Absence of the psbH gene product destabilizes photosystem II complex and bicarbonate binding on its acceptor side in Synechocystis PCC 6803

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The PsbH protein, a small subunit of the photosystem II complex (PSII), was identified as a 6-kDa protein band in the PSII core and subcore (CP47–D1–D2–cyt b-559) from the wild-type strain of the cyanobacterium Synechocystis PCC 6803. The protein was missing in the D1–D2–cytochrome b-559 complex and also in all PSII complexes isolated from IC7, a mutant lacking the psbH gene. The following properties of PSII in the mutant contrasted with those in wild-type: (a) CP47 was released during non-denaturing electrophoresis of the PSII core isolated from IC7; (b) depletion of CO2 resulted in a reversible decrease of the QA reoxidation rate in the IC7 cells; (c) light-induced decrease in PSII activity, measured as 2,5-dimethyl-benzoquinone-supported Hill reaction, was strongly dependent on the HCO3− concentration in the IC7 cells; and (d) illumination of the IC7 cells lead to an extensive oxidation, fragmentation and cross-linking of the D1 protein. We did not find any evidence for phosphorylation of the PsbH protein in the wild-type strain. The results showed that in the PSII complex of Synechocystis attachment of CP47 to the D1–D2 heterodimer appears weakened and binding of bicarbonate on the PSII acceptor side is destabilized in the absence of the PsbH protein.

Keywords: cyanobacteria; D1 protein; photosystem II; psbH gene; Synechocystis PCC 6803.

The core of photosystem II (PSII) complex of higher plants, algae and cyanobacteria consists of large central subunits D1, D2, CP47, CP43 and a number of low molecular mass proteins. It is believed that with an exception of cytochrome b-559, the small proteins do not participate directly in the transfer of electrons within PSII but they are important for the optimization of electron transfer processes and for the proper assembly of the complex (reviewed in [1]).

Two different strategies are used to get information about the role of small PSII subunits. One approach is based on the functional comparison of the intact and detergent treated PSII complex missing a specific subunit. Resumption of the particular function after reconstitution of the complex with this subunit is considered as evidence for its role. For example, using this approach the role of the PsbL subunit in the QA binding has been proposed [2]. The second, more frequent approach is based on the deletion of the gene encoding a studied protein followed by a detailed characterization of PSII complex in the resulting mutant. This strategy has been very often successful in cyanobacteria, namely in the strain Synechocystis PCC 6803, which can grow photoheterotrophically and is easily transformable. In this way, mutants of Synechocystis with deleted psbK, psbH, psbI [3–5] and other genes were constructed. These mutants contained assembled PSII complexes and after their functional characterization possible functions were ascribed to these subunits. Interestingly, in algae and higher plants this approach is useful only rarely as deletions of PSII subunits usually lead to disappearance of the whole PSII complex from thylakoids [6–8].

The PsbH protein, a product of the psbH gene, was initially found as the 10-kDa phosphoprotein in thylakoids of higher plants by Bennett [9]. From that time its homologues have been found in more than 15 photosynthetic organisms including cyanobacteria. The first partial sequence of the cyanobacterial PsbH protein was obtained in the thermophilic cyanobacterium Synechococcus vulcanus [10], but the complete gene was sequenced in the strain Synechocystis PCC 6803 by Abdel-Mawgood & Dilley [11] and Mayes & Barber [12]. Construction of the Synechocystis psbH-less mutant and its characterization in vivo provided the first more solid basis for the elucidation of the role of the protein in PSII [4]. The mutant was more sensitive to photoinhibition in comparison with the wild-type [4,13] and this sensitivity has been mostly attributed to perturbations in the electron flow between QA and QB on the acceptor side of PSII. In the present paper we have conducted a more detailed analysis of the effects of the PsbH absence on the structure and function of PSII both in vivo and in vitro. Our results indicated a stabilizing role of the protein for CP47 binding to the D1–D2 heterodimer and showed its importance for bicarbonate binding and preventing oxidative stress in PSII.
MATERIALS AND METHODS

Strains and growth of organisms

The glucose tolerant strain *Synechocystis PCC 6803* [14], referred to as wild-type (WT), and its *psbH* deletion mutant IC7 [4] were grown in BG-11 medium with (photomixotrophic growth) or without (photoautotrophic growth) glucose (10 mM final concentration). The plate medium contained BG-11, 10 mM Tris/NaOH, pH 8.2, 1.5% agar and 0.3% sodium thiosulphate [15] and in the case of the IC7 mutant also kanamycin (25 μg·mL⁻¹) and atrazine (5·10⁻⁶ M) were added. Liquid cultures (100–200 mL) in conical flasks were aerated using an orbital shaker, irradiated with 50–70 μmol photons·m⁻²·s⁻¹ of white light at 29 °C and diluted every day to maintain the chlorophyll concentration at ≈ 8 μg·mL⁻¹. Cultures of *Chlorella sorokiniana*, *Scenedesmus quadricauda* and *Chlamydomonas reinhardtii* were grown under the same conditions and their density was maintained at D₅₇₃ = ≈ 1.

Photoinhibitory treatment of the *Synechocystis* cultures was performed at a chlorophyll concentration of 6 μg·mL⁻¹ in 18-mm thick plate-parallel cuvettes placed in a temperature controlled bath. Cultures were bubbled with air containing 2% CO₂ (CO₂-enriched air), with air bubbled through 40% NaOH (CO₂-depleted air) or with pure nitrogen. In some experiments, the cell suspension was supplemented with 10 mM NaHCO₃. The light source was a 500-W tungsten filament bulb mounted in an aluminium reflector. In the experiments with a protein-synthesis inhibitor lincomycin (Sigma, USA, 100 μg·mL⁻¹ final concentration) the culture was incubated for 10 min in the dark before the start of light treatment.

Phosphorylation of membrane proteins in algal and *Synechocystis* strains was induced in the cell suspensions diluted to D₅₇₃ = 0.2. They were exposed to 250 μmol photons·m⁻²·s⁻¹ of white light for 30 min either in the absence or in the presence of 3 μM LiCl, 0.01 M Pi, 0.01 M PO₄. Thylakoids isolated from the cells were analysed by SDS/PAGE and Western blotting using rabbit polyclonal antiphosphothreonine antibody (Zymed, USA) or by autoradiography.

Preparation of membranes and PSII complexes and their trypsinization

Cyanobacterial membranes were prepared by breaking the cells with glass beads (150–200 μm in diameter) at 4 °C followed by differential centrifugation. For small scale preparation, the cells (approx. 150 μg of chlorophyll) were washed and resuspended in 150 μL of 25 mM Tris/HCl buffer, pH 7.5 containing 1 mM aminocaproic acid. The beads were added to the suspension and the mixture was vortexed twice for 1 min with 2 min interruption for cooling on ice. Beads were then washed four times with 200 μL of buffer. Aliquots were pooled and centrifuged at 3000 g for 1 min to remove unbroken cells. Membranes were collected from the supernatant at 20 000 g for 10 min. The final sediment was resuspended in 25 mM Tris/HCl buffer, pH 6.8 containing 1 M sucrose (final chlorophyll concentration 400–600 μg·mL⁻¹) and stored at −75 °C. Large scale preparation of membranes for isolation of PSII was performed according to Tang & Diner [16] using a beadbeater (Biospec Products, USA) for breaking the cells. Isolation of PSII complexes from the wild-type and mutant thylakoids was conducted according to the modified procedure of Ritter et al. [17]. Briefly, membranes were spun down, resuspended in 25 mM Mes/NaOH, pH 6.5 and solubilized with dodecylmaltoside (DM)/chlorophyll = 20, w/w) for 15 min. Unsolubilized material was removed by centrifugation (40 000 g, 15 min). The supernatant was applied on the column of chelating Sepharose (Amershams Pharmacia, Sweden) with bound Cu²⁺ ions and imidazole equilibrated with two column volumes of 25 mM Mes/NaOH, pH 6.5 containing 200 mM NaCl and 0.03% DM. PSII and carotenoid fraction did not bind to the column and went through directly into the second column of Q Sepharose (Amershams Pharmacia, Sweden). This was washed with several volumes of 25 mM Mes/NaOH, pH 6.5 containing 200 mM NaCl and 0.03% DM. During this step, carotenoids and remaining small amounts of phycocyanobiliproteins and PSI were removed from the column. Finally, the PSII core complex was eluted from the column by 25 mM Mes/NaOH, pH 6.5 containing 250 mM NaCl and 0.03% DM. The preparation was concentrated in Centricon 30 spin columns (Millipore, USA).

Trypsinization of membranes was performed at chlorophyll concentration 200 μg·mL⁻¹ and trypsin concentration 50 μg·mL⁻¹ (Serva, Germany). After 5, 15 and 30 min incubation at 25 °C, aliquots were withdrawn and proteolysis was stopped by transfer to ice and addition of 2 μM Pefabloc SC (Merck, Germany).

Analysis of proteins

Isolated PSII complexes or membranes solubilized with DM (DM/chlorophyll = 20, w/w) were analysed by nondenaturing electrophoresis at 4 °C in 5–10% polyacrylamide gel according to Laemmli [18] except that the electrophoretic buffers contained 12.5 mM Tris, 98 mM glycine and 0.1% Deriphat 160, and the gel contained 0.1 M Tris/HCl, pH 8.8 without detergent.

Protein composition of membranes and pigment protein complexes obtained by Deriphat electrophoresis was assessed by electrophoresis in a denaturing 12–20% linear gradient polyacrylamide gel containing 7 M urea [18]. The membranes were solubilized in 25 mM Tris/HCl, pH 6.8, containing 2% SDS (w/v) and 2% dithiothreitol (w/v) at laboratory temperature for 60 min. Samples were loaded with equal amount of chlorophyll as indicated in figure legends. Analysis of pigment proteins was performed either by re-electrophoresis of individual pigment protein bands or the whole lane from the native gel was excised and placed on the top of the SDS gel (diagonal PAGE). The gels with pigment proteins were incubated for one hour in the same solubilization solution as thylakoids prior to SDS/PAGE. Proteins separated in the gel were either stained by Coomassie Blue or transferred onto nitrocellulose membrane (0.1 μm, Schleicher-Schuell, Germany) by semidry blotting. Membrane was incubated with specific antibodies and then with alkaline phosphatase conjugated secondary antibody (Sigma). Proteins were visualized by colorimetric reaction using BCPIP-NBT system. Antibodies used in the study were raised against: (a) residues 2–17 of the *Synechocystis PCC 6803* D1 protein (D1-Nt); (b) residues 58–86 of the spinach D1 protein (D1-Mp); (c) the last 29 residues of the pea...
D1 precursor (D1-Ct); (d) the last 14 residues of the
Synechocystis D2 (D2-Ct) and (e) the isolated α subunit of the
cytochrome b-559 from Synechocystis PCC 6803 (cyt b-559). For autoradiography, the membrane with labelled proteins was exposed to X-ray film at laboratory temperature for 2 days.

Oxidation of proteins

Oxidation of the D1 protein was determined using the detection kit Oxyblot (Intergen, USA). Solubilized thylakoid membrane proteins were derivatized using dinitrophenylhydrazine, which reacts with carbonyls present on oxidized proteins. After protein separation by SDS/PAGE and transfer onto the membrane, dinitrophenyl (DNP)-proteins were detected by Western blotting using anti-DNP Ig. The whole procedure was performed according to manufacturer’s instructions.

N-terminal protein sequencing

N-terminal sequence of proteins was analysed performing eight cycles of automated Edman degradations using Protein sequencer LF3600D (Beckman, USA) and program 2–39 according to manufacturer’s instructions. Amino-acid sequence was called from the comparisons of chromatograms. Protein in the gel was blotted onto poly(vinylidene difluoride) membrane, prewetted with acetonitrile, and then deblocked by treatment with 0.6 M HCl for 20 h at 25 °C. HCl was then evaporated and the membrane was inserted into cartridge of the sequencer.

Measurement of oxygen evolution

Light-saturated steady-state rates of oxygen evolution (Hill reaction activity, HRA) in cell suspensions were measured at 30 °C using a temperature controlled chamber [19] equipped with a Clark-type electrode (YSI, USA). Artificial electron acceptors 2,5-dimethyl-p-benzoquinone (DMBQ) or 2,6-dichloro-p-benzoquinone (DCBQ) (0.5 mm final concentration each) were added 1 min before the measuring illumination (3500 μmol photons m⁻² s⁻¹, 30 s) was switched on.

Chlorophyll fluorescence measurement

The rate of \( Q_{\text{r}} \) reoxidation was measured with the P.S.I. double-modulated fluorimeter FL-100 (P.S.I., Czech republic). Short, nonactivating pulses of blue light were used as the measuring light and \( F_{\text{Mr}} \) reflecting fully reduced \( Q_{\text{A}} \) was elicited by the strong saturating red flash. Cells were incubated for 5 min in the dark before measurements.

Pigment analyses

For the routine measurements of chlorophyll concentration, the cells were collected by centrifugation and extracted with 100% methanol. The concentration of chlorophyll was calculated from the absorbance values of the extract at 666 and 720 nm according to Wellburn and Lichtenthaler [20]. Detailed analysis of pigments was performed by HPLC (Beckman, USA) using procedure of Gilmore and Yamamoto [21].

RESULTS

Identification of the PsbH protein in photosystem II complexes of Synechocystis

PSII core complexes from wild-type and IC7 strains of Synechocystis were isolated by a combination of metal affinity and ionex chromatography. Absorption spectra of preparations from each strain exhibited similar absorption maxima at 673 nm, typical for PSII complex from this cyanobacterial species [16]. The preparations were then subjected to the nondenaturing electrophoresis in the presence of Deriphat 160 (Fig. 1). In the case of wild-type, we obtained two prominent green bands (Fig. 1A). The first band was ascribed to the monomeric PSII core consisting of CP47, CP43, D2, D1, both cytochrome b-559 subunits, a 6-kDa protein and other smaller proteins (Fig. 1B, WT: A). The second band represented PSII core lacking CP43 (PSII subcore, Fig. 1B, WT: B). There were also two low molecular mass pigment-containing bands that were ascribed to free CP43 based on its protein composition (Fig. 1B, WT: D) and free carotenoids (Fig. 1A, FP) based on its absorption spectrum (data not shown). Similar electrophoretic pattern of the pigment proteins was obtained from the IC7 strain with the exception that: (a) the band of the PSII subcore was much weaker than in wild-type (b) there was an additional band identified as the D1–D2–cyt b-559 complex (PSII RC) (Fig. 1B, IC7: C), and (c) the lower green band contained both CP47 and CP43 (Fig. 1B, IC7: D). The results suggest that during electrophoresis the PSII subcore from IC7 became unstable and decomposed into CP47 and PSII RC complex.

Comparison of the protein composition of the PSII cores and subcores (Fig. 1B) from both strains showed that there was a protein with \( M_r \) of ≈ 6 kDa in the complexes from wild-type that was absent in IC7. The band was subjected to the automated Edman degradation. The obtained sequence DILRPLNS corresponding to the internal sequence 8–15 of the PsbH protein from Synechocystis PCC 6803 (SWISS-PROT accession number P14835) confirmed the identity of the protein.

Analysis of protein composition of PSII complexes from wild-type revealed that PsbH protein was present in the core as well as in the subcore complex lacking CP43. Evaluation of its presence in PSIIIRC was allowed by the treatment of the wild-type preparation with SDS in the ratio SDS/chlorophyll = 10. Deriphat PAGE of this preparation led to the generation of PSIIIRC that was devoid of the PsbH protein (Fig. 2). It means that this subunit was released from PSII subcore together with CP47, again suggesting a close structural relationship between PsbH and CP47.

Effect of the PsbH absence on the accessibility of the D1 protein to trypsin

The effect of the PsbH absence on the structure of the PSII core complex was further probed by trypsinization of the D1 protein in isolated membranes of wild-type and IC7 (Fig. 3). The initial trypsin-induced cut of the D1 protein occurred at the N-terminus and was documented by a small increase of the electrophoretic mobility and by a loss of reactivity with the D1-Nt Ig (data not shown). As in Synechococcus PCC 7942, this cutting occurred concomi-
D1-Nt represented the D1 subfragment between residues R8 and K238. However, in the IC7 mutant, a 16-kDa Nt2 fragment reacting with D1-Mp Ig also appeared. As judged from the amino-acid sequence of the protein, the Nt2 fragment originated from the cut at R225. In summary, trypsinization of thylakoids showed that in the absence of the PsbH protein the accessibility of the D1 protein to trypsin was changed and also mutual position between D1 and D2 was modified as indicated by inhibition of the D1–D2 adduct formation in the IC7 strain.

The PsbH protein affects the bicarbonate binding on the acceptor side of PSII

A characteristic feature of PSII in the IC7 strain is a slow electron transfer between QA and QB [4]. This was demonstrated in the Fig. 4 (compare solid lines in the left and right panels). Additional retardation of the electron transfer could be induced by removal of CO2 from the medium during dark incubation of the mutant cells. This retardation was fully reversed after subsequent addition of bicarbonate and/or bubbling with the CO2-enriched air (Fig. 4, right panel). In contrast, removal of CO2 and its subsequent addition did not affect the \( Q^-_A \) reoxidation rate in the wild-type strain (Fig. 4, left panel). It indicated that the binding of bicarbonate to the PSII acceptor side was weakened in the IC7 mutant as a consequence of the missing PsbH protein. This conclusion was supported by the following experiment. We have shown previously that after exposure to high irradiance, the Hill reaction activity of the IC7 cells measured using 2,5-dimethyl-benzoquinone as an artificial electron acceptor (DMBQ-HRA) was very quickly inhibited. In contrast, the decline of activity measured using 2,6-dichloro-benzoquinone (DCBQ-HRA), was much slower [13]. We found that this difference was further enhanced when the illuminated IC7 cells were bubbled with the CO2-depleted air (Fig. 5, closed symbols). However, when the suspension was supplemented with 5 mM bicarbonate and bubbled with CO2-enriched air, the decline of DMBQ- and DCBQ-HRA was parallel (Fig. 5, open symbols). The rate of DMBQ- and DCBQ-HRA decline in the wild-type cells was not dependent on the CO2 and/or bicarbonate concentration (not shown, see [13]).

The D1 protein is extensively photooxidized in the mutant

It was shown previously that the turnover of the D1 protein in the illuminated IC7 cells is retarded and also the recovery from photoinhibition is slow as compared with the wild-type cells [13]. Possible explanation for this feature of the IC7 strain could be an increased formation of reactive oxygen species (ROS) in PSII that may inhibit the D1 replacement process [23]. The first supporting evidence for this came from the analysis of pigment content in the autotrophically grown wild-type and IC7. We assumed that increased formation of ROS could lead to increased content of cellular carotenoid content, as these pigments are able to eliminate to some extent the ROS effect. HPLC analysis revealed almost four times higher ratio of myxoxanthophyll/chlorophyll in the IC7 cells as compared to the wild-type cells (Table 1). The increase in content of other carotenoids was not as significant. Increased generation of
ROS in the PSII complex of IC7 was further supported by the results of the D1 analysis in cells exposed to high irradiance. The Western blot showed, in addition to the typical 32-kDa D1 band, formation of a 40-kDa band that also reacted with the antibody raised against the α subunit of cytochrome b-559 (Fig. 6). Although this band was present even in control cells, high irradiance induced formation of an additional, slightly smaller D1-cytochrome b-559-reactive band. We propose that this band was identical to that found by Barbato et al. [24] in illuminated plant thylakoids which seems to be induced by the action of ROS [25]. Effect of high irradiance was further accompanied by decreased intensity of the original 32-kDa band and in the case of IC7 mobility of the remaining protein was decreased in an oxygen-dependent manner. Such a shift often reflects protein oxidation [26] and this was confirmed by Oxyblot, a commercially available kit developed to detect oxidized proteins. Indeed, after light treatment of IC7 cells the D1 protein with lower mobility exhibited significant oxidation that was partially inhibited in the cells bubbled with nitrogen during illumination. In addition, a 23-kDa N-terminal D1 fragment was detected in the IC7 cells and its mobility was also shifted by high irradiance in the presence of oxygen. As showed by Miyao [25], also fragmentation of the D1 protein may be induced by ROS. Taken together, the above results provide strong experimental support for enhanced generation of ROS in the PSII complex lacking the PsbH protein.

The PsbH of *Synechocystis* is not phosphorylated *in vivo*

The PsbH protein has originally been identified in higher plants due to its phosphorylation in light [9]. This phosphorylation also exists in green algae and two N-terminal threonine residues seem to be phosphorylated in these organisms [27,28]. However, the question concerning phosphorylation of the cyanobacterial PsbH remains still open. There is a single report documenting *in vitro* phosphorylation of PsbH in *Synechocystis* by Race & Gounaris [29]. However, in this report identification of the phosphorylated band as the PsbH protein was ambiguous as in thylakoids there is a dozen of polypeptides below...
10 kDa. In addition, the cyanobacterial PsbH lacks the N-terminal sequence with threonine 2 and 4 residues phosphorylated in plants and algae [30,31]. To clarify this point, we attempted to identify phosphorylation of the PsbH in vivo using antiphosphothreonine Ig that proved to react well with PSII phosphoproteins in plant chloroplasts [32]. For comparison, we also analysed PsbH phosphorylation in green algae Scenedesmus quadricauda, Chlorella sorokiniana and Chlamydomonas reinhardtii. A single 6.5-kDa band in Scenedesmus and two closely migrating 8 and 9-kDa bands in Chlorella and Chlamydomonas could be detected in the PSII cores (Fig. 7A). In contrast, absolutely no reaction of cyanobacterial proteins with the antibody in membranes and PSII core complexes of both wild-type and IC7 suggested that the threonine phosphorylation does not occur in thylakoids of Synechocystis. In order to extend this conclusion for phosphorylation of other residues, the cells of Synechocystis were labelled with $^{33}$P-H$_3$PO$_4$. We found weak phosphorylation of two bands with $M_r$ values of 3 and 4 kDa clearly distinct from the PsbH protein (Fig. 7B). In addition, these two bands were present in both wild-type and the IC7 mutant. In summary, we did not obtain any experimental evidence for the phosphorylation of the PsbH protein in Synechocystis cells.

**DISCUSSION**

Packham [33] proposed that the PsbH protein of photosystem II is a functional homologue of the H subunit from the reaction centre of photosynthetic bacteria. This is in line with the effect of the protein on the herbicide binding in PSII [34] and also in line with the data in this paper. The recent 3.8 Å model of PSII [35] tentatively situated the membrane helix of PsbH on the side of CP47 and D2 in the proximity of the QA–QB region. This position provides a good justification for the stabilizing effect of PsbH on the binding of CP47 to the heterodimer D1–D2 as well as on the bicarbonate binding to the acceptor side of PSII. Nevertheless, there is also recent report showing the PsbH protein in Chlamydomonas on the periphery of the PSII dimeric core [36]. However, detection of the protein was based on the attachment of gold particles to His-tagged N-terminus that can be positioned at the different region of the core than the membrane helix.

In Synechocystis, the PsbH protein was found not only in the PSII core but also in the subcore (CP47–D1–D2–cyt)

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Table 1. Carotenoid composition in cells of wild-type and IC7 strains grown in the absence of glucose. Numbers represent the percentage of the total carotenoids, numbers in parenthesis represent the percentage of the particular carotenoids taking content per chlorophyll unit in wild-type cells as 100%.

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Wild-type cells</th>
<th>Zeaxanthin</th>
<th>Echinon</th>
<th>β-caroten</th>
</tr>
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<tbody>
<tr>
<td>Myxoxanthophyll</td>
<td>22 (100)</td>
<td>35 (100)</td>
<td>19 (100)</td>
<td>23 (100)</td>
</tr>
<tr>
<td>IC7 cells</td>
<td>47 (347)</td>
<td>23 (107)</td>
<td>13.5 (115)</td>
<td>16 (115)</td>
</tr>
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complex. In contrast, the PsbH protein was not detected in the subcore isolated from spinach [37]. The reason for this discrepancy is unclear, but it could be related to the difference either between the species or between the methods of the subcore preparation.

The instability of the IC7 subcore during electrophoresis of the isolated PSII core can be relevant to the situation in vivo during the PSII core assembly. Weak binding of CP47 to the D1–D2 heterodimer may destabilize these subunits to the extent that they are degraded before the whole complex can be assembled. This proteolysis seems to be less efficient in cyanobacteria than that in algae and therefore the assembly of PSII complexes occurs in the psbH-deletion mutant of Synechocystis, but not in the similar mutant of Chlamydomonas [6,7]. On the other hand, the PsbH protein may also represent an important factor regulating process of PSII repair. Its removal from PSII could result in a complete disassembly of PSII during the D1 replacement while in its presence the D1 replacement could proceed in the subcore complex as suggested by Zhang et al. [38].

We have identified formation of the D1–cytochrome b-559 adduct and the D1 fragments together with the apparent oxidation of the D1 protein in the cells of IC7. This shows that the impaired function of PSII in IC7 leads to increased probability of the formation of ROS. These species oxidize the D1 protein which can be subsequently cross-linked with the α subunit of cytochrome b-559, or even fragmented. However, ROS may also attack other PSII subunits as well as protein synthesis apparatus and then the recovery from photoinhibition is slow as observed in IC7 [13]. Oxidative damage was also implicated in the slow restoration of PSII activity after photoinhibition of Synechocystis [39] and Synechococcus elongatus cells [23]. We were not able to accelerate recovery from photoinhibition in IC7 by bubbling the cell suspension with nitrogen during high irradiance treatment. Nevertheless, this does not negate our hypothesis as even under these conditions, oxidation of the D1 protein still occurred although to a lesser extent (Fig. 6).

Importance of the PsbH protein for the proper functioning of the PSII complex in higher plants and algae is closely related to phosphorylation of its threonine residues on the N-terminus. However, in Synechocystis we did not find any evidence for the phosphorylation of this protein. Looking at the N-terminal sequences of PsbH in organisms containing phycobilisomes attached to the stromal side of the membrane (e.g. Synechocystis, Synechococcus, Porphyra and Cyanidium), it is apparent that they contain the PsbH protein with shorter N-terminal part without the phosphorylatable threonines. As the common feature of these organisms is the absence of grana, it is possible that the
phosphorylation of PsbH is important for the function of PSII in the Se appressed regions of the membrane. In line with this, Giardi et al. [40] showed that after PsbH dephosphorylation by alkaline phosphatase an extremely fast inactivation of the PSII activity occurred in isolated spinach membranes while the phosphatase had no effect on the activity of the cyanobacterial membranes.

Xiong et al. [41] postulated a hypothesis suggesting that arginine residues of the D1 protein (especially Arg257) stabilize binding of bicarbonate on the PSII acceptor side. However, similar role could be fulfilled by arginines of the PsbH protein as suggested by Sundby et al. [42]. They found that phosphorylation of the PsbH protein is indirectly proportional to the binding of bicarbonate on the acceptor side of PSII. Based on this correlation they proposed that the basic residues on the stromal side of the PsbH protein are involved in the bicarbonate binding. From this point of view it is interesting that our results indicated destabilization of the bicarbonate binding in PSII as a consequence of the missing PsbH protein. However, it is not clear if the protein binds bicarbonate directly or whether it has long-distance effect on the conformation of D1 and/or D2 that is important for the binding of this anion. It is worth to note that fast light-induced inactivation of DMBQ-HRA, which most probably reflects release of bicarbonate, has been also found in the PEST-deletion mutant of Synechocystis by Nixon et al. [43]. It may indicate that the PEST sequence of the D1 protein is in close contact with PsbH and also contributes to the formation of the bicarbonate binding site. In line with this hypothesis our trypsinization experiment showed that in the absence of the PsbH protein the PEST region of the D1 protein was more exposed to stroma. The fact that the release of bicarbonate completely inhibited the DMBQ-HRA but only slowed down the DCBQ-HRA suggests that DCBQ may accept electrons before the bicarbonate binding site. Therefore, it is tempting to speculate that the difference between the active (Qₐ-reducing) and inactive (Qₐ-nonreducing) PSII centres, having distinct affinity to DMBQ and DCBQ [44], is given by the occupancy of the bicarbonate binding site and/or the state of the PsbH protein (e.g. phosphorylation) that affects the bicarbonate binding.

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