Role of the PSII-H Subunit in Photoprotection

NOVEL ASPECTS OF D1 TURNOVER IN SYNECHOCYSTIS 6803*

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Elisabetta Bergantino, Alessia Brunetta, Eleftherios Touloupakis‡, Anna Segalla, Ildikó Szabó§, and Giorgio Mario Giacometti¶

From the Department of Biology, University of Padova, Viale G. Colombo 3, 35121 Padova, Italy

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 QA/herbicide-binding site. The different turnover of the D1 protein and chlorophyll photobleaching displayed by mutant cells in response to photo-inhibitory 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) II is the pigment-protein complex, of both prokaryotic and eukaryotic thylakoid membranes, which is devoted 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, PsbTn, 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 QA to QB (4), protection from photoinhibition (5), contribution of important structural features to the QA/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 QA site, which were hypothetically ascribed to its distinctive N-terminus 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 QA/herbicide-binding niche and the QA to QB 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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‡ Present address: Dept. of Chemistry, University of Iraklion, 71 409 Iraklion, Crete, Greece.
¶ Recipient of a Young Researcher Grant of the University of Padova.
* To whom correspondence should be addressed: Dept. of Biology, University of Padova, Viale G. Colombo 3, 35121 Padova, Italy. Tel.: 39-049-827-6342; Fax: 39-049-827-6300; E-mail: giorgiomario.giacometti@unipd.it.

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 (psaA′) (12) and PSI-less/psbH double mutant strains were grown at 30 °C and 5 μE 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 106 cells ml−1. Lincymycin was used at a final concentration of 1 mM.

Construction and Genomic Analysis of Mutants—Construction of plasmids pSH235k and pMH264k has been described elsewhere (6). Their parent plasmid, containing a 1319-bp fragment of the Synechocystis plasmids pSH235k and pMH264k has been described elsewhere (6). Recombinant DNA from selected clones was extracted and analyzed by PCR with primers syn7 (5′-CGG ACT GGC GCA GG-3′) and syn8 (5′-GAT GGA TCA GTG CTT GCC AGG-3′) into the obtained ends. ii. pPH282 was constructed by substituting the HindIII-psiH-BamHI fragment of the parent vector with a fragment obtained by PCR, using primers sr-delta (5′-AAG CTT GAT CCT GAA GAC TCG AGA CCT AAC CCG ACT CGG TTA GGA GAT GC ATG-3′) and syn14 (5′-ATC TCC TAA CGG AGT GCG CCT AGG CCT AGG TCT GCT CCT GCA GTC ACG GTG ACT AGG CAT A-3′) into the obtained ends. (ii) pPH282 was constructed by substituting the HindIII-psiH-BamHI fragment of the parent vector with a fragment obtained by PCR, using primers sr-delta (5′-AAG CTT GAT CCT GAA GAC TCG AGA CCT AAC CCG ACT CGG TTA GGA GAT GC ATG-3′) and syn2 (5′-ATT GAT CCA AAA ACT ATG AAG TC-3′) on template pMH264k (Chiaromante et al., Ref. 6), cut by the same enzymes, and (iii) pH-less, obtained by substituting the HindIII-psiH-BamHI fragment of the parent plasmid with a cohesive-end adaptor duplex formed by annealing undecamers H3P1 (5′-CTG TTT TTT TTT GGG-3′) and H228k (Amersham Biosciences) was cloned into the single BamHI sites of (i), (ii), and (iii), and generating the final constructs pCH269k, pCH228k, and pH-less, 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 (Chiaromante et al., Ref. 6). Recombinant colonies were subcloned 7–9 times in BG11 containing both 5 mM glucose and 100 μg ml−1 kanamycin. Genomic DNA from selected clones was extracted and analyzed by PCR with primers syn7 (5′-TTATCCAGACCTTCTTGCGGC-3′) and syn8 (5′-CAAGGACATTTTACGTGCGA-3′). The kanamycin cassette derived from pU4K (Amersham Biosciences) was cloned into the single BamHI sites of (i), (ii), and (iii), and generating the final constructs pCH269k, pCH228k, and pH-less, respectively. All constructs were completely sequenced to ensure that no undesirable mutation had occurred during the cloning procedure.

Preparation of Thylakoid Membranes and MALDI Mass Spectrometry—For SDS-PAGE and Western blotting, thylakoids were prepared from 5 ml of cell culture (~2 μg of chlorophyll) following the procedure of Komenda and Barber (15). Final pellets were resuspended in 50–100 μl of 50 mM Tris, pH 7.5, 1 mM succinate and quantified by both chlorophyll and protein dosage.

For MALDI mass spectrometry, thylakoids from 4 liters of cell culture (OD750 = 0.8) were prepared following the procedure described in Szabó 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 μl/min, and cell counts were determined by the intrinsic chlorophyll fluorescence (λexc = 485 nm, λem >670 nm).

Oxygen Evolution Measurements—Cells collected from solid BG11 medium were suspended in liquid medium to a initial OD750 = 0.4–0.5. Cultures were grown for 2 days in liquid BG11 medium, supplemented with 10 mM glucose, 25 μg ml−1 kanamycin, 2.5 μg ml−1 chloramphenicol, at 5 μE 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 μg 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 L0. 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 μM s−2 m−2 intensity and 30 °C, and the final value was calculated by subtraction of the oxygen consumption measured in the dark (prevale).

Fluorescence Measurements—Cells were cultured in the conditions described above and suspended at a concentration of 2 μg 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 μM s−2 m−2 intensity and 1-s duration was applied. To determine fluorescence decay, single turnover flashes of 10000 μM s−2 m−2 intensity and 8-μs duration were applied every 18 s using a xenon lamp (XST 103). When specified, fluorescence decay was measured in the presence of 40 μM 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 μM urea and 0.1% SDS, according to Laemmli (17). 15, 7.5, or 1.5 μg 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 immunodetected 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 chemiluminescence kit (Pierce) for peroxidase-conjugated secondary antibodies (Kirkegaard and Perry Laboratories). When indicated, thylakoid membranes (6 μg of protein) and respective soluble fractions were incubated with 0.012 units of Lys-C for 15/60 min, in the presence of 25 μM 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 μE 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-psbH1-petC-petA gene cluster (19) centered around one out of five differently engineered psbH genes, followed by the same kanamycin resistance gene (Km′). 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 chomosome (4). For the same reason, the position and orientation of the Km′ 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/1H2, PSI-less/MH264, PSI-less/CH269, PSI-less/ΔH228 were examined for proper integration of the artificial genes and for achievement of the target expression level. In the following text we omit the indication PSI-less in the name of the 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. 2A), with

2 In a previous report (21) mutants were, respectively, indicated with the names 233k, H1, 264k, 269k, and 228k.

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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 rectangle, Synechocystis gene; grey rectangle, maize gene. Lightest grey area, region coding N-terminal, extramembrane, maize extension, kan': kanamycin resistance marker gene. Restriction sites: B, BamHI; C, ClaI; H, HindIII; N, NcoI; S, SalI. 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 gene. 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 stands for presence of Km' 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, 228 bp insert; and pJH268k, with a second artificial gene coding for a shortened maize PsbH, lacking 12 N-terminal residues (228 bp insert).

MALDI-MS was used to verify the correct expression of the mutated PsbH proteins and assembly in PSIII 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 PSIII preparations from Synechocystis (13). Fig. 3, A and B show the representative MALDI spectra of PSI 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 QA firmly bound to the D1 subunit, to the second plastoquinone molecule, reversibly bound to the D1 subunit, is highly sensitive to the protein environment of the QA site. Perturbation of this site is reflected in a change of the QA → QB electron transfer. Thus, various herbicide-resistant mutants, in which the QA site is modified, are impaired in QA → QB 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 QA and QB, on the acceptor side of PSII. An initial fast decay phase (a few hundred microseconds) after flash excitation reflects reoxidation of QA through electron transfer to the quinone bound at the QB site. An intermediate phase (millisecond range) derives from QA 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 QA oxidation
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occurs with a rate coefficient between $k$ and $k + dk$. This may be obtained by fitting experimental time courses with the simple power law in Equation 1,

$$N(t) = (1 + k_d t)^{-n}$$

(Eq. 1)

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

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 $\Delta H228 < MHI264 < CH269 < H-less$. 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 $Q_a$.

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: $Q_a \rightarrow Q_a$. Fig. 4C 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 $\sim 2$.

**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 to 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

$$\frac{N(t) - Q_a(t)}{Q_a(0)} = \int_0^t p(k)e^{-kt}dk$$

(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,

$$p(k) = \frac{k^{n-1}}{\Gamma(n)} \exp(-k/k_o)$$

(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.

$$N(t) = \int [p(k)] = (1 + k_d t)^{-n}$$

(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 $f(k) = k \, p(k)$.

![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 insertion, and by Southern blotting, after Neo1 digestion with (B) *Synechocystis* psbH or (C) *Zea mays* psbH probes. Calculated sizes of amplified and hybridized DNA fragments are indicated.](http://www.jbc.org/)

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 insertion, and by Southern blotting, after Neo1 digestion with (B) *Synechocystis* psbH or (C) *Zea mays* psbH probes. Calculated sizes of amplified and hybridized DNA fragments are indicated.
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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 μE 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 PSI 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 PSIII content, cell counting indicates that the mutated strains assemble the same amount of PSI 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. 5A. 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. 5B). 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 (~9000 μE 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
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 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. 6B). The presence of other proteins of the PSII core was also examined: the D2 protein and the \( \alpha \)-subunit of the cytochrome b\(_{559} \) were immunodetected, but no trace of the inner antenna CP47 was found (Fig. 6C). 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 \( \Delta H228, CH269, \) 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.

### Table II

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

| Strain   | \(-\)DCMU | + DCMU |
|----------|------------|--------|
|          | n  | \( k_0 \) | n  | \( k_0 \) |
| SH233    | 0.63 | 13.1 | 1.46 | 2.52 |
| \( \Delta H228 \) | 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   | DCMU | Atrazine | Ioxynil |
|----------|------|----------|---------|
| SH233    | \( 0.12 \pm 0.01 \) | \( 0.01 \) | \( 1.35 \pm 0.19 \) |
| \( \Delta H228 \) | \( 0.08 \pm 0.00 \) | \( 0.75 \pm 0.15 \) | \( 1.35 \pm 0.05 \) |
| MH264    | \( 0.09 \pm 0.01 \) | \( 0.74 \pm 0.08 \) | \( 0.88 \pm 0.37 \) |
| CH269    | \( 0.07 \pm 0.01 \) | \( 0.48 \pm 0.12 \) | \( 0.55 \pm 0.09 \) |
| H-less   | \( 0.08 \pm 0.02 \) | \( 0.63 \pm 0.09 \) | \( 0.24 \pm 0.05 \) |
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.

Fluorescence analysis of QA 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 QA 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 QA 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 para-

**TABLE IV**

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

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

![Fig. 5](http://www.jbc.org/)

**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 µg/lane for control mutant SH233; 7.5 µg/lane for mutants SH264, ΔH228 and CH269; and 15 µg/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).
The situation is different in the absence of DCMU. In this case, reoxidation of Q_\text{A} can proceed through two different pathways, i.e. electron transfer to Q_\text{B} (or Q_\text{M}), 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_\text{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_\text{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_\text{B}, indicating extensive perturbation of the Q_\text{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_\text{A} to Q_\text{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_\text{B}; (iii) deletion of the PsbH subunit or introduction of an extra N-terminal extension strongly affects the transfer rate, increasing the non-Q_\text{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_\text{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 PSI 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_\text{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_\text{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 MIH264 (37, 38). In these strains in fact, during prolonged photo-inhibition 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-
sis (5, 39, 40), perhaps because of limiting amounts of the specific protease (Var2-PsH 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 Qb 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. 5A). 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.

![Fig. 7. Spectra of pigments extracted from control and photoinhibited cell cultures.](http://www.jbc.org/Downloaded from)

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

It is noteworthy that the strain ΔH228, which contains a shortened version of maize PsbH (transmembrane portion), is less perturbed, in the QA to QB electron transfer, than 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 (7). PsbH plays at least three important functions, separately

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Elisabetta Bergantino, Alessia Brunetta, Eleftherios Touloupakis, Anna Segalla, Ildikò Szabò and Giorgio Mario Giacometti

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