Synechocystis 6803 mutants expressing distinct forms of the Photosystem II D1 protein from Synechococcus 7942: relationship between the psbA coding region and sensitivity to visible and UV-B radiation

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Abstract

Synechocystis PCC 6803 mutants expressing either the “low light” (D1:1) or the “high light” (D1:2) form of the Photosystem II (PSII) D1 protein from Synechococcus PCC 7942 were constructed and characterized with respect to properties of PSII and sensitivity to visible and UV-B radiation. The AI and AIII mutants (containing only the D1:1 and D1:2 forms, respectively) exhibited very similar PSII characteristics as the control strain and they differed only in the accelerated decay kinetics of flash-induced variable fluorescence measured in the presence of DCMU. However, the mutants showed increased sensitivity to photodamage induced by visible and UV-B radiation, with higher loss of PSII activity in the AI than in the AIII strain. Thus, the difference between strains containing D1:1 and D1:2 found previously in Synechococcus 7942 is maintained after transfer of corresponding psbA genes into Synechocystis 6803 and is directly related to the coding region of these genes. The higher light sensitivity of the AI mutant is caused partly by the higher rate of photodamage and partly by the less efficient PSII repair.

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1. Introduction

The Photosystem II (PSII) D1 protein in cyanobacteria is usually encoded by the psbA multigene family (for review see Ref. [1]). The cyanobacterium Synechocystis PCC 6803 contains two functional genes psbA2 and psbA3 encoding proteins with the same amino acid sequence [2] while in Synechococcus PCC 7942 there are three genes, psbAI, psbAII and psbAIII, encoding two different forms of the D1 protein [3]. As a response to increased irradiance, the “low light” D1:1 form, which is encoded by psbAI, is depleted from the cells and it is replaced by the “high light” D1:2 form encoded by psbAII and psbAIII [4,5]. On the other hand, when the high-light-treated cells are transferred back to low irradiance, D1:2 is quickly replaced by D1:1 [5].

The interchange of two D1 forms in Synechococcus is a complex process regulated at both transcriptional and translational levels. High irradiance [6], low temperature [7], oxygen removal [8] or blue light [9] causes rapid loss of the psbAI transcript while transcription of psbAII/III genes is strongly induced. Extensive accumulation of the psbAII/III transcripts occurs also after exposure to UV-B light. Although this effect is not accompanied by psbAII transcript depletion, the D1:1 isoform is exchanged with D1:2 [10]. During the response to high irradiance, the stability of the psbAI and psbAIII transcripts is significantly decreased due to a newly synthesized protein factor while the stability of the psbAII remains unchanged [11]. Transcriptional induction of psbAII is achieved by the binding of high-light-
induced proteins to the DNA segment upstream of the gene [12]. Studies using photosynthetic inhibitors and disulfide reducing agents showed that thiol-disulfide exchange reactions, driven by electrons from Photosystem I, are involved in the transcriptional control of psbA genes [13]. Regulation of translation ensures that only D1:2 can be detected in thylakoids even after prolonged exposure to high light although there are comparable amounts of both psbAI and psbAII/III transcripts accumulated [14,15]. Similarly, in anoxia or in the presence of DCMU and dithiothreitol, there is no appearance of D1:2 in thylakoids despite the presence of psbAII/III transcript [8,13].

The D1 protein exhibits fast, light-dependent turnover that is usually related to the repair of photoactivated PSII complexes [16]. Both D1 forms in Synechococcus PCC 7942 exhibit a similar rate of turnover under low light conditions [17]. However, our recent study showed that under increased irradiance, turnover of D1:1 cannot match the rate of PSII photoinactivation (PI) and this seems to be a primary reason for its replacement by D1:2, the D1 form exhibiting more efficient PSII repair in high light [18]. In agreement with this, a strain over expressing D1:2 is less susceptible to photoinhibition under high-light conditions [19], as well as under UV-B radiation [10]. The replacement of D1:1 by D1:2 seems to be triggered by PSII PI, while the reverse process in low light occurs in intact PSII complexes, suggesting that it is a regulatory process independent of PSII PI [20].

In previous studies the differences in the coding region of psbAI and psbAII/III genes were assigned to different intrinsic sensitivity of PSII complexes containing D1:1 or D1:2 to photodamage [21]. On the other hand, transcriptional regulation mostly dependent on noncoding regions was proposed to determine the distinct capability of each D1 form to repair PSII complexes under high irradiance and UV-B [21] while the effect of differences in the coding region on the PSII repair has not been seriously considered. The aim of this study was to clarify the role of the psbAI and psbAII/III coding regions in the differential high-light and UV-B sensitivity of PSII. Therefore, Synechocystis PCC 6803 mutants were constructed containing either the psbAI or psbAIII gene from Synechococcus PCC 7942 under the regulation of the psbA2 promoter. In this way differences in the untranslated regulatory regions of both psbA genes were eliminated. The results show that the coding regions influence light sensitivity of PSII not only at the level of photodamage, but also at the level of repair and are responsible for the distinct ability of the proteins to counteract PSII damage under increased visible or UV-B radiation.

2. Materials and methods

2.1. Culturing conditions

Cyanobacterial strains Synechocystis PCC 6803 and Synechococcus PCC 7942 were grown in BG-11 medium. On agar plates, BG-11 plus glucose contained, in addition, 10 mM Tris/HCl, pH 8.2, 1.5% agar and 0.3% sodium thiosulfate [22]. One-hundred or one-thousand-milliliter liquid cultures in conical flasks were shaken using a rotary shaker and irradiated with 50–70 μE m⁻² s⁻¹ of white light at 29 °C. The culture was diluted every day to maintain the chlorophyll concentration at about 6–8 μg ml⁻¹. The concentration of chlorophyll was assayed in 100% methanol [23].

2.2. Mutant construction

The Synechocystis 6803 psbA triple deletion strain has been constructed by transformation of the psbA1/psbA3 double deletion mutant carrying spectinomycin/chloramphenicol cassettes (gift from Prof. Vermaas) with chromosomal DNA isolated from the psbAII-KS strain, where the whole psbA2 gene is replaced by kanamycin-resistance cartridge [24]. Segregation of the resulting non-autotrophic psbA deletion strain was checked by PCR (not shown). psbAI and psbAII genes from the Synechococcus 7942 coding for the D1:1 and D1:2 proteins and psbA2 gene from Synechocystis 6803 were amplified by PCR using the mix of Taq and Pfu DNA polymerases and gene-specific primers with artificially generated restriction sites for NdeI and BamHI. After restriction, the PCR fragments were cloned into NdeI and BamHI sites of the pSBA2 plasmid [24] containing the upstream and downstream regions of the Synechocystis 6803 psbA2 gene. Resulting plasmids were then used to transform the psbA deletion strain. Autotrophic transformants were selected on agar plates. Sequencing of the psbA2 region confirmed that in all strains the particular gene has been correctly inserted under the control of the Synechocystis 6803 psbA2 promoter.

2.3. Steady-state oxygen evolution

Light-saturated (3500 μE m⁻² s⁻¹) steady-state rate of oxygen evolution in cell suspensions was measured at 29 °C using a temperature-controlled chamber [25] equipped with a Clark-type electrode (YSI, USA). For measurement of photosynthesis, 10 mM sodium bicarbonate was added to the suspension; for measurement of Hill reaction activity (HRA) artificial electron acceptors p-benzoquinone (0.5 mM final concentration) and potassium ferricyanide (1 mM final concentration) were added just prior to measurement.

2.4. Flash-induced oxygen evolution

Flash-induced oscillations of oxygen evolution from cyanobacterial cells were detected by using a home-built amplifier connected to a computer. Cells at concentration of 40 μg Chl ml⁻¹ were dark-adapted for 3 min and then subjected to a sequence of 20 exciting flashes of frequency 1 Hz as described previously [26].
2.5. Variable fluorescence

The initial \( F_0 \), maximum \( F_M \) and variable \( F_V = F_M - F_0 \) components of fluorescence were measured using a modulation PAM101 fluorometer (Walz, Germany) with an ED-101US cuvette as described in Ref. [27].

Effective absorption cross section was determined from the shape of the fluorescence induction curve of the sequential QA reduction. After 5-min dark adaptation the cells were exposed to a train of 15-\( \mu \)s subsaturating flashes 500 \( \mu \)s apart supplied by red (650 nm) LEDs in the presence of 10 \( \mu \)M DCMU. The fluorescence induction curve was fitted by cumulative one-hit Poisson function \( F = F_M - (F_M - F_0) \exp (-\sigma t) \) where \( F \) is fluorescence reflecting fully reduced QA was elicited by the strong saturating red flash. The measurements were performed in the 150 \( \mu \)s–100 s time range.

### 2.6. Decay of flash-induced fluorescence

The rate of QA reoxidation after single saturating flash was measured with the P.S.I. double-modulated fluorometer FL-100 (P.S.I., Czech Republic). Short, non-actinic pulses of orange light were used as the measuring light and \( F_M \) reflecting fully reduced QA was elicited by the strong saturating red flash. The measurements were performed in the 150 \( \mu \)s–100 s time range.

### 2.7. Effective absorption cross section

Effective absorption cross section was determined from the shape of the fluorescence induction curve of the sequential QA reduction. After 5-min dark adaptation the cells were exposed to a train of 15-\( \mu \)s subsaturating flashes 500 \( \mu \)s apart supplied by red (650 nm) LEDs in the presence of 10 \( \mu \)M DCMU. The fluorescence induction curve was fitted by cumulative one-hit Poisson function \( F = F_M - (F_M - F_0) \exp (-\sigma t) \) where \( F \) is fluorescence reflecting fully reduced QA was elicited by the strong saturating red flash. The measurements were performed in the 150 \( \mu \)s–100 s time range.
Table 2
The fluorescence decay parameters in the absence (A) and presence (B) of DCMU measured in the Synechocystis 6803 strains A2K, AI, AIII and in Synechococcus PCC 7942

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fast phase</th>
<th>Middle phase</th>
<th>Slow phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_1$ (%)</td>
<td>$T_1$ (ms)</td>
<td>$A_2$ (%)</td>
</tr>
<tr>
<td>A2K</td>
<td>72</td>
<td>0.29</td>
<td>20</td>
</tr>
<tr>
<td>AI</td>
<td>72</td>
<td>0.33</td>
<td>20</td>
</tr>
<tr>
<td>AIII</td>
<td>75</td>
<td>0.31</td>
<td>17</td>
</tr>
<tr>
<td>S. 7942 control</td>
<td>61</td>
<td>0.46</td>
<td>26</td>
</tr>
<tr>
<td>S. 7942 illuminated</td>
<td>57</td>
<td>0.32</td>
<td>29</td>
</tr>
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</table>

(B) + DCMU

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fast phase</th>
<th>Slow phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_1$ (%)</td>
<td>$T_1$ (ms)</td>
</tr>
<tr>
<td>A2K</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>AI</td>
<td>3</td>
<td>1.9</td>
</tr>
<tr>
<td>AIII</td>
<td>7</td>
<td>2.5</td>
</tr>
<tr>
<td>S. 7942 control</td>
<td>11</td>
<td>10.9</td>
</tr>
<tr>
<td>S. 7942 illuminated</td>
<td>19</td>
<td>8.8</td>
</tr>
</tbody>
</table>

The fluorescence traces were resolved into two exponential (fast and middle phases) and one hyperbolic component (slow phase) when measured in the absence of DCMU, and into one exponential (fast phase) and one hyperbolic component (slow phase) when measured in the presence of DCMU. $A$ indicates contribution and $T$ half-time of the particular fluorescence component.

measured after actinic flash, $n$ is number of the flash and $\sigma$ is the effective absorption cross section) from which the effective absorption cross section was calculated using SigmaPlot 5.0 software.

2.8. Light treatments

Before the experiments with high visible irradiance, the culture was adjusted to a chlorophyll concentration of 6 $\mu$g ml$^{-1}$ and placed in 18-mm-thick optical cuvettes in a temperature-controlled bath and bubbled with air containing 2% CO$_2$. Illumination was provided by tungsten filament bulbs. When the inhibitor lincomycin (LIN; 100 $\mu$g ml$^{-1}$ final concentration) was used, the cells were incubated for 5 min before the start of the light treatment. UV-B light was provided by a Vilbert-Lourmat VL-215M lamp, in combination with a 0.1-mm cellulose acetate filter yielding 6 $\mu$E m$^{-2}$ s$^{-1}$ intensity at the surface of the samples, which were suspended at 10 $\mu$g Chl/ml in a 2-cm-thick layer.

2.9. Pulse-chase experiments

The set-up for the radioactive labeling was dependent on the type of the experiment. For the study of the D1 degradation under visible light, the setup was the same as applied for the photoinhibitory treatment. A mixture of radiolabeled methionine and cysteine was added to the suspension in planparallel cuvettes (final activity 2 $\mu$Ci ml$^{-1}$) and cells were pulse-labeled at 200 $\mu$E m$^{-2}$ s$^{-1}$ for 20 min. Chase was followed after addition of cold 1.8 mM methionine and 0.2 mM cysteine in cells subjected to 1000 $\mu$E m$^{-2}$ s$^{-1}$ of UV-B radiation 6 $\mu$E m$^{-2}$ s$^{-1}$ in the background of the continuing visible light. For the assembly studies under increased visible irradiance, cells containing 75 $\mu$g of Chl were resuspended in 250 $\mu$l of BG 11 in an Eppendorf tube, shaken at 60 $\mu$E m$^{-2}$ s$^{-1}$ for 60 min and then a mixture of L-$[^{35}S]$-methionine and L-$[^{35}S]$-cysteine (>1000 Ci mmol$^{-1}$, Trans-label, ICN) was added (final activity 400 $\mu$Ci ml$^{-1}$). The suspension was exposed to 500 $\mu$E m$^{-2}$ s$^{-1}$ either at 29 or 20 $^\circ$C and, after 15 min of incubation, the cells were either frozen in liquid nitrogen (pulse) or washed twice with BG11 and exposed for additional 30 min to illumination in the presence of cold methionine and cysteine (final concentration 4 and 1 mM, respectively; chase).

2.10. Thylakoid preparation and protein analyses

Thylakoid membranes were prepared by breakage of the cells with glass beads (150–200 $\mu$m in diameter) at 4 $^\circ$C followed by differential centrifugation as described in [27]. The protein composition of thylakoids was assessed by electrophoresis in a denaturing 12–20% linear gradient...
polyacrylamide gel containing 7 M urea [28]. The thylakoids were solubilized as described in Ref. [26] and loaded with the equal amount of chlorophyll (2.5 μg). The gel was run overnight at 18 °C and proteins in the gel were transferred onto nitrocellulose membrane (0.2 μm, Schleicher-Schuel, Germany) by semi-dry blotting. Membrane was incubated with specific antibodies and then with secondary antibody–alkaline phosphatase conjugate. Proteins were visualized by colorimetric reaction using BCPIP–NBT system. The following D1-specific antibodies were used: (i) antibody raised against the first 25 amino acid residues of D1:1 from Synechococcus 7942 [5] (D1:1 antibody); (ii) antibody raised against residues 2–16 of the Synechocystis D1 also recognizing the D1:2 from Synechococcus 7942 (D1-N antibody); (iii) antibody raised against residues 58–86 of the spinach D1 (D1-M antibody). For autoradiography, the membrane was exposed to the sensitive film for 48–96 h. Assembly of the PSII complexes was assessed by diagonal electrophoresis consisting of the Blue Native PAGE (BN-PAGE) in one direction and standard SDS–PAGE described above in the second dimension. For the analysis by BN-

Table 3
Parameters of flash-induced oxygen evolution measured in the Synechocystis 6803 strains A2K, AI, AIII and in Synechococcus PCC 7942

<table>
<thead>
<tr>
<th>Strain</th>
<th>S₀ (%)</th>
<th>Miss (%)</th>
<th>D hit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2K</td>
<td>33.3</td>
<td>19.3</td>
<td>4.1</td>
</tr>
<tr>
<td>AI</td>
<td>37.6</td>
<td>24.1</td>
<td>4.8</td>
</tr>
<tr>
<td>AIII</td>
<td>37.7</td>
<td>27.1</td>
<td>4.4</td>
</tr>
<tr>
<td>S. 7942</td>
<td>100</td>
<td>28.1</td>
<td>19.0</td>
</tr>
</tbody>
</table>

*S₀* indicates proportion of PSII RC with manganese cluster in the S₀ state in the dark.

*Miss* indicates proportion of PSII RC not responding to the flash.

*D hit* indicates proportion of PSII RC responding to the flash by transfer from S₀ into Sₙ₊₂ state.

Fig. 4. Time course of the PSII photoinactivation in cells of the Synechocystis 6803 mutants subjected to high irradiance in the absence or presence of lincomycin (A) and the recovery of the PSII activity after the photoinhibitory treatment (B). (A) Cells of A2K (circles), AI (squares) and AIII (triangles) were illuminated with 500 μEm⁻²s⁻¹ of white light for 120 min in the absence (left panel) or presence (right panel) of 100 μg/ml lincomycin. Aliquots of the suspensions were taken during illumination at the times indicated and HRA was assayed in whole cells as described in Materials and methods. Values in the plot represent mean of three to five measurements. Initial values were in the range as shown in Table 1. (B) Cells were illuminated either at 1000 μEm⁻²s⁻¹ (A2K and AIII) or 750 μEm⁻²s⁻¹ (AI) for 45 min (PI), then the cells were transferred to low irradiance of 50 μEm⁻²s⁻¹ and incubated for another 120 min (REC). Aliquots of the suspensions were taken during illumination at the times indicated and HRA was assayed in whole cells as described in Materials and methods. Values in the plot represent mean of two measurements. Initial values were in the range as shown in Table 1.
PAGE, the thylakoids (8 μg of Chl) were spun down and resuspended in 30 μl of 50 mM Bis-Tris/HCl buffer, pH 7.0 containing 750 mM aminocaproic acid and 0.5 mM EDTA, then 1.6 μl of 10% DM was added and the thylakoids were spun down again. The obtained supernatant was mixed with 3 μl of loading buffer containing CBB G-250 and loaded into the 6–12% Blue-native polyacrylamide gel according to Refs. [29,30]. When the front reached about 1/3 of the resolving gel, upper buffer containing Coomassie blue G-250 was replaced by the buffer without the stain and separation continued until the front reached 2/3 of the gel. Then individual lanes were cut out and incubated for 20 min in the solubilization buffer containing 25 mM Tris/HCl, pH 6.8, 2% SDS and 1% 2-mercaptoethanol. Afterwards the lanes were placed on the top of the 12–20% linear gradient polyacrylamide gel containing 7 M urea as described above. The gels were stained with Coomassie blue R, destained, dried and then exposed to phosphorimager plate. In some cases the gel was transferred onto nitrocellulose membrane and correct identification of the radiolabeled D1, D2, CP43 and CP47 was confirmed by Western blotting using specific antibodies raised against C-terminal parts of D1, D2 and CP47 proteins and against the whole CP43 protein isolated from the gel.

![Fig. 5. Degradation of the D1 and D2 proteins in cells of the Synechocystis 6803 mutants under increased visible radiation as detected by pulse chase radiolabeling (A, C) and Western blotting (B, D). Cells were subjected to 200 μE m⁻² s⁻¹ of white light for 20 min in the presence of radiolabeled mixture of methionine and cysteine (P), then cold methionine and cysteine were added and the cells were subjected to 1000 μE m⁻² s⁻¹ of white light for 180 min (HL chase). Aliquots of the suspensions were taken during illumination at the times indicated for isolation of thylakoids. After analysis of thylakoid proteins by SDS-PAGE and transfer onto the nitrocellulose membrane, the membrane was exposed to X-ray film (A) and subsequently the D1 and D2 proteins were detected by specific antibodies (B). Autoradiograms (C) and immunoblots (D) were scanned and quantified using a SigmaGel software.](image-url)
3. Results

3.1. Overall phenotype of the mutants

The constructed strains of *Synechocystis* 6803 differ by the presence of different psbA coding regions under the control of the psbA2 promoter. Western blot using specific D1 antibodies confirmed that the strain A2 contains original *Synechocystis* D1 form while the strain AI only D1:1 and strain AIII only D1:2 form from *Synechococcus* 7942 (Fig. 1). It was also confirmed that the high-light treatment in our experiments induces the replacement of the D1:1 by D1:2 form in the *Synechococcus* cells. Measurement of the growth rate under autotrophic conditions at 50 μE m⁻² s⁻¹ showed approximate doubling time of all three mutants around 9 h. They also exhibited practically identical absorption and fluorescence spectra in vivo, indicating the same cellular content of phycobilisomes, PSII and PSI complexes.

3.2. Characterization of the PSII complex in the mutants

Testing the properties of PSII complex revealed similar rates of oxygen evolving activity measured in the presence of bicarbonate or benzoquinone, similar values of Fv/FM and effective absorption cross section (Table 1) and almost identical rates of QA reoxidation assessed by the decay of variable fluorescence after a short, saturating flash (Fig. 2, − DCMU; Table 2A). This shows that the quantum yield of the PSII photochemistry as well as the QA to QB electron transfer step are not modified by the replacement of native *Synechocystis* protein with D1:1 or D1:2 of *Synechococcus*. For comparison, we also measured the QA reoxidation kinetics in the strain *Synechococcus* 7942 and this was apparently slower compared with the *Synechocystis* 6803 strains. However, in the cells containing D1:1 as well as in high-light-treated cells with D1:2 (see Fig. 1) the reoxidation rate was almost identical.

Clear difference among the *Synechocystis* strains was found in the rate of decay of variable fluorescence in the presence of DCMU (Fig. 2, + DCMU), which reflects the recombination of the reduced QA with positive charges located on the donor side of PSII. The fluorescence decay was accelerated only slightly in the AI strain with the “low light” form D1:1 but in the strain AIII containing the “high light” form D1:2 the decay was much faster (Table 2B). Similarly as in the constructed strains, replacement of D1:1 for D1:2 by increased irradiance lead to faster QA reoxidation in the presence of DCMU also in the strain *Synechococcus* 7942 (Fig. 2).

Flash-induced oxygen evolution showed the usual pattern in the control *Synechocystis* 6803 strain with the first maximum appearing after the third flash (Fig. 3). In contrast, in the *Synechococcus* 7942 cells the first maximum appeared after the fourth flash and the sequence was highly dampened. The flash pattern in the AI and AIII mutants of *Synechocystis* 6803 was similar to that observed in the *Synechococcus* 7942 cells, i.e. showed increased dampening, as well as increased proportion of the S0 state in the dark (Table 3). The reason for unusual flash oxygen

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**Fig. 6.** Analysis of the PSII assembly in the cells of the *Synechocystis* 6803 mutants by two-dimensional Blue Native/SDS-PAGE in combination with 35S radiolabeling (A, B) and D1 immunodetection (C). (A) Cells of A2K, AI and AIII were illuminated with 500 μE m⁻² s⁻¹ of white light for 15 min in the presence of a mixture of radiolabeled methionine and cysteine at 29 °C before they were broken for isolation, 2-D analysis and autoradiography of thylakoid proteins. (B) Cells of AIII were illuminated with 500 μE m⁻² s⁻¹ of white light for 15 min in the presence of a mixture of radiolabeled methionine and cysteine at 29 °C before they were broken for isolation, 2-D analysis and autoradiography of thylakoid proteins. (C) Cells of AII were illuminated with 500 μE m⁻² s⁻¹ of white light for 15 min at 20 °C before the cells were used for isolation and 2-D analysis of thylakoids. After electrophoresis the proteins from the gel were transferred onto nitrocellulose membrane and D1 protein was detected by D1-M antibody. CC(1) and CC(2): monomeric and dimeric PSII core complex; RC47: CP47-D1-D2-cytochrome b-559 complex; *: RC47 in AI and AIII containing pD1 and iD1 instead of D1; RC: iD1(pD1)-D2-cytochrome b-559 complex; u. prot.: unassembled proteins.
sequence seen in Synechococcus 7942 is not known, but most likely related to the D1 protein sequence, which is different from that in Synechocystis 6803 and higher plants. Thus, the modified flash pattern in the AI and AIII mutants of Synechocystis 6803 should be related to the Synechococcus D1:1 and D1:2 sequences in these mutants.

3.3. Effect of increased visible radiation

Measurement of PSII activity in the constructed strains during exposure to increased visible radiation (500 μE m⁻² s⁻¹) showed that the mutants are sensitive to photoinhibition to various extent. In A2K this irradiance caused only limited decrease of the activity. This decrease was greater in the AIII cells and the AI strain was inhibited even more (Fig. 4A, − LIN). To assess if these differences were caused by faster PSII PI or by inefficient repair ability, we also measured the PSII activity during illumination in the presence of protein synthesis inhibitor lincomycin (LIN). In this case the rate of inactivation was higher in AI in comparison with AIII and especially with A2K, indicating that the amino acid sequence directly influences the rate of PSII PI (Fig. 4A, + LIN). Moreover, the small difference between the rate of PSII PI in the presence and absence of lincomycin observed in the AI strain shows that under increased irradiance the repair of PSII in AI is insufficient to counteract PSII PI. Interestingly, when the cells photoinhibited to the approximately same extent were transferred to low irradiance (50 μE m⁻² s⁻¹), restoration of the activity reached approximately the same rate in all three strains (Fig. 4B). It shows that the photoinhibition is reversible even in AI, and repair cannot match the rate of PI only in high light. This conclusion was further confirmed by the radiolabeling pulse-chase experiment in combination with immunoblotting (Fig. 5). Pulse using radioactive methionine at 200 μE m⁻² s⁻¹ led to the labeling of the D1, D2 and CP43 polypeptides that was similar in all three strains (Fig. 5A). The rate of the chase at 1000 μE m⁻² s⁻¹ was also similar in all three strains although there was slower degradation at the beginning of the light treatment in AI and AIII (Fig. 5A and C). However, the D1 degradation in AI was apparently accompanied by the degradation of the D2 protein while in the other strains the D2 protein remained almost stable. This shows that in the AI strain the process of degradation was qualitatively different. Result of blotting (Fig. 5B and D) confirmed that the steady state...
level of D1 and D2 remained constant in the A2K and AIII strains, while a significant decrease was found in AI, indicating insufficient synthesis of both proteins in this strain.

In order to reveal details in PSII assembly under increased irradiance that could be important for explanation of the repair differences among strains, we performed high-intensity protein radiolabeling with $^{35}$S-methionine and the labeled complexes were analyzed by diagonal electrophoresis consisting of Blue Native and SDS-denaturing electrophoresis. After the 15-min pulse at 500 $\mu$E m$^{-2}$ s$^{-1}$ the 2-D electrophoretic patterns showed that the labeled D1 as well as other PSII proteins were predominantly present in four protein complexes. Based on general protein staining and Western blotting, these complexes were attributed to the dimeric and monomeric PSII core complex (CC), PSII core lacking CP43 (RC47) and PSII reaction center (RC) consisting of D1, D2 and cytochrome b-559 proteins (Fig. 6A). Some D2 and CP43 proteins were also found in the region at the right edge of the gel belonging to unassembled proteins. Comparison of the labeling intensities showed lower incorporation of the label into the D1 band and almost no radioactivity in the D2 band of the PSII core complex in AI. These data suggest that the PSII repair in AI is limited by insufficient synthesis and/or PSII core integration of the D1 and D2 proteins that are needed for formation of new RCs. Interestingly, unlike the protein labeling in diluted cultures with low amount of radioactivity, during this high-intensity labeling the D1 protein could be found in two additional forms representing most probably unprocessed (pD1) and incompletely processed D1 (iD1) [31]. This assignment was confirmed by the chase of these two bands into matured D1 in AIII cells pulse-labeled at low temperature. These cells accumulate low level of matured D1 and

![Figure 8](image_url)

**Fig. 8.** Degradation of the D1 proteins in the *Synechocystis* 6803 mutant cells under UV-B radiation as detected by pulse chase radiolabeling (A) and Western blotting (B). Cells were subjected to 100 $\mu$E m$^{-2}$ s$^{-1}$ visible light for 20 min in the presence of a mixture of radiolabeled methionine and cysteine (P), then cold methionine and cysteine were added and the cells were subjected to UV-B radiation (6 $\mu$E m$^{-2}$ s$^{-1}$) in the background of the continuing visible light. Aliquots of the suspensions were taken during illumination at the times indicated for isolation of thylakoids. After analysis of thylakoid proteins by SDS-PAGE and transfer onto the nitrocellulose membrane, the membrane was exposed to X-ray film (A) and subsequently the D1 protein was detected by specific D1-M antibody (B). D1 radioactive and immunoreactive bands and (C) were scanned and quantified using a SigmaGel software.
higher level of pD1 and especially iD1, but after the transfer of the cells to growth temperature the pD1 and iD1 forms disappear with concomitant appearance of the label in the D1 band (Fig. 6B). In addition, immunodetection using D1-M antibody recognized iD1 in the RC complex of AIII (Fig. 6C). We could not detect pD1 by this antibody, most probably due to its very low steady-state level. Nevertheless, taking into account our data from chase and data from literature [31], we can ascribe this band to the pD1 protein. Only a small amount of the iD1 was present in the RC and RC47 complexes of A2K, while larger amounts of this protein and traces of pD1 were found in RC and in a small but distinct RC47 band (*) of AIII and especially of AI.

3.4. Sensitivity to the UV-B treatment

Campbell et al. [10] showed that the Synechococcus 7942 strain containing only D1:1 is not able to efficiently counteract the UV-B induced damage. In contrast, WT cells in which D1:1 was exchanged for D1:2, and the mutant which contained only D1:2 were more tolerant to UV-B. We observed the same difference in the analogous Synechocystis strains. The UV-B irradiance applied together with weak visible light induced about 70% decrease in the PSII activity in AI and 50% in AIII (Fig. 7A, – LIN). Similarly to that seen in high light, the rate of UV-B-induced damage in the presence of LIN was faster in AI then in AIII (Fig. 7A, + LIN). However, in contrast to the efficient recovery from visible light-induced damage, recovery from UV-B induced damage was very slow in AI under 100 μE m⁻² s⁻¹ visible light. Labeling of the cells (Fig. 7B) confirmed higher requirement of the D2 protein relative to the D1 protein in AI in comparison with AIII and especially with A2K during the recovery from UV-B stress. Pulse-chase experiments showed the same rate of UV-B-induced D1 degradation in all three strains (Fig. 8A and C), but this degradation was accompanied only in AI by the decrease in the steady-state level of the protein (Fig. 8B and C). It is important to note that the A2K cells in which PSII contained the native D1 protein were less sensitive to UV-B than both the AI and the AIII cells. This difference could be observed both in the absence and presence of LIN (Fig. 7).

4. Discussion

Replacement of the native D1 protein in Synechocystis 6803 with each of the two D1 isoforms from Synechococcus 7942 resulted in fully functional cells, whose overall physiological parameters, including various PSII functions, are the same as in the control strain. Nevertheless, there is only about 90% identity between the Synechocystis and Synechococcus psbA coding regions and we cannot exclude that the D1:1 and D1:2 forms do not completely fit into the PSII RC complex of Synechocysts. However, this possible consequence of the heterologous gene expression does not mask the difference in the sensitivity to visible and UV-B radiation between AI and AIII that remains similar as in the Synechococcus strains containing corresponding psbA genes [10,20,21]. Thus, the constructed mutants provide a useful system to study the mechanistic background of visible- and UV-light sensitivity of PSII.

Differences in this sensitivity are manifested partly at the level of PSII damage, as shown by the inactivation curves obtained in the presence of lincomycin, and partly at the level of PSII repair. The rate of damage must be directly related to the amino acid sequence of each D1 form. On the other hand, there are more possibilities how the coding region can affect the PSII repair. It is known that efficiency of translation may be dependent on the presence of rare codons or on interaction of specific elements within the coding region with UTR of the transcript or with protein factors. In this way D1 synthesis can be modified by its coding sequence. Our assembly study showed lower level of radioactive amino acid incorporation into the D1 and D2 proteins of the PSII core complex in illuminated AI cells. However, there is relatively high level of labeled iD1 in the RC47 and RC complexes, and D2 in the part of the gel containing unassembled protein. It suggests that instantaneous synthesis of both proteins is not significantly affected. Therefore, we propose that an inhibition of the PSII core assembly from newly synthesized D1 and D2 proteins is the reason for less efficient PSII repair in high-light-treated AI cells. Recently, Zak et al. [32] proposed that in cyanobacteria a formation of new RC complexes occurs in plasmatic membrane that is the first target of the visible and UV-B radiation as it forms the first membrane layer. One possible explanation for the D1:1 repair insufficiency would be that increased irradiance inhibits translocation of RC complex from plasmatic membrane to thylakoids in which it probably assembles with CP47, CP43 and other PSII subunits to form RC47 and CC [32]. In this case some specific D1:1 residues could mediate this inhibition. In the case of UV-B-induced and largely irreversible PSII damage, one can assume that reactive oxygen species formed during this treatment may inactivate the translation apparatus needed for D1 and D2 resynthesis [33]. Presently, we are exploiting advantages of Synechocystis system (transformation of the heterothrophically grown psbA deletion strain and subsequent selection for autotrophy) over Synechococcus by constructing chimeric and site-directed mutants to identify sequences responsible for the differences in the PSII damage and repair.

Accelerated decay of flash-induced fluorescence in the presence of DCMU observed especially in AIII mutant indicates lower stability of the S₂Q₅ charge pair. Similar result has been also obtained with the Synechocystis mutant expressing the D1 protein from Poa annua [34]. In this plant D1 as well as in D1:2 glutamine residues 130 are replaced by glutamate, and recent reports on specific Synechocystis mutants differing in this residue indicate its importance for the kinetics of the PSII charge recombination [35]. We assume that increased contribution of the fast phase in the
fluorescence decay observed in D1:2 containing *Synechocystis* and *Synechococcus* cells (Fig. 2, Table 2) can be ascribed to the presence of Glu130.

On the other hand, in the absence of DCMU, Q\textsubscript{A} reoxidation rate was identical in all three *Synechocystis* strains as well as in D1:1 containing control and D1:2 containing high-light-treated cells of the strain *Synechococcus PCC 7942* (see Fig. 2). This result is in an apparent contradiction with recent data of Sane et al. [36] who reported that the replacement of D1:1 for D1:2 results in the destabilization of Q\textsubscript{B} almost to the level of Q\textsubscript{A} as concluded from thermoluminescence measurements. Such a destabilization of Q\textsubscript{B} should be necessarily accompanied by a significant slowing down of the electron transfer between Q\textsubscript{A} and Q\textsubscript{B}. The cause of this discrepancy is not completely clear, but our experience shows that *Synechococcus 7942* cells are sensitive to freezing damage, which seriously modifies the TL characteristics and abolishes flash-induced oxygen release (data not shown). Thus, the freezing of *Synechococcus 7942* cells to about -40 °C in the experiments of Sane et al. could induce an artifact into their TL measurements. Another discrepancy exists with respect to the PSII photochemical quantum yield. This parameter is identical in AI and AIII while in native *Synechococcus* system it is lower in mutant containing D1:1. This discrepancy could be explained by much greater difference in the PSII quantum yield between the State I and II in *Synechococcus* when compared with *Synechocystis*. Cells expressing D1:1 are more in State II than cells containing D1:2 [21] and consequently the difference in the quantum yield between these cells will be more pronounced in *Synechococcus* than in *Synechocystis*.

The rate of Q\textsubscript{A} reoxidation in the *Synechococcus 7942* cells is slower than in the AI and AIII mutants both in the absence and presence DCMU (Fig. 2) despite the presence of the same D1 protein form in the PSII complex. It is known that stability of S\textsubscript{2}Q\textsubscript{B} and S\textsubscript{2}Q\textsubscript{A} pairs is increased in thermophilic species due to the optimization of charge stabilization at higher growth temperature as judged from the position of thermoluminescence bands [37]. Therefore, the difference in the reoxidation rates can be ascribed to higher optimal growth temperature of *Synechococcus* (about 34 °C) compared with *Synechocystis* (about 30 °C). Result also shows that the rate of Q\textsubscript{A} reoxidation is determined not only by the D1 protein sequence, but also by the other proteins and by lipids of the PSII RC complex.

The overall similarity of the *Synechocystis 6803* D1 is larger to the high-light-induced D1:2 form (identity 92%) of *Synechococcus 7942* than to the D1:1 form (identity 89%) that prevails in low light, or under low UV levels. Therefore, *Synechocystis 6803* appears to be in continuous alert for high-light/UV conditions by producing more phototolerant PSII complexes. Thus, in contrast to *Synechococcus 7942*, this cyanobacterial species does not bank on the replacement of its PSII with a structurally different, more phototolerant complex during acclimation to high visible and UV light. Instead, the only possibility is to enhance transcription of psbA genes already encoding the more tolerant D1 form and, in this way, to increase its availability for the PSII repair. It is possible that this different strategy is based on the different habitats in which these two species live. *Synechococcus 7942* cells are keeping the D1:1 form that seems to maintain the PSII complex more in State 2 [21] in order to better meet metabolic requirements in shaded, rarely light-exposed living niche. On the other hand, the presence of the phototolerant D1:2-like protein in *Synechocystis 6803* may be related to its life in the environment more exposed to high visible or UV radiation.

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