\mu$l/min, and cell counts were determined by the intrinsic chlorophyll fluorescence ($\lambda_{exc}$ 488 nm, $\lambda_{em}$ >670 nm).

*Oxygen Evolution Measurements*—Cells collected from solid BG11 medium were suspended in liquid medium to a initial $OD_{750}$ = 0.4–0.5. Cultures were grown for 2 days in liquid BG11 medium, supplemented with 10 mM glucose, 25 $\mu$g/ml kanamycin, 2.5 $\mu$g/ml chloramphenicol, at 5 $\mu$E m$^{-2}$ s$^{-1}$ light intensity and 30 °C. Before each measurement, cells were collected and resuspended in BG11 to a final concentration of 2 $\mu$g of chlorophyll/ml and then incubated at 30 °C in the same growth condition, up to the time of addition of the specific drug. Herbicides DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), atrazine, and ioxynil were tested over a range of concentrations suitable for calculation of the $I_{50}$. Samples were preincubated with the specific drug for 15 min in the dark, in order to reach the specific binding site and equilibrate. Oxygen evolution was recorded on a Clark-type electrode (Hansatech CB1D) at a light intensity of 1000 $\mu$E m$^{-2}$ s$^{-1}$ and 30 °C, and the final value was calculated by subtraction of the oxygen consumption measured in the dark (prevalue).

*Fluorescence Measurements*—Cells were cultured in the conditions described above and suspended at a concentration of 2 $\mu$g of chlorophyll/ml. Samples were dark-adapted for 5 min prior to measurements. Fluorescence induction kinetics were obtained using a pulse amplitude modulated fluorimeter (PAM 101, Walz). Actinic light of 3000 $\mu$E m$^{-2}$ s$^{-1}$ intensity and 1-s duration was applied. To determine fluorescence decay, single turnover flashes of 10000 $\mu$E m$^{-2}$ s$^{-1}$ intensity and 8-$\mu$s duration were applied every 18 s using a xenon lamp (XST 103). When specified, fluorescence decay was measured in the presence of 40 $\mu$M DCMU. Data were recorded and analyzed using the 4.5 Fluorescence Induction Program (QA Finland).

*SDS-PAGE and Immunoblotting*—Thylakoid proteins were resolved by denaturing 12% polyacrylamide gel containing 6 M urea and 0.1% SDS, according to Laemmli (17). 15, 7.5, or 1.5 $\mu$g of total thylakoid proteins (depending on strain, see "Results") were loaded per lane, in gels used for staining or immunoblotting. For the latter procedure, proteins were electrophoretically transferred onto polyvinylidene difluoride membranes using the carbonate/bicarbonate buffer of Dunn (18). Blots were immunodecorated by polyclonal antibodies against the D1 polypeptide of *Synechocystis*, raised in rabbit by subcutaneous injection using poly(A)·poly(U) as adjuvant. Detection was made using the SuperSignal chemoluminescence kit (Pierce) for peroxidase-conjugated secondary antibodies (Kirkegaard and Perry Laboratories). When indicated, thylakoid membranes (6 $\mu$g of protein) and respective soluble fractions were incubated with 0.012 units of Lys-C for 15/60 min, in the presence of 25 mM Tris, pH 8.8. Reactions were directly stopped by addition of loading buffer for SDS-PAGE.

## RESULTS

*Genomic and Expression Analysis of PSI-less/psbH Mutants*—To generate the PSI-less/*psbH* mutants we took advantage of a PSI-less strain of *Synechocystis* 6803, which lacks the *psaA* and *psaB* genes and is tolerant to low light intensities, growing reasonably well in photoheterotrophic conditions at 5 $\mu$E m$^{-2}$ s$^{-1}$ (12). This strain (a kind gift of Prof. W. Vermaas) was transformed in separate experiments with the five plasmids shown in Fig. 1. Each plasmid contains a wide segment of the *Synechocystis psbN-psbH-petC-petA* gene cluster (19) centered around one out of five differently engineered *psbH* genes, followed by the same kanamycin resistance gene (Km$^r$). To avoid variability in the expression of the different PsbH proteins, all plasmids maintained the original upstream regulatory sequences of the *psbH* gene present in the bacterial chromosome (4). For the same reason, the position and orientation of the Km$^r$ cassette were the same with regard to the gene cluster.

Following transformation, the obtained control strain PSI-less/SH233 and the double mutant strains PSI-less/H-less, PSI-less/MH264, PSI-less/CH269, PSI-less/ΔH228 [2] were examined for proper integration of the artificial genes and for achievement of homozygous clones (for the sake of simplicity, in the following text we omit the indication PSI-less in the name of mutants). Correct integration of the five *psbH* versions, together with the common kanamycin marker, in the gene cluster of the mutant strains was verified both by PCR (Fig. 2*A*), with

[2] In a previous report (21) mutants were, respectively, indicated with the names 233k, H$^-$k, 264k, 269k, and 228k.

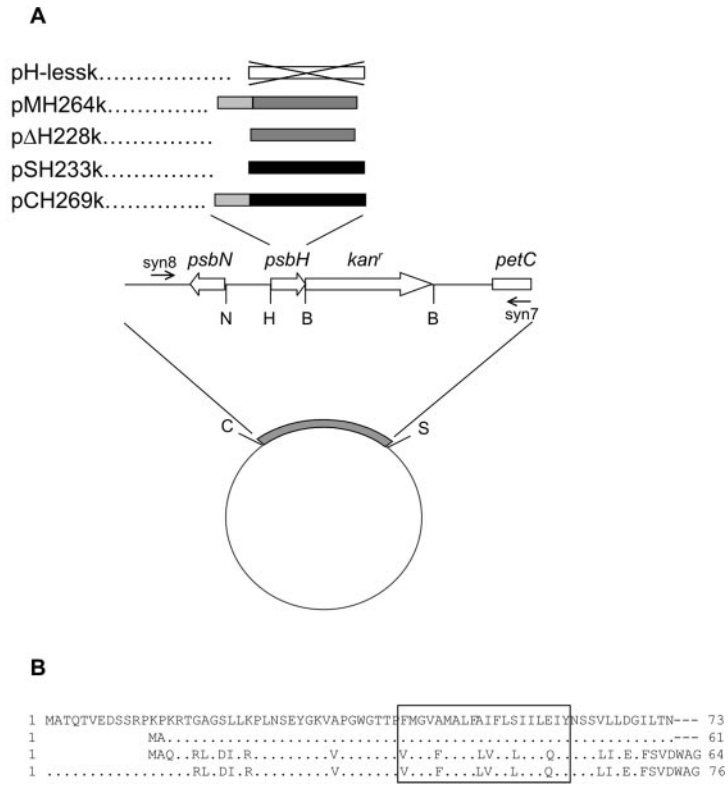


FIG. 1. **Physical and restriction map of plasmids used for mutagenesis of PSI-less *Synechocystis* 6803 strain.** Diagram of modified *psbH* genes is shown: *open rectangle*, null gene; *black rectangles*, *Synechocystis* gene; *gray rectangles*, maize gene. *Lightest gray area*, region coding N-terminal, extramembrane, maize extension, kan$^r$: kanamycin resistance marker gene. Restriction sites: *B*, *Bam*HI; *C*, *Cla*I; *H*, *Hin*dIII; *N*, *Nco*I; *S*, *Sac*I. Also indicated are positions of primers syn8 (sense) and syn7 (antisense) used for PCR analysis of mutant genomes (see text and Ref. 6 for other details). *Lower part*, alignment of translation products corresponding to each *psbH* genes. Identical residues are indicated by *points*; *box*, hydrophobic regions corresponding to putative membrane-spanning domains. Rationale for names assigned to obtained plasmids is encoded PsbH product and size of the cloned sequence; letter *k* stands for presence of Km$^r$ gene. Names are: pH-lessk for plasmid in which *psbH* gene was completely deleted; pSH233k for plasmid containing insert corresponding to wild-type *Synechocystis* (S) *psbH* gene, 233 bp long (6); pMH264k for plasmid containing *Zea mays* (M) *psbH* gene insert, 264 bp long (6); pCH269k, with an artificial chimerical (*C*) gene coding for a protein in which the first 12 extramembrane amino acids of maize PsbH are fused to N terminus of cyanobacterial PsbH (269 bp insert); and pΔH228k, with a second artificial gene coding for a shortened maize PsbH, lacking 12 N-terminal residues (228 bp insert).

primers bounding regions of recombination (Syn 7, Syn 8), and by Southern blotting (Fig. 2, *B* and *C*). Neither experiment could detect residual copies of the wild-type DNA in any of the clones.

MALDI-MS was used to verify the correct expression of the mutated PsbH proteins and assembly in PSII complexes. This technique has recently been used to identify the main, as well as many of the minor, components of photosystem II in both thylakoid and PSII preparations from *Synechocystis* (13). Fig. 3, *A* and *B* show the representative MALDI spectra of PSII cores isolated from the original PSI-less strain, possessing the wild-type copy of PsbH, and from H-less cells respectively. A protein of 6982 Da mass is present in the former but lacking in the latter. Identification of this peak with PsbH is in accordance both with the predicted molecular mass of PsbH for cyanobacteria (6985 Da) and with our previous results (13). The MALDI spectra of thylakoid membranes prepared from control SH233 (wild type) and CH269 cells are shown in Fig. 3, *C* and *D*. The former contains a 7088 Da protein; the latter exhibits a large peak at 8138 Da. The appearance of the 8138 Da peak is in agreement with the expected mass for the chimerical PsbH. The results, reported in Table I, indicate the substitution of the wild-type PsbH copy with the mutated one in the engineered strains.

*Fluorescence Analysis*—Electron transfer rates from the first stable acceptor, the plastoquinone $Q_A$ firmly bound to the D2 subunit, to the second plastoquinone molecule, reversibly bound to the D1 subunit, is highly sensitive to the protein environment of the $Q_B$ site. Perturbation of this site is reflected in a change of the $Q_A \rightarrow Q_B$ electron transfer. Thus, various herbicide-resistant mutants, in which the $Q_B$ site is modified, are impaired in $Q_A \rightarrow Q_B$ electron transfer (22). Single turnover flash fluorescence decay kinetics are useful in providing information on how an electron generated by charge separation is equilibrated between $Q_A$ and $Q_B$ on the acceptor side of PSII. An initial fast decay phase (a few hundred microseconds) after flash excitation reflects reoxidation of $Q_A^-$ through electron transfer to the quinone bound at the $Q_B$ site. An intermediate phase (millisecond range) derives from $Q_A^-$ reoxidation in centers with an empty $Q_B$ site at the instant of the flash (kinetics of PQ binding from the PQ pool). Lastly, a slow phase (time range of seconds) reflects $Q_A^-$ reoxidation *via* recombination with the S states (mainly S2) of the manganese cluster of the oxygen-evolving complex. These complex kinetics have been described as the sum of two or three exponentials (23) or, in some cases, by two exponentials plus one hyperbolic component (24, 25). However, in view of the complexity of the kinetic system and its intrinsic microheterogeneity, other decay components may also be present, and any description in terms of a discrete set of components may be arbitrary and approximate. For this reason, we prefer a description in terms of a rate distribution $p(k)$, such that $p(k)dk$ is the probability that $Q_A^-$ oxidation

occurs with a rate coefficient between $k$ and $k + dk$.[3] This may be obtained by fitting experimental time courses with the simple power law in Equation 1,

$$N(t) = (1 + k_0 t)^{-n} \quad \text{(Eq. 1)}$$

and distribution $p(k)$ can be obtained from fitting parameters $k_0$ and $n$.[4]

As shown in Fig. 4*A*, Equation 1 describes the experimental data quite accurately for all our mutants. The average value of rate constant $<k> = nk_o$ and standard deviation $\sigma^2 = nk_0^2$ of the distribution can be evaluated from fitting parameters $k_o$ and $n$ (Table II). Fig. 4*B* shows the rate distribution function $f(k)$ for the different mutants on a logarithmic scale.[5]

This type of analysis clearly shows how electron transfer rates in PSII are affected by modifications to the PsbH subunit. It may be observed that perturbation in electron transfer at the acceptor side gradually becomes more extended in the various mutant strains in the order ΔH228 < MH264 < CH269 < H-less. Besides a small decrease in the probability of reoxidation at the highest rate, the main effect is clearly that of increasing the probability of the lowest rate (recombination to the donor side). This corresponds to an increase in the fraction of centers that are not able to reduce $Q_B$.

In a separate experiment, we measured $Q_A^-$ reoxidation in the presence of DCMU. In these conditions, the only pathway open to $Q_A^-$ reoxidation is recombination with the Mn cluster: $Q_A^-$ S2 → $Q_A$ S1. Fig. 4*C* shows the results: it is evident from both time courses and rate distributions that all mutants are grouped, with little differences among them, around recombination rates lower than the control by a factor of ~2.

*Oxygen Evolution Measurements*—In a previous paper on the characterization of a *Synechocystis* mutant expressing the PsbH protein of maize in a PSI-containing strain, we showed that substitution of this subunit was accompanied by modifications in the sensitivity of the hybrid PSII toward herbicides, with particular regard to the cyanophenol ioxynil. We tentatively indicated the longer N terminus of the chimeric protein as the domain responsible for this effect (6). To check this hypothesis, all the new strains expressing a mutated PsbH in the PSI-less context were tested in oxygen evolution experiments with herbicides DCMU, atrazine, and ioxynil. Titration curves were drawn using increasing concentrations of each herbicide, and $I_{50}$ values were calculated as the average $I_{50}$ from repeated experiments ($n = 3$). As shown in Table III, all mutants exhibited higher sensitivity than the control strain toward the three herbicides used. However, comparisons with the parent PSI-less strain revealed that, although $I_{50}$ values for DCMU and atrazine were only slightly reduced, sensitivity

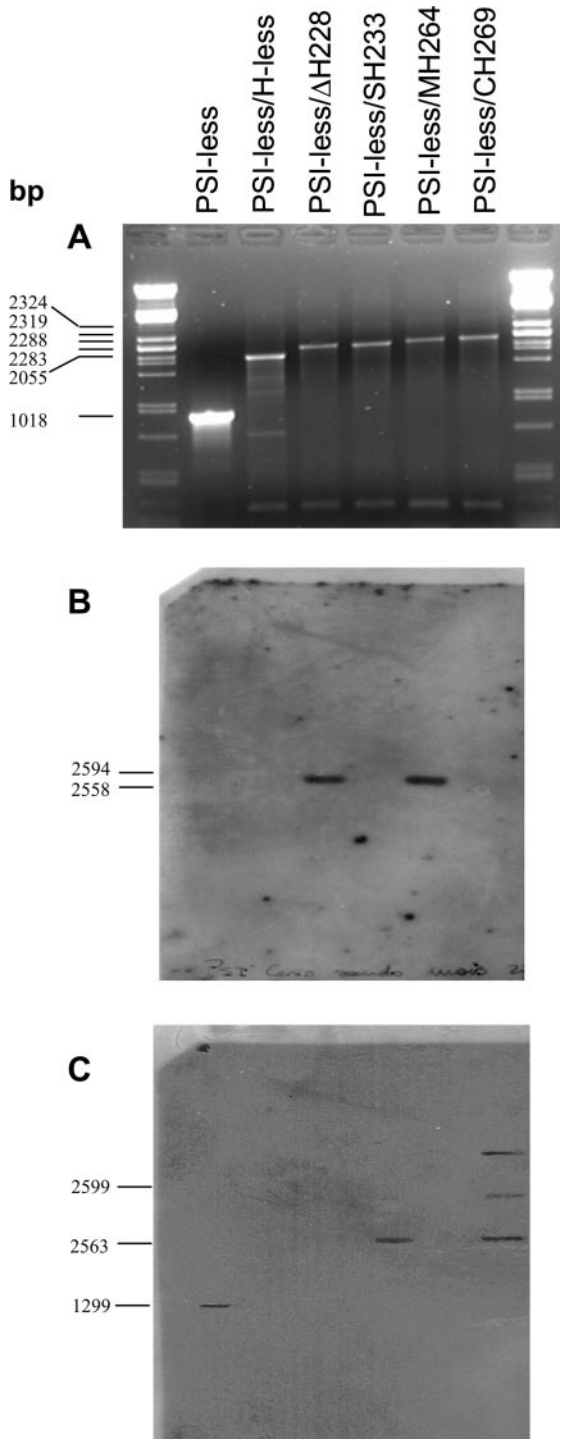


FIG. 2. **PCR and Southern blot analysis of mutant *Synechocystis* genomes.** Genomic DNA from original PSI-less strain and PSI-less/*psbH* double mutants was analyzed (*A*) by PCR with primers syn7 and syn8, annealing to regions of *psbN-psbH-petC-petA* cluster at opposite sides of *psbH*+*kan*$^r$ insertion, and by Southern blotting, after *Nco*I digestion with (*B*) *Synechocystis psbH* or (*C*) *Zea mays psbH* probes. Calculated sizes of amplified and hybridized DNA fragments are indicated.

[3] In the continuous limit, rate distribution p($k$) is defined by Equation 2,

$$N(t) = \frac{Q_A^-(t)}{Q_A^-(0)} = \int_0^\infty p(k) e^{-kt} dk \quad \text{(Eq. 2)}$$

where $N(t)$ represents the fraction of centers with $Q_A$ still reduced at time $t$.

[4] Equation 1 has often been used in analysis of multi-exponentials (stretched exponentials) (26, 27). In principle, rate distribution $p(k)$ can be obtained by the inverse Laplace Transform of experimental data set $N(t)$. However, we prefer to use a model function and fit the data in the time domain. Assuming a unimodal rate distribution, we can approximate it with a gamma distribution in Equation 3,

$$p(k) = \frac{k^{n-1}}{k_0^n - \Gamma(n)} \exp(-k/k_0) \quad \text{(Eq. 3)}$$

where $\Gamma(n)$ is the gamma function. The advantage of this distribution is that it gives a simple description in the time domain, as its Laplace Transform is simply Equation 4.

$$N(t) = l[p(k)] = (1 + k_0 t)^{-n} \quad \text{(Eq. 4)}$$

[5] The relation between $p(k)$ and $f(k)$ plotted on a logarithmic scale is $f(k)d \log k = p(k)\, dk$ and hence $f(k) = k\, p(k)$.

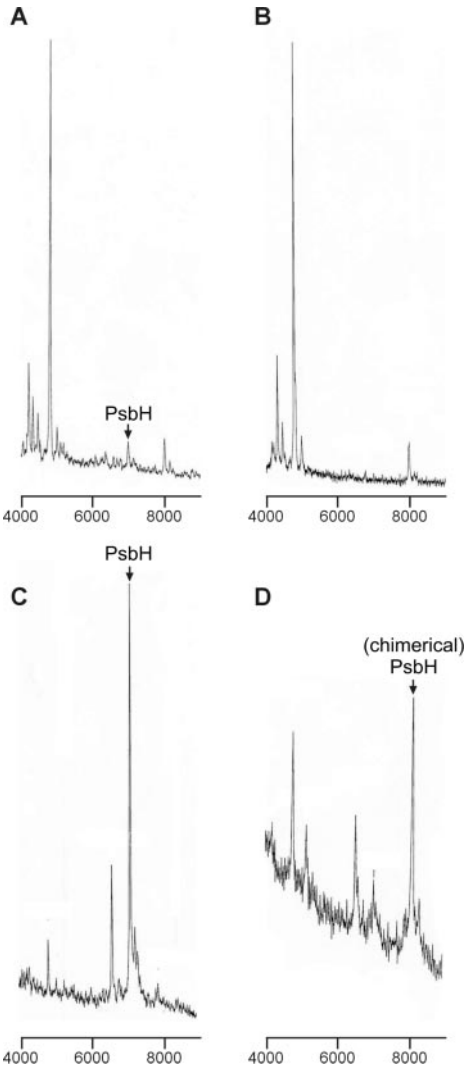


FIG. 3. **MALDI spectra of photosystem II (*A* and *B*) prepared from PSI-less/SH233 (control strain) (*A*) and H-less (*B*) cells and thylakoid membranes (*C* and *D*) obtained from PSI-less/SH233 (*C*) and CH269 mutant (*D*) cells.** *m/z* values, corresponding to molecular masses, are shown on abscissa, ordinates are arbitrary units. Note disappearance of PsbH peak in *B* and *D* and appearance of a new peak corresponding to chimeric PsbH in *D*. Ionization and consequently position and intensity of *m/z* peaks may vary depending on different environments between spectra from thylakoid and PSII preparations.

toward ioxynil increased more than ten times. In particular, the sensitivity of mutants ΔH228, MH264, and CH269 were, respectively, about 30, 12, and 18 times higher than that of the control strain. The almost 70-fold lower $I_{50}$ value of the H-less mutant suggests that the absence of this subunit allows easier docking of ioxynil to its binding site on D1.

At variance with a previous hypothesis (6), these results indicate that the sensitivity of the MH264 mutant toward ioxynil is not due to the addition of an extra N-terminal extension but, rather, the whole protein is involved, and the entire subunit plays a role in setting up the correct structure of the $Q_B$/herbicide-binding site.

*D1 Turnover and Chlorophyll Photobleaching*—It has been shown that, in a *Synechocystis* strain devoid of PsbH, photosystem II undergoes faster photoinactivation than in wild-type, but that D1 protein degrades at a lower rate. Moreover, in the same conditions, degradation of the D1 protein is significantly slowed down by the inhibitor of protein synthesis chloramphenicol (5). To better understand this point, we examined the effects of photoinhibitory treatment on our mutants, in terms of

TABLE I
*Molecular mass of PsbH subunit in WT and mutated strains of Synechocystis 6803*

| Strain | Expected mass | Measured mass |
|---|---|---|
| SH233 (WT) | 6985 | 6982[a] 7094[b] |
| ΔH228 | 6329 | 6335[b] |
| MH264 | 7656 | 7692[b] |
| CH269 | 8312 | 8138[b] |

[a] Measured in purified PSII cores.
[b] Measured in whole thylakoid membranes.

D1 degradation in the absence and presence of lincomycin, which abolishes protein synthesis. Samples from different mutants were analyzed for the D1 content of the thylakoid membrane after exposure to photoinhibitory light of 1000 $\mu$E $m^{-2}$ $s^{-1}$. Cell cultures were light-treated in both the absence and presence of the antibiotic. Aliquots of each strain were taken at different times of light treatment and thylakoid membranes were analyzed by Western blotting with specific antisera. For some mutants, a significant change in the color of the culture was observed during light exposure, indicating chlorophyll bleaching (see below). For this reason, preparations of thylakoid membranes were quantified for total protein contents prior to immunochemical analysis, and equal amounts of total proteins were loaded in SDS-PAGE. The possibility that mutant strains could assemble less PSII than wild-type PSI-less strains also had to be considered. To this aim, samples of the different strains were analyzed, at two times of growth (24 and 48 h), for the number and dimension (FSC) of cells. From the results, reported in Table IV, it may be seen that no significant differences are present in the chlorophyll content after 48 h of culture growth. Since in these PSI-less mutants chlorophyll concentration is proportional to PSII content, cell counting indicates that the mutated strains assemble the same amount of PSII than the wild type. Therefore, assuming that the total protein contents of the membrane do not change significantly during light treatment (except for the D1 protein), light-induced changes in the amount of D1 may safely be evaluated by Western blotting with reference to total protein contents.

Results of experiments on light-induced degradation of D1 are presented in Fig. 5*A*. In the absence of lincomycin, all mutants showed progressive reduction in the amount of D1 during high light treatment, indicating that D1 degradation was faster than the insertion of newly synthesized protein. It is also shown that the D1 protein is lost at different rates in the mutant strains, and, within the errors intrinsic to the method used, we may observe that the D1 protein is lost fastest in SH233 and MH264. In particular, the strains with the most severe modification or even elimination of PsbH (ΔH228 and H-less, respectively) lose D1 more slowly.

An unexpected result was obtained in experiments performed in the presence of lincomycin: the thylakoid membrane from cells expressing the wild-type bacterial copy of PsbH, photoinhibited in the presence of the antibiotic, did not lose but rather acquired D1 protein. This effect is clearly evident in the SH233 strain; instead, the H-less strain shows a clear loss of D1 (Fig. 5*B*). In order to better understand this point we performed exactly the same experiment using the control strain 233K (6), obtained from the wild-type *Synechocystis* 6803 parent. In this case, stronger light was used ($\approx$ 9000 $\mu$E $m^{-2}$ $s^{-1}$) because of the higher resistance of the PSI competent strain to photoinhibitory light. No increase in the content of D1 was observed either in the absence or presence of lincomycin (data not shown).

The fact that the D1 protein was found to increase in the thylakoid membrane under conditions in which protein synthesis was disabled, had to be interpreted by assuming insertion

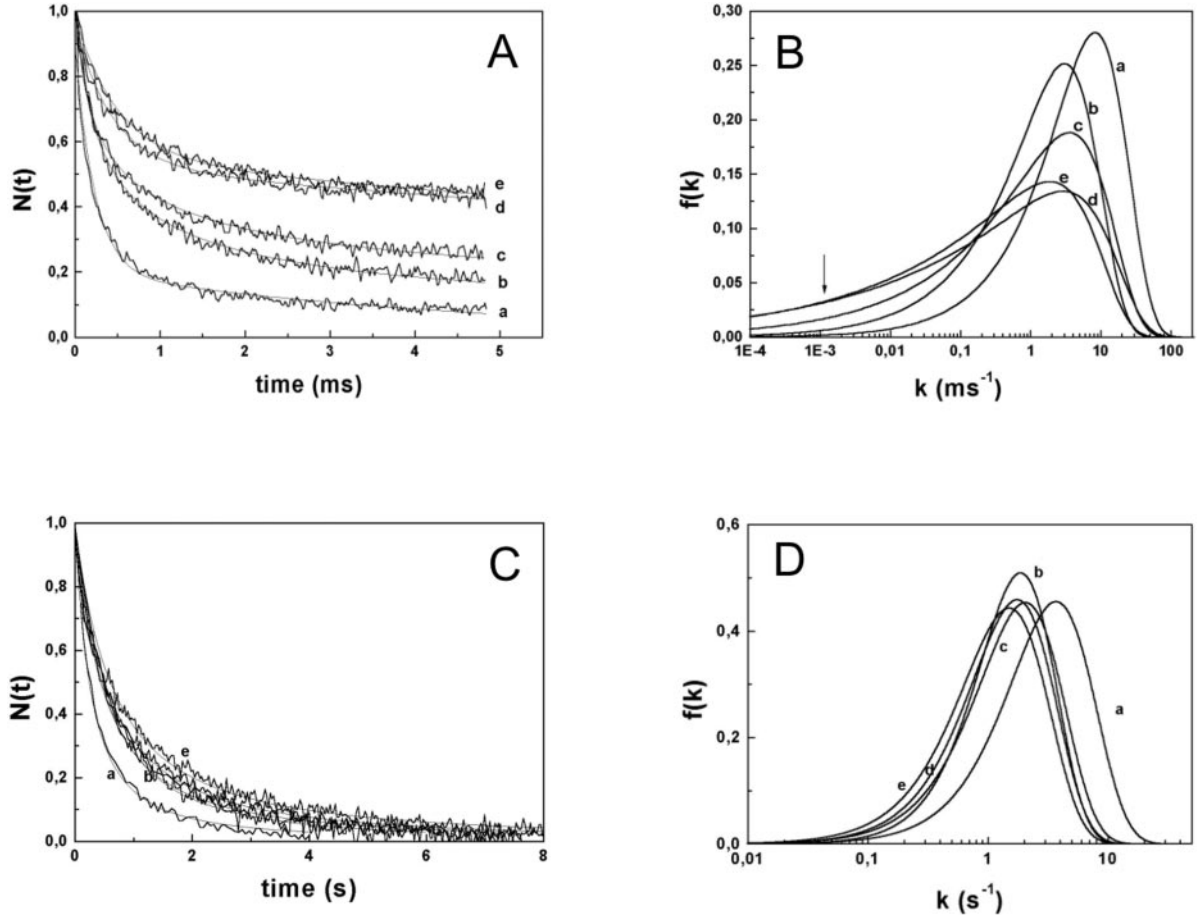


FIG. 4. **Kinetics of $Q_A^-$ reoxidation in the absence (*A* and *B*) and presence (*C* and *D*) of DCMU.** Time courses (*A* and *C*) represent time dependence of fraction of centers in which $Q_A$ is still reduced at time *t* after a single turnover flash: $N(t) = (F(t) - F_0)/F_0$, where $F_0$ and $F(t)$ are fluorescence levels of dark-adapted sample and at time *t* after flash, respectively. *Solid lines* through data: best fits with Equation 1. *Arrow* in *panel B*, region of k for recombination to S states (see text). *a*, SH233 (control strain); *b*, ΔH228; *c*, MH264; *d*, CH269; *e*, H-less.

TABLE II
*Analysis of fluorescence decay kinetics after single turnover flash*

Average value of rate constant $\langle k \rangle = nk_0$ and standard deviation of distribution $\sigma^2 = nk_0^2$ can be evaluated from fitting parameters $k_0$ and $n$.

| Strain | −DCMU | | + DCMU | |
|---|---|---|---|---|
| | n | $k_0$ | n | $k_0$ |
| | | $ms^{-1}$ | | $s^{-1}$ |
| SH233 | 0.63 | 13.1 | 1.46 | 2.52 |
| ΔH228 | 0.53 | 5.71 | 1.79 | 1.04 |
| MH264 | 0.34 | 10.5 | 1.45 | 1.40 |
| CH269 | 0.21 | 13.3 | 1.48 | 1.18 |
| H-less | 0.23 | 8.10 | 1.39 | 1.08 |

TABLE III
*Inhibition of oxygen evolution by herbicides*

Concentrations necessary to inhibit 50% oxygen evolution ($I_{50}$) are reported. Values result from three complete and independent series of measurements for each herbicide.

| Strain | $I_{50}$ | | |
|---|---|---|---|
| | DCMU | Atrazine | Ioxynil |
| | | μM | |
| SH233 | 0.12 ± 0.01 | 1.35 ± 0.19 | 16.26 ± 1.68 |
| MH264 | 0.08 ± 0.00 | 0.75 ± 0.15 | 1.35 ± 0.05 |
| CH269 | 0.09 ± 0.01 | 0.74 ± 0.08 | 0.88 ± 0.37 |
| ΔH228 | 0.07 ± 0.01 | 0.48 ± 0.12 | 0.55 ± 0.09 |
| H-less | 0.08 ± 0.02 | 0.63 ± 0.09 | 0.24 ± 0.05 |

into the thylakoid membrane of an extra amount of D1 protein, already synthesized before the photoinhibitory treatment (with addition of the antibiotic) and present in a different cell compartment. For this reason, we analyzed the supernatant fractions of our thylakoid preparations by Western blotting. Fig. 6*A* shows that the D1 protein was clearly detected in the supernatant of all mutants. Lys-C digestion confirmed the identity of the polypeptide by producing a 12 kDa C-terminal fragment, as expected on the basis of the primary sequence (Fig. 6*B*). The presence of other proteins of the PSII core was also examined: the D2 protein and the α-subunit of the cytochrome $b_{559}$ were immunodetected, but no trace of the inner antenna CP47 was found (Fig. 6*C*). We can therefore exclude the presence of residual thylakoid membrane in our supernatant fractions, and conclude that the PSII proteins detected were contained in the plasma membrane, some residues of which were certainly present in the supernatant. The presence in this compartment of PSII subcomplexes containing D1, D2, and cytochrome $b_{559}$ but not CP43 or CP47 has recently been shown by Zak *et al.* (28).

Separately, control aliquots of each treated culture were analyzed for chlorophyll content. The spectra of pigments extracted by methanol from samples before and after 6 h of photoinhibitory treatment are shown in Fig. 7. A reduction in chlorophyll content was the general effect of light treatment, probably due to the formation of reactive oxygen species (7) and consequent photobleaching of pigments. The reduction was remarkably severe in mutants ΔH228, CH269, and H-less, in which chlorophyll could no longer be detected after treatment. Interestingly, only a limited loss of chlorophyll was observed in SH233 and MH264.

## DISCUSSION

The present study focused on PsbH, first with the aim of revealing the different contributions of the intra- and extramembrane domains of the molecule to the structure of the $Q_B$ site of photosystem II. An additional aim was to extend information so far collected in various studies on the involvement of this protein subunit to photoprotection and D1 turnover (4, 5, 29).

For the construction of the new *Synechocystis* mutants, we chose to adopt a strain devoid of PSI, which had been successfully used in other studies (13–15). Its advantage is that biophysical and biochemical investigation of PSII is favored compared with wild-type strain, since only about 100 chlorophyll molecules are present per PSII reaction center and the PSII complex is the only major chlorophyll-binding complex (12). Using this PSI-less strain as background, we produced three mutants by substitution of the endogenous *psbH* gene with genes coding for the PsbH protein of maize or combination of this gene with the endogenous one. We also produced a PsbH-less mutant and a control strain carrying the antibiotic marker gene cloned in the same position as in the other mutants. All mutants were checked for correct integration into the genome and sufficient segregation. Moreover, proper expression of recombinant proteins and assembly in PSII complexes were verified by MALDI-MS. The functional properties of the mutants were studied by analysis of electron transfer kinetics and by titration of oxygen evolution with herbicides.

TABLE IV
*Forward scattering parameter (FSC) and number of cells per microgram of chlorophyll at two growth times*

| Strain | FSC | | (Cells/$\mu$g chl) $\times 10^{-6}$ | |
|---|---|---|---|---|
| | 24 h | 48 h | 24 h | 48 h |
| SH233 | 663 ± 50 | 828 ± 20 | 375 ± 25 | 228 ± 20 |
| CH269 | 556 ± 25 | 574 ± 10 | 338 ± 30 | 302 ± 20 |
| MH264 | 746 ± 35 | 847 ± 10 | 414 ± 50 | 253 ± 15 |
| ΔH228 | 611 ± 15 | 666 ± 50 | 297 ± 30 | 254 ± 35 |
| H-less | 833 ± 20 | 979 ± 30 | 373 ± 10 | 258 ± 10 |

Fluorescence analysis of $Q_A^-$ reoxidation after a single turnover flash extended the results reported so far (4, 5, 6, 21). We analyzed reoxidation time courses, assuming a unimodal distribution of rate constant $k$, with which $Q_A^-$ is reoxidized. For some of the mutants, a bimodal distribution would probably have been more appropriate. However, we preferred to limit the number of fitting parameters to a minimum and to look at the effects caused by the mutations on the rate distribution with reference to the control strain. Thus, in the presence of the herbicide DCMU, when the only pathway for $Q_A^-$ reoxidation was recombination to S states at the donor side, we observed a shift in the average value of the recombination rate toward lower values, whereas the shape of the distribution remained essentially the same as that of the control strain. A decrease in the rate of recombination by a factor of 2 is not a very large effect. Nonetheless, the fact that all the mutant strains underwent approximately the same effect, independently of the par-

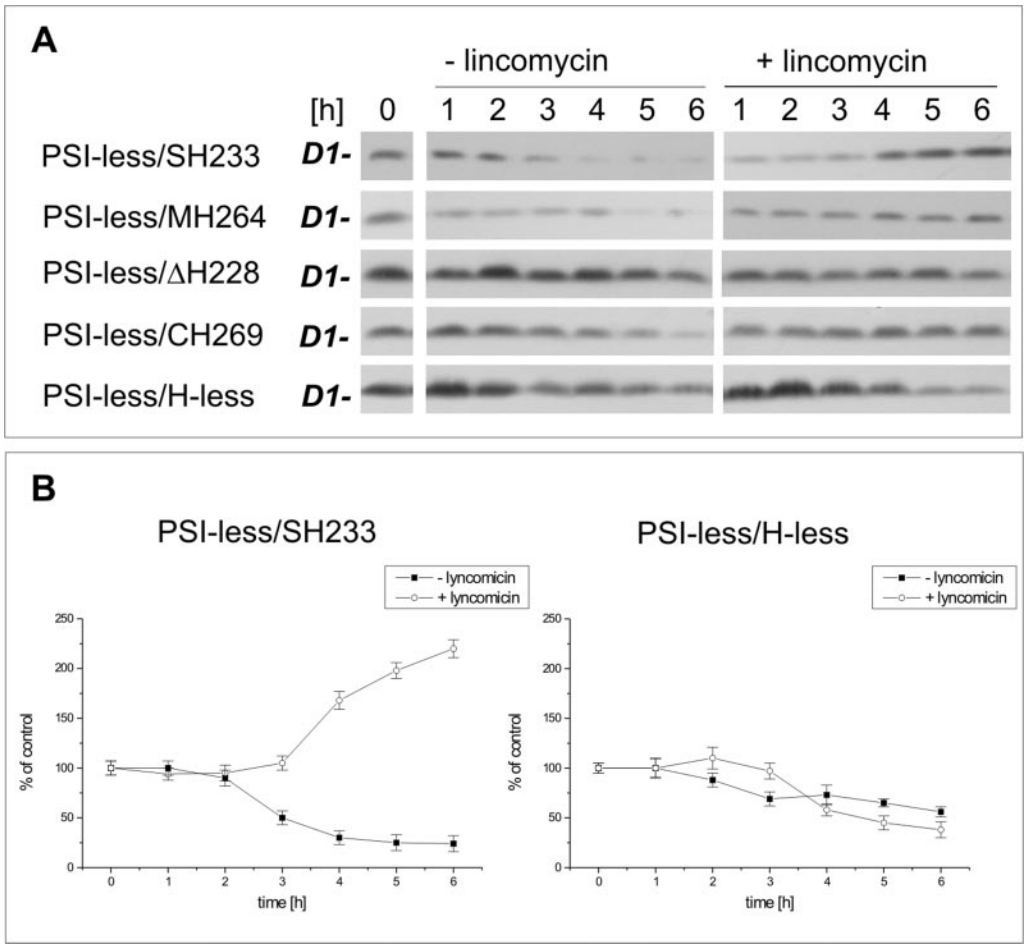


FIG. 5. **Western blot analysis of D1 degradation during photoinhibition.** *A*, contents of D1 protein in thylakoid membrane fraction were checked at regular intervals (up to 6 h) during photoinhibitory treatment, in absence or presence of lincomycin, by detection with anti-*Synechocystis* D1 antibodies. Gels were loaded, on basis of protein concentration, as follows: 1.5 $\mu$g/lane for control mutant SH233; 7.5 $\mu$g/lane for mutants SH264, ΔH228 and CH269; and 15 $\mu$g/lane for H-less. *B*, comparison of D1 degradation time courses, in absence or presence of lincomycin, between SH233 and H-less strains by densitometric analysis of respective blots ($n = 3$).

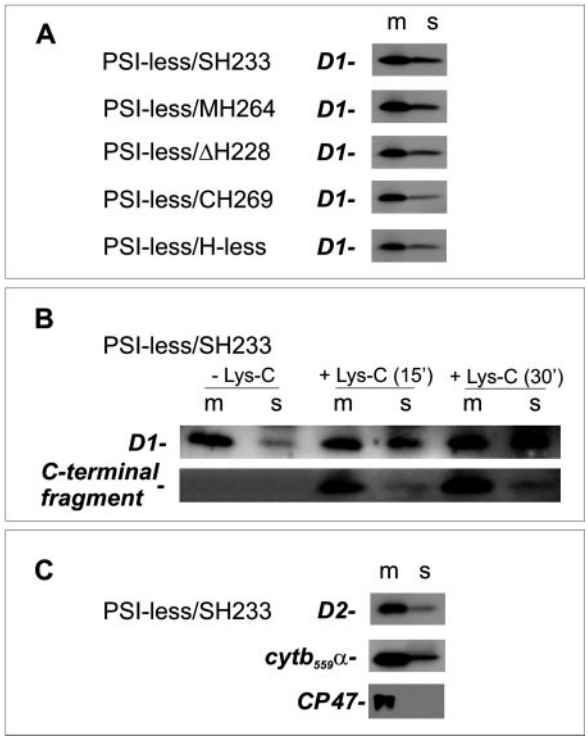


FIG. 6. **Western blot analysis of thylakoid membrane and supernatant fractions from control and mutant strains.** *A*, thylakoid membrane (*m*) and supernatant (*s*) fractions of $t_0$ samples of each mutant were tested for the presence of D1 protein. See text for further details. *B*, thylakoid membrane (*m*) and supernatant (*s*) fractions from control mutant SH233 were immunodecorated before (−Lys-C) and after digestion (+Lys-C) for various times (15 and 30 min) with Lys-C, a lysine-specific endoprotease. *C*, same fractions were tested for presence of D2, CP47, and Cytb$_{559}\alpha$ proteins with specific antibodies.

ticular change in the PsbH protein (including its absence), indicates that long-range electron transfer through the PSII complex is modulated by structural features of the entire complex rather than by specific interactions with the H-subunit.

The situation is different in the absence of DCMU. In this case, reoxidation of $Q_A^-$ can proceed through two different pathways, *i.e.* electron transfer to $Q_B$ (or $Q_B^-$), or recombination to the donor side. In normal conditions, the first pathway is greatly favored to ensure high photosynthetic yield. Indeed, the probability of recombination in the control strain is very small. Electron transfer to $Q_B$ is characterized by complex kinetics, as several processes are involved in the equilibration of the redox state of the two quinones, including the equilibrium of binding of the plastoquinone pool to the $Q_B$ site. Accordingly, rate distribution is sharply limited upward but broadens out toward low rate values. In mutant strains, rate distribution broadens progressively and the probability of recombination with S states increases. Thus, in CH269 and H-less mutants, a significant fraction of centers cannot reduce $Q_B$, indicating extensive perturbation of the $Q_B$ site. Milder perturbation is undergone by the ΔH228 mutant, which contains the shortened maize protein. We may therefore conclude that: (i) the transmembrane portion of the PsbH subunit plays a role in facilitating electron transport from $Q_A$ to $Q_B$; (ii) substitution of the endogenous transmembrane portion with that of maize slows down the rate of transfer but does not significantly increase the number of centers which are unable to reduce $Q_B$; (iii) deletion of the PsbH subunit or introduction of an extra N-terminal extension strongly affects the transfer rate, increasing the non-$Q_B$ reducing fraction of centers.

Experiments on oxygen evolution in the presence of herbicides confirmed the conclusion regarding the involvement of PsbH in determining $Q_B$ site conformation (6). With all herbicides used, the presence of a modified PsbH protein caused a change in sensitivity. Mutants appeared slightly more sensitive than controls to the classical herbicides DCMU and atrazine; instead, sensitivity to the cyanophenol ioxynil was considerably increased in all mutants, with $I_{50}$ values much lower than those of controls. The fact that the H-less mutant displays the highest affinity for ioxynil suggests that the absence of this subunit somehow clears the way to its binding site possibly loosening the compactness of the general structure of the PSII core. A major effect is also observed for ΔH228, in which the transmembrane portion of maize protein can only partially mimic the endogenous protein. Less increased affinity for the herbicide is displayed by the strains bearing the exogenous N-terminal extra portion of the protein which likely counteracts a more loosely packed structure of the core with a partial steric obstruction exerted by the N-terminal domain.

Experiments on D1 turnover in PsbH mutants resulted in the most interesting part of our work. The PSI-less background of the mutants allowed us to observe an accumulation of mature D1 protein in the thylakoid membrane of bacterial cells during photoinhibitory treatment, in the absence of protein synthesis. This feature was evident in control strain SH233 and was shared, although to a lesser extent, by other mutants but not by the H-less strain. In order to explain this distinctive behavior, some already acquired aspects of D1 turnover in cyanobacteria must be considered.

First of all, expression of *psbA* isogenes is mainly regulated at the level of transcription (30, 31, 32, 33). In particular, it has been shown that accumulation of $Q_A^-$ specifically activates transcription (34). In our PSI-less strains, reoxidation of electron carriers is strongly inhibited and we expect an anomalously high fraction of centers in which $Q_A$ is reduced at steady state. In these conditions, the signal for *psbA* transcription is always turned on and we expect a high concentration of messenger.

Second, in a recent work it was elegantly shown that the synthesis of D1 is regulated at the level of translation elongation rather than initiation (35). In conditions of excess *psbA* mRNA, cytosolic ribosomes and membrane-bound polysomes are found to pause at two precise and different positions, corresponding to detectable intermediates. A number of unknown components seems to be required for completion of the chain, maturation, targeting, insertion (35) and eventual translocation (28, 36). We deduce that, in our PsbH mutants, synthesis of D1 was initiated before light treatment, with or without the addition of lincomycin.

Third, it has been demonstrated that chlorophyll availability is necessary not only for translation of the D1 pre-peptide but also for its maturation; pulse-chase experiments showed that the D1 precursor processing rate decreases in conditions in which little chlorophyll is available, while the unprocessed or non-stabilized D1 rapidly degrades (within half an hour) (14). This means that, during our photoinhibitory treatment, in those mutants in which chlorophyll content was rapidly reduced (CH269, ΔH228, H-less), stabilization and maturation of pre-synthesized D1 precursor was prevented. Instead, maturation by the C-terminal protease CtpA could be accomplished in SH233 and MH264 (37, 38). In these strains in fact, during prolonged photoinhibition in the presence of lincomycin, incorporation of D1 protein continues and progressively prevails over removal. The incorporated protein has the size of the mature D1 (Fig. 6), indicating that processing of the precursor molecule was completed before or during light treatment.

Lastly, it is well established that no efficient degradation of damaged D1 subunits occurs in the absence of protein synthe-

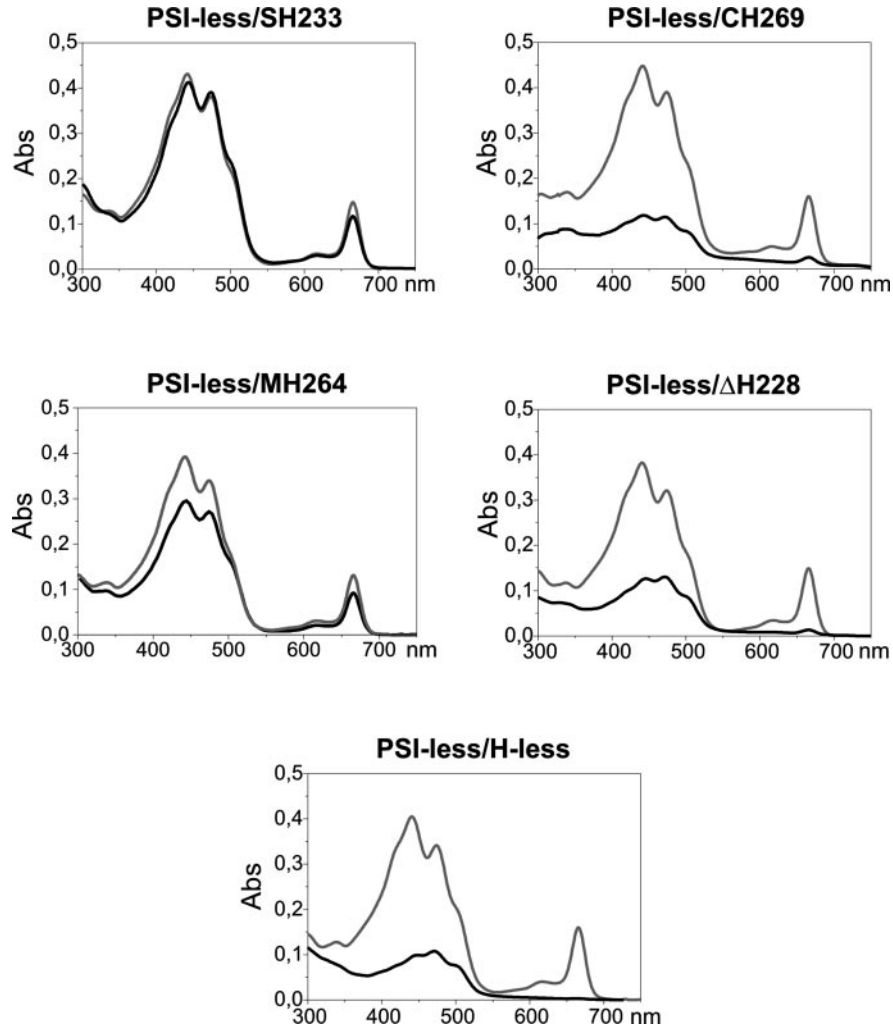


FIG. 7. **Spectra of pigments extracted from control and photoinhibited cell cultures.** $t_0$ (*gray*) and $t_{6\,h}$ (*black*) samples from photoinhibition experiments (see Fig. 5, no lincomycin added) were analyzed for pigment contents, after methanol extraction, by registering absorption spectra in 300–750 nm wavelength range.

sis (5, 39, 40), perhaps because of limiting amounts of the specific protease (Var2-FtsH homologue) (41).

The above information, together with a great deal of other data, was combined in a model for PSII repair in cyanobacteria in which D1 degradation and synthesis are closely synchronized (42, 43) and a conformational modification in the $Q_B$ site region of the protein is suggested as the signal controlling D1 degradation (5, 44, 45). On the basis of this model, we can reasonably explain the different response of our mutants to prolonged photoinhibitory treatment (Fig. 5*A*). In the absence of lyncomicin, prompt degradation of damaged D1 protein occurs only in the presence of a functional PsbH subunit (SH233). Modification of this subunit or its removal slow down or stop degradation. Therefore, we propose that the interaction with the PsbH polypeptide is important for the damaged D1 protein to assume the correct conformation for its rapid degradation.

Conversely, during photoinhibition in the presence of lincomycin, an increase in D1 protein was observed in the thylakoid membrane of the same strains which, in the absence of the antibiotic, permit D1 degradation. In mutants ΔH228 and CH269, no significant accumulation of D1 protein is detectable, and in the H-less strain loss of D1 is evident. The increase, which can be interpreted only by incorporation of D1 protein, which was synthesized before the addition of lincomycin, is only observable in these PSI-less strains because of the high reduction level, which promotes an overaccumulation of stock D1. This supply of D1 protein may be stored in the PSII subcomplexes of the plasma membrane, believed to be the locus of initial biogenesis of PSII cores (28). Alternatively, precursor D1 protein may be directly incorporated into the thylakoid membrane when the light-activated repair cycle of PSII is turned on, as recently proposed (46). In any case, our results indicate that, either for translocation from the plasma to the thylakoid membrane or for insertion of new D1 subunits into the thylakoid membrane, a functional PsbH protein must be present. The role of PsbH seems to be that of rendering the structure of the accepting site correct, so that new D1 molecules can be incorporated.

Finally, the strong chlorophyll photobleaching observed in some mutant strains is not surprising: in the absence of PSI activity, high photon flux brings about overreduction of the acceptor side of PSII, with the generation of various forms of reactive oxygen species able to attack and destroy the chlorophylls. More puzzling is the different resistance to photobleaching of the strains bearing a different copy of PsbH. The great majority of chlorophyll molecules is coordinated by the internal antennae CP43 and CP47. However, the prevalent origin of activated oxygen species is charge recombination at triplet P680 with formation of singlet oxygen (20). It is interesting to observe that the strains in which the D1 protein is degraded faster (SH233, MH264) are the more resistant to chlorophyll photobleaching. Prompt degradation of the D1 protein, to which the P680 chlorophyll dimer is coordinated, stops the production of singlet oxygen, thus preserving the integrity of antenna chlorophylls. During photoinhibition, the role of D1 may be compared with that of a "fuse"; its degradation is necessary in order to preserve the overall structure of the PSII core, which would be lost by the destruction of chlorophylls,

with consequent destabilization of antenna proteins and of the whole PSII complex.

It is noteworthy that the strain ΔH228, which contains a shortened version of maize PsbH (transmembrane portion), is less perturbed, in the $Q_A$ to $Q_B$ electron transfer, than the MH264 which contains the entire subunit of maize. A stronger perturbation at the acceptor side surface brought about by the exogenous N terminus is not surprising in view of the localization of the electron transfer pathway, which involves the non-heme iron close to this surface. On the other hand, since chlorophyll bleaching is correlated to the rate of D1 degradation, as pointed out above, we may infer that D1 degradation is not strictly correlated to the electron transfer between the acceptor quinones. We might speculate that the same perturbation induced by the extra portion of the maize subunit on the acceptor side can make the D1 protein more susceptible to the attack of the proteases, the action of which protects chlorophylls from photooxidation by singlet oxygen.

In conclusion, the PsbH protein appears now to be much more than an auxiliary subunit for the cyanobacterial PSII, necessary to optimize its activity (5) and stabilize its structure (7). PsbH plays at least three important functions, separately observable in our mutants. The first one, that we confirmed and further described, is its role in determining the structure of the $Q_B$ site and optimizing the electron transfer rate between the two plastoquinone acceptors. The second function is that of determining the right structure of the damaged D1 polypeptide which allows its prompt degradation. The third is that of being required for inserting new synthesized D1 proteins into the thylakoid membrane and, thus, for completing the PSII repair cycle.

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# Role of the PSII-H Subunit in Photoprotection: NOVEL ASPECTS OF D1 TURNOVER IN SYNECHOCYSTIS 6803

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