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The role of the PsbU subunit in the light sensitivity of PSII in the cyanobacterium *Synechococcus* 7942

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ABSTRACT

In the present study we investigated the role of the PsbU subunit in the electron transport characteristics and light sensitivity of the Photosystem II complex. The experiments were performed by using an earlier characterized PsbU-less mutant of the cyanobacterium *Synechococcus* PCC 7942, which has enhanced antioxidant capacity (Balint et al. FEBS Lett. 580 (2006) 2117–2122). Flash induced Chl fluorescence measurements in the presence and absence of the electron transport inhibitor DCMU showed that both the $S_2Q_A^-$ and the $S_2Q_B^-$ recombination is slowed down in the PsbU mutant relative to the WT strain. Thermoluminescence measurements confirmed the increased stability of the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs by showing an increased peak temperature of Q and B bands, which were measured in the presence and absence of DCMU, respectively. In addition, the intensity of the TL bands is also increased in the PsbU mutant (≈1.7 times for the B band), as compared to the WT. The PsbU mutant shows enhanced loss of Photosystem II activity under exposure to high light intensity both in the absence and presence of the protein synthesis inhibitor lincomycin. It is concluded from the data that the lack of the PsbU subunit in *Synechococcus* PCC 7942 affects the energetic stability of the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs by modifying both the PSII donor and acceptor side components. This effect is most likely caused by structural changes in the vicinity of the Mn cluster and in the inner part of the PSII complex, which are induced by the lack of the PsbU subunit from the lumenal part of the complex. The light sensitivity of Photosystem II in *Synechococcus* 7942 in the absence of the PsbU subunit is likely due to reactive oxygen species, which are produced as a consequence of disturbed donor side structure and/or due to the modified energetic properties of the primary radical pair.

1. Introduction

Cyanobacteria use light energy to convert water into molecular oxygen. Oxygen evolution is catalyzed by the Photosystem II (PSII) pigment protein complex, which is embedded in the thylakoid membrane. This catalytic function is performed by light driven electron transfer reactions through redox cofactors of the PSII reaction center. The initial photoact is charge separation between the excited reaction center Chl (P_{680}) and the first acceptor, a pheophytin (Phe). The primary charge separated state, $P_{680}^+Phe^-$, is stabilized by secondary electron transfer reactions. On the acceptor side of PSII the electron is transferred from Phe^- to a permanently bound plastoquinone, called Q_A, and Q_A^- is reoxidized by the second quinone electron acceptor called Q_B (see Ref. [1]). On the donor side the first step of charge stabilization is the reduction of P_{680}^+ by a redox active tyrosine, called Y_Z, which serves as intermediate electron carrier between P_{680} and the water oxidizing complex. The PSII complex contains another redox active tyrosine, called Y_D, as well, which can serve as alternative electron donor to P_{680}^+. The final electron donor is the catalytic Mn cluster of water oxidation, which can exist in five oxidation states denoted as $S_0,\ldots,S_4$ (for reviews see [2,3]).

The protein backbone of the PSII complex consists of more than 15 membrane intrinsic protein components. In addition, there are extrinsic proteins located on the lumenal surface of PSII. Cyanobacterial PSII complexes contain five extrinsic proteins, PsbO, PsbP, PsbQ, PsbU, and PsbV. Structural studies of PSII from the cyanobacterium *Thermosynechococcus elongatus* have confirmed the association of PsbO, PsbU and PsbV with the oxygen evolving complex [4–6]. Biochemical and genetic studies have shown that these proteins function to stabilize the manganese cluster and minimize Ca^{2+} and Cl^- requirements for oxygen evolution [7–9].

The function of the PsbU protein has been studied and was found that it stabilizes the PSII complex in several ways. Based on the crystal structure the role of PsbU protein is generally assigned to shield the manganese cluster from the aqueous phase. In fact, the absence of PsbU resulted in the reduction of oxygen

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evolution [10], high sensitivity of cells to photodamage [11,12], and dark inactivation of PSII activity [8]. Additionally, PsbU was found to be required to stabilize the ion environment for oxygen evolution [10]. The presence of PsbU contributes to the heat stability of the oxygen-evolving machinery during the acclimation to high temperature [13,14]. Furthermore, it has been reported that removal of PsbU affects the stromal surface of the PSII complex and leads to accumulation of uncoupled phycobilisomes in Synechocystis 6803 [15]. Recently, flash-induced Chl fluorescence decay and thermoluminescence (TL) measurements performed with a PsbU mutant of *Synechococcus* PCC 7942 showed that the rate of charge recombination from the $S_2Q_A^-$ state is slowed down, as shown by the retarded rate of fluorescence decay and upshifted peak position of the TL band in the presence of the electron transport inhibitor DCMU [12].

In the present study we show by flash induced Chl fluorescence and TL measurements that in the PsbU-mutant of *Synechococcus* PCC 7942 not only the $S_2Q_A^-$, but also the $S_2Q_B^-$ recombination is slowed down. Moreover, TL measurements both in the presence and absence of DCMU showed a significant increase in the TL signal intensity, which is accompanied by the shift of the position of the peaks to higher temperatures. The PsbU mutant also shows increased light sensitivity both in the absence and presence of protein synthesis inhibitors.

2. Materials and methods

2.1. Propagation of cells

Synechococcus sp. PCC 7942 (WT) and the PsbU mutant cells, whose creation was described earlier [12], were grown in BG-11 medium in a rotary shaker under air enriched with 3% CO_2. The intensity of irradiation during growth was 40 μE m^{-2} s^{-1} at 30 °C. Cells were used in the exponential growth phase (A_{580} = 0.8–1), which corresponds to 4.7–5.8 μg Chl/ml.

2.2. Light treatment

Cells were harvested by centrifugation at 8000*g* for 5 min and resuspended in 100 mL of fresh BG-11 medium at concentration of 7 μg of Chl a mL^{-1}. Before starting high light treatment cells were left for one hour under 40 μE m^{-2} s^{-1} light at continuous stirring followed by measurement of control value of oxygen evolution, which was used as zero time point for the high light treatment. For photoinhibitory treatment cells were illuminated with 500 μmol photons m^{-2} s^{-1} in the presence and absence of protein synthesis inhibitor lincomycin (300 μg/ml).

2.3. Oxygen evolution measurements

Oxygen evolution was measured with Hansatech DW2 O_2 electrode at saturating light intensity in the presence of 0.5 mM DMBQ, as an artificial electron acceptor. 1 mL of cells at 7 μg Chl mL^{-1} was used in each measurement, and three replicates were measured. When light treatment was performed in the absence of protein synthesis inhibitor lincomycin cells were brought back to 40 μE m^{-2} s^{-1} light intensity after 1.5 h of high light exposure to monitor their ability for repairing PSII activity.

3. Thermoluminescence measurements

TL glow curves were measured with a home-built apparatus as described earlier [16]. Cells were harvested on a filter paper disk to achieve 50 μg Chl. Samples were dark adapted for 3 min at 0 °C. TL was excited either in the absence or in the presence of the electron transport inhibitor DCMU. In the absence of DCMU dark-adapted samples were excited with a single turnover saturating flash at +5 °C followed by TL measurement from 0 to +80 °C. In other experiments 10 μM DCMU was added to the sample in the dark, and after excitation of the sample at −2 °C TL was detected during heating from −4 to +80 °C.

The peak position of a TL band reflects the energetic stability of the recombining charge pair, i.e. higher peak temperature shows deeper stabilization, whereas the amplitude of a TL band is proportional to the amount of the recombining centers. However, the intensity of the B and Q bands is modulated also by the free energy difference between the excited reaction center Chl (P_{680}^*) and the primary charge separated state ($P_{680}^+Phe^-$) [17]. This phenomenon can be used to estimate the modification of the free energy change between P_{680}^* and $P_{680}^+Phe^-$, which is induced by the lack of PsbU, by using the $\Delta G = kT\ \ln(TL_{PSBU}/TL_{WT})$ formula [17] (where k, T, and TL are the Boltzmann constant, the temperature and TL intensities, respectively).

4. Flash-induced fluorescence relaxation kinetics

Flash-induced fluorescence increase and the subsequent decay of Chl fluorescence yield were measured by using a double-modulation fluorometer (PSI Instruments, Brno) [18] in the 150 μs–100 s time range as described earlier [19]. Charge separation between Q_A and the PSII donor side was induced by a single saturating flash typically from the red LED of the PSI machine. For most experiments the measuring light was also red. However, for some experiments blue excitation was used from an external Xenon lamp equipped with a blue filter, as well as the blue measuring flashes of the PSI machine in order to ensure that only Chls, but not phycobilisomes, are involved in the fluorescence emission. The sample concentration was 5 μg Chl mL^{-1}. Multicomponent deconvolution of the measured curves was done by using a fitting function with three components. The fast and middle phases were simulated with exponential components. However, slow recombination of Q_A^- via charge recombination has been shown to obey hyperbolic kinetics corresponding to an apparently second-order process [20]. Therefore, the slow phase was simulated with a hyperbolic component:

$$F(t) - F_0 = A_1\exp(-t/T_1) + A_2\exp(-t/T_2) + A_3/(1 + t/T_3) + A_0 \quad (1)$$

where $F(t)$ is the variable fluorescence yield at time t, F_0 is the basic fluorescence level before the flash, A_1–A_3 are the amplitudes, T_1–T_3 are the time constants of the decay phases, and A_0 is the fluorescence level which does not decay after the flash. The nonlinear correlation between fluorescence yield and the redox state of Q_A was corrected for by using the Joliot model [21] with a value of 0.5 for the energy-transfer parameter between the PSII units. The time constant of the slow phase reflects the rate of charge recombination from the $S_2Q_B^-$ or $S_2Q_A^-$ states, depending on the absence or presence of DCMU, respectively. This effect can be used to calculate the changes in the energetic stabilization of the respective charge pairs by assuming that the decay of the charge separated states is dominated by a thermally activated process, which obeys Boltzmann equilibrium between the free energy levels of the charge separated state and of the primary radical pair. The relative change of the stabilization free energy of the $S_2Q_B^-$ charge pair in the mutant as compared to the WT can be obtained by using the $\Delta G_{PSBU(-)WT}(S_2Q_B^-) = kT\ \ln(T_{3noadd,PSBU}/T_{3noadd,WT})$ formula, where k and T correspond to the Boltzmann constant and the temperature, respectively. Whereas, the T_3 values denote the time constants of the respective decay processes in the absence of DCMU. Similarly, the relative change of the stabilization free energy of the $S_2Q_A^-$ charge pair in the mutant as compared to the WT can be obtained

by using the $\Delta G_{PSBU\langle-\rangle WT}$ $(S_2Q_A^-) = kT\ln(T_{3DCMU,PSBU}/T_{3DCMU,WT})$ formula with the T_3 values obtained in the presence of DCMU.

The free energy (redox) gap between Q_A and Q_B can be estimated from the ratio of the time constants of the $S_2Q_B^-$ and $S_2Q_A^-$ charge recombination processes ($\Delta G(Q_A \langle-\rangle Q_B) = kT\ln(T_{3,noadd}/T_{3,DCMU})$), provided that the $Q_A^-Q_B \langle-\rangle Q_AQ_B^-$ charge equilibrium is maintained during the time course of the flash fluorescence measurement.

5. Results

5.1. Relaxation kinetics of flash-induced fluorescence

Forward electron transfer at the acceptor side of PSII, as well as charge recombination processes of the reduced acceptors with oxidized donor side components were monitored by using flash-induced Chl fluorescence decay measurements. Previous data have indicated that the rate of the forward electron transport from Q_A^- to Q_B was not affected in the absence of the PsbU subunit, while the $S_2Q_A^-$ charge recombination process is slowed down in *Synechococcus* 7942 [12]. However, the earlier presented flash fluorescence data were rather noisy, and the different phases of the fluorescence decay could not be clearly resolved. Therefore, we have aimed at the clarification of the fluorescence kinetics with more precise measurements. Illumination of dark-adapted samples with a single turnover saturating flash forms Q_A^-, which results in a rise of variable fluorescence. The amplitude of the fluorescence signal reflects the number of PSII centers capable of reducing Q_A, which was only 70% in the PsbU mutant of that obtained in the WT strain (Fig. 1A inset). This shows that the amount of functional PSII centers is significantly smaller in the PsbU mutant (≈70%) than in the WT. It has been previously reported that the lack of PsbU leads to disruption of energy transfer between phycobilisomes and PSII in *Synechocystis* 6803 [15]. If such effect exists also in *Synechococcus* then the decreased flash fluorescence amplitude in the PsbU mutant could be due to the lack of saturation of PSII since we used the red actinic and measuring flashes in our experiments, which excite mainly the phycobilisomes. In order to clarify this question we performed experiments also with blue actinic flashes (from an external Xenon flash lamp through a blue filter) in combination with the blue measuring flashes of the fluorescence machine. These experiments showed the same ca. 70% initial fluorescence amplitude in the PsbU mutant relative to the WT as obtained with the red actinic and measuring flashes. We have also performed 77 K fluorescence measurements, which showed no sign of energy transfer disruption in the *Synechococcus* PsbU mutant (not shown). These data confirm that in our case the decreased flash fluorescence amplitude in the PsbU mutant is not caused by a disturbed energy transfer between the phycobilisomes and PSII, and therefore it reflects the decrease of functional PSII centers.

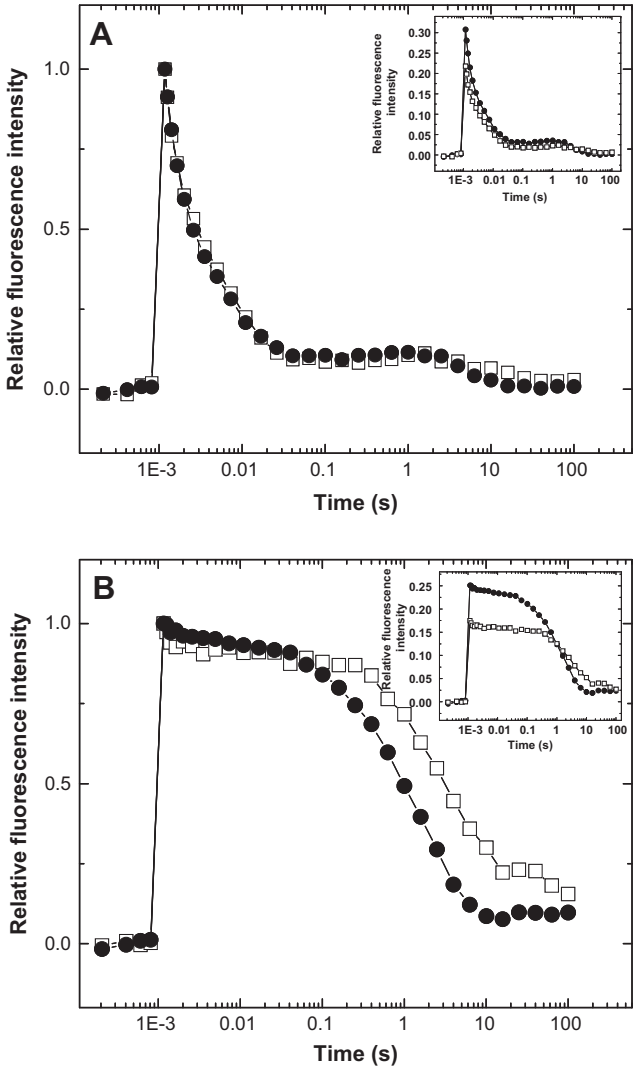

Fig. 1. Relaxation of flash-induced Chl fluorescence. WT (full circles) and PsbU mutant cells (empty squares) were excited with a single turnover saturating flash at the 1 ms time point. The measurements were performed in the absence of electron transport inhibitors (A), or in the presence of 10 μM DCMU (B). The fluorescence traces were measured at the same amount of Chl (5 μg mL^{-1}) and shown after normalization to identical initial amplitudes. The insets show the same traces with their original amplitudes, but after shifting the Fo values to 0.

The relaxation of the fluorescence kinetics exhibits three main decay phases, which reflect the reoxidation of Q_A^- in the dark via different routes (Fig. 1, Table 1) (see [19]). The fast ($T_1 \sim 700$ μs) and middle ($T_2 \sim 9$–11 ms) phases reflect forward electron transport from Q_A^- to PQ which occupies the Q_B site at the time of the flash, or binds after the flash, respectively. Whereas, the slow phase of the decay ($T_3 \sim 7$–10 s) originates from back reaction of Q_B^- with the oxidized S_2 state. The fast phase is similar in the WT and the PsbU mutant in terms of time constant and relative amplitude (Table 1), while the middle phase is somewhat slower in the PsbU-less mutant. In contrast, the slow phase is about 50% slower in the mutant than in the WT (Table 1).

Fluorescence kinetics of back electron transfer reactions were also monitored in the presence of 10 μM DCMU, which inhibits the Q_A^- to Q_B electron transfer step, as confirmed by oxygen evolution measurements. In this case the fluorescence relaxation is dominated by a slow phase, which reflects the recombination of Q_A^- with the S_2 state of the water oxidizing complex. In the presence of DCMU, the main fluorescence decay component is slower in the mutant ($T_3 \sim 3.4$ s) than in the wild type ($T_3 \sim 1.6$ s) (Fig. 1B), which is in agreement with previous results [12]. An approximately 3–8% fast component also remained in the presence of DCMU. We have confirmed that this fast component is not due to a flash artifact, and could not be abolished by further increase of DCMU concentration up to 50 μM. Although at this stage we can not exclude that DCMU binding may not be complete in a small fraction of PSII centers, it is more likely that the fast phase arises from the recombination of Q_A^- with $Y_Z^{+\cdot}$ (see [22]).

Fluorescence relaxation in the presence of DCMU exhibits a non-decaying fraction, which is small in the WT ($A_0 \sim 6\%$), but significant in the PsbU mutant ($A_0 \sim 25\%$) (Fig. 1B). The initial amplitude of the fluorescence signal is smaller in the PsbU mutant (≈70%) than in the WT (Fig. 1B inset) as observed in the absence of DCMU. This difference in the fluorescence amplitude of the DCMU treated mutant and WT cells was confirmed also by using blue actinic and measuring flashes, which excite only Chls (not shown).

Table 1
Kinetic parameters of flash-induced fluorescence traces. Fluorescence was measured and analyzed as described in Section 2. The standard errors were obtained from averaging three different measurements obtained on biologically different cultures. The $T_1, \ldots, T_3$ values are the time constants of the decay phases, whereas the $A_1, \ldots, A_3$ values are the relative amplitudes. The A_0 values shows the fraction of fluorescence, which does not decay after the flash.

Strain	Fast phase T_1 (ms)/A_1 (%)	Middle phase T_2 (ms)/A_2 (%)	Slow phase T_3 (s)/A_2 (%)	Non-decaying phase A_0 (%)
No addition				
WT	0.73 ± 0.01/30.9 ± 1.7	8.6 ± 0.3/51.2 ± 2	6.9 ± 0.6/17.9 ± 0.7	0 ± 0
PsbU mutant	0.71 ± 0.1/36.1 ± 4.7	10.8 ± 1.3/47.3 ± 4	10.1 ± 3.8/16.1 ± 1.7	0.5 ± 0.5
+DCMU				
WT	0.6 ± 0.2/3.5 ± 0.23	–/–	1.6 ± 0.1/90 ± 3.3	6.5 ± 3.3
PsbU mutant	0.4 ± 0.1/8 ± 1.1	–/–	3.4 ± 0.31/68 ± 1.6	24 ± 1.3

5.2. Thermoluminescence characteristics

Backward electron transfer reactions were also checked by TL measurements. The usual protocol for measuring TL is to give a single saturating flash at +5 °C in the absence, or at −10 °C in the presence of 10 μM DCMU, and to record the emission curves over a temperature range starting from −40 °C [16]. However, *Synechococcus* cells appeared to be sensitive to freezing, which leads to the loss of TL signal intensity (not shown), and makes it even undetectable in the PsbU mutant in the absence of DCMU [12]. In order to avoid the loss of the TL signal due to the freezing effect, the TL curves were recorded from 0 to 80 °C (with a saturating flash at +5 °C) in the absence of DCMU, and from −4 to +80 °C (with saturating flash at −2 °C) in the presence of DCMU.

The main TL band (B band) arises from the $S_2Q_B^-$ recombination in the absence of DCMU [23,24], which appeared at 29 °C in the WT cells (Fig. 2A). In the PsbU mutant the position of this band was upshifted to higher temperatures (33 °C). In the presence of DCMU, which blocks the Q_A to Q_B electron transfer, the main TL band (Q band) arises from the $S_2Q_A^-$ recombination [23,25]. The Q band was observed at around 10 °C in the WT (Fig. 2B). In the mutant the peak position was shifted to higher temperatures and appeared at ≈29 °C. It is important to note that the lack of the PsbU subunit induced not only an increased TL peak temperature, but increased the TL intensity as well. In case of the Q band the TL peak position in the WT (ca. +10 °C) is not very far from the excitation temperature (−2 °C) and from the starting temperature of the measurement (−4 °C). Therefore part of the TL signal intensity could be lost in the initial phase of the measurement, which decreases the apparent Q band amplitude in the WT. However, such an effect is not expected to occur in case of the mutant, or in the absence of DCMU, where the TL bands are at around or above +30 °C, which allows stabilization of the separated charges at the excitation temperature. As a consequence, the increased intensity of B band in the PsbU mutant reflects a real effect of modified charge recombination characteristics. Whereas, in case of the Q band part of the intensity difference in the WT and mutant can be related to the destabilization of the $S_2Q_A^-$ charge pair in the WT during the initial phase of the TL measurement. When the true extent of TL intensity increase is calculated we have to take into account that the amount of functional PSII centers is only about 70% in the PsbU mutant as compared to the WT as shown by the initial amplitude of the flash fluorescence signal. When the TL intensity is normalized to the flash fluorescence amplitude of the particular strain, the B band intensity appears to be ca. 1.7-fold higher in the PsbU mutant than in the WT on the basis of equal number of functional PSII centers.

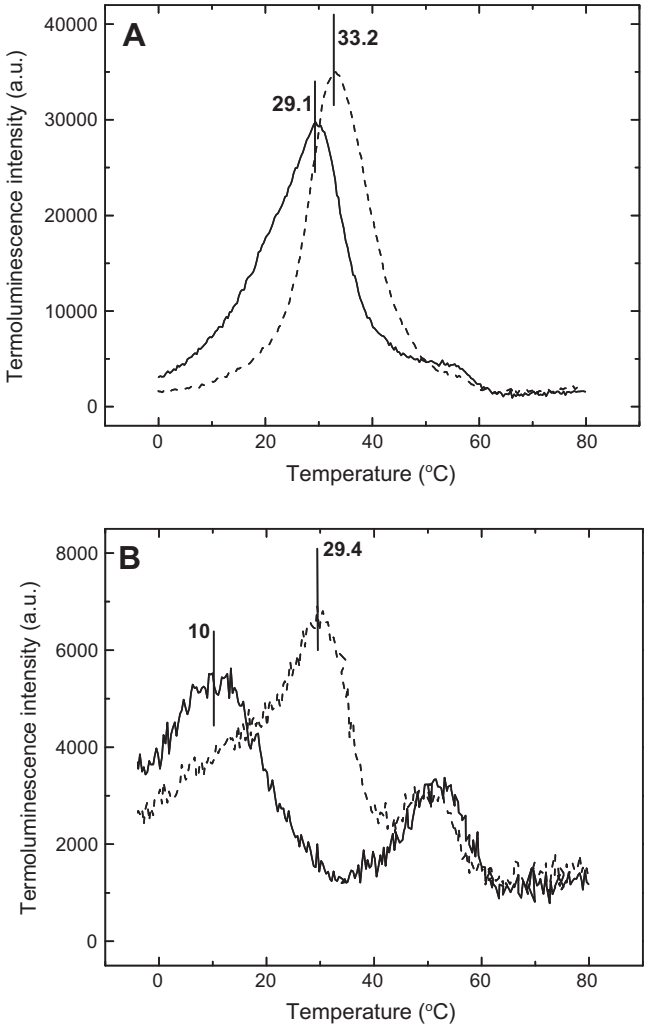

Fig. 2. Flash-induced thermoluminescence. WT (full line) and PsbU mutant cells (dashed line) of *Synechococcus* 7942 were excited with a single turnover saturating flash either at $T = 5$ °C without addition followed by TL measurement from 0 to +80 °C (A), or at $T = -2$ °C in the presence of electron transport inhibitor 10 μM DCMU followed by TL measurement from −4 to +80 °C (B). The TL curves were measured at the same amount of Chl (50 μg). The vertical lines show the peak position of the corresponding TL peaks.

5.3. High light sensitivity

The sensitivity of the WT and the PsbU strains to photoinhibition was checked by exposing cells to strong light (500 μE m^{-2} s^{-1}) for 1.5 h. In the presence of lincomycin, which prevents *de novo* protein synthesis, the decrease of oxygen-evolving activity was greater in the PsbU mutant, indicating its increased sensitivity to high light relative to the WT strain (Fig. 3A).

We also measured the loss of oxygen-evolving activity during illumination in the absence of lincomycin to compare the PSII repair ability of the WT and PsbU strains. Exposure of cells to high light showed that the rate of inactivation was higher in the PsbU

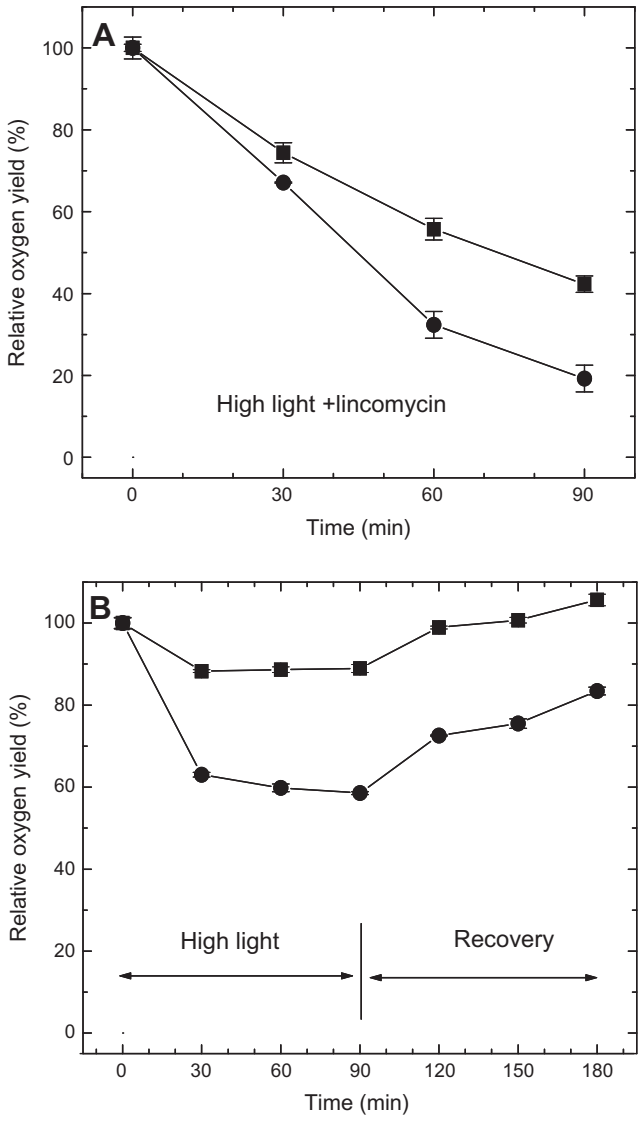

Fig. 3. Light induced damage of PSII activity. WT (full squares) and PsbU mutant cells (full circles) of *Synechococcus* 7942 were exposed to 500 μmol m^{-2} s^{-1} visible light either in the presence of 200 μg mL^{-1} lincomycin (A), or without addition (B). After 90 min high light treatment the cells were transferred back to growth light intensity 40 μmol m^{-2} s^{-1} in order to let the PSII activity recover.

mutant than in the WT. However, both strains were able to restore the inhibited PSII activity when brought back to growth light conditions (Fig. 3B). The larger extent of high light induced loss of PSII activity in the PsbU mutant as compared to the WT was also confirmed by variable fluorescence data, calculated from the initial amplitude of flash-induced fluorescence decay (not shown), which confirm the earlier findings obtained by steady-state variable Chl measurements [12].

6. Discussion

6.1. Electron transport characteristics

Our high resolution flash fluorescence data confirm the earlier findings that the absence of the PsbU subunit does not affect forward electron transport from Q_A^- to Q_B, but induces a significant retardation of Q_A^- reoxidation via charge recombination with the S_2 state [12]. In addition, we could also identify a slower rate of Q_A^- reoxidation in the absence of DCMU in the slow phase of the fluorescence relaxation, which reflects the $S_2Q_AQ_B^-$ recombination via charge equilibrium with the $S_2Q_A^-Q_B$ state (Fig. 1, Table 1). The time constant of the $S_2Q_A^-$ decay was ~2 times slower in the PsbU mutant than in the WT, whereas the ratio of the $S_2Q_B^-$ time constants was 1.5, as calculated from the data in Table 1. These values reflect 18 and 10 meV increase in the free energy of stabilization of the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs, respectively, in the PsbU mutant relative to the WT.

The energetic stability of the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs is determined by the free energy span between the $P_{680}^+Phe^-$ radical pair state and the $S_2Q_A^-$ and $S_2Q_B^-$ charge separated states, respectively. Therefore, stabilization of $S_2Q_B^-$ and $S_2Q_A^-$ can arise either from the decreased free energy level of the $P_{680}^+Phe^-$ radical pair, or from the increased free energy level (redox potential) of the S_2, or the Q_A, Q_B states. The $P_{680}^+Phe^-$, as well as the S_2 states are shared by the recombination pathways of both $S_2Q_A^-$ and $S_2Q_B^-$, but the two acceptor states, Q_A and Q_B, are different. As a consequence, differential stabilization of $S_2Q_A^-$ and $S_2Q_B^-$ is indicative of a free energy (redox potential) change at the level of the quinone electron acceptors. This effect is also reflected by the modification of the free energy gap between the Q_A and Q_B acceptors, which can be estimated from the ratio of the time constants of the $S_2Q_B^-$ and $S_2Q_A^-$ charge recombination processes (see Materials and Methods). This calculation resulted in 37 and 28 meV gap between Q_A and Q_B in the WT and PsbU mutant cells, respectively. These values are smaller than those reported in the literature previously for higher plants (≈70 meV [26,27]) and cyanobacteria (60–70 meV for *Synechocystis* 6803 [22,28,29], 56 meV for *Acaryochloris marina* [28]). The small free energy gap in *Synechococcus* 7942 represents a decreased driving force for the Q_A^- to Q_B electron transfer, which is consistent with the slower rate of this process in *Synechococcus* (≈700 μs) as compared to other cyanobacterial species (450–600 μs) [22,28,29].

The decrease of the Q_A ⟨−⟩ Q_B free energy (redox) gap in the mutant as compared to the WT is in agreement with previous findings [15], which showed that an increased fraction of PSII centers is present in the closed state in the dark. The closed state of PSII centers in the dark is formed via charge equilibrium between the $Q_A^-Q_B$ and $Q_AQ_B^-$ acceptor states, i.e. via reduction of Q_A by electron from Q_B^- and the PQ pool. Therefore, decrease of the free energy difference between the Q_A and Q_B acceptors should increase the amount of closed PSII centers in the dark provided that the reduction level of Q_B and the PQ pool is not affected.

Although most of the consequences, which are induced by the lack of PsbU have been reported to occur at the donor side of PSII [8,10,14], our data show, in agreement with previous findings [15], that the absence of the PsbU subunit modifies not only the donor, but also the acceptor side components of PSII (Fig. 4). Similar examples, showing that structural changes at the PSII donor side can affect the redox potential of acceptor side components have been reported after removal of the PsbO subunit [16], or of the Mn cluster [30].

A characteristic feature of the PsbU mutant is the increased level of non-decaying fluorescence in the presence of DCMU (Fig. 1B, Table 1). This phenomenon indicates that in part of the PSII centers the electrons, which reduce Q_A originate from a very stable donor instead of the S_2 state of the Mn cluster. This stable donor can be Y_D, which acts as alternative electron donor to P_{680}^+, and is usually in the oxidized radical state ($Y_D^{+\bullet}$), but may become more easily reduced in the PsbU mutant than in the WT. In normally functioning PSII Y_D is kept in the oxidized state for tens of minutes through charge equilibrium with the P_{680}, Y_D and the Mn cluster [31] and therefore inactive in light induced electron transport. The large stability of $Y_D^{+\bullet}$ is due to the highly hydrophobic nature of the protein environment around the Y_D pocket [31]. In the absence of the PsbU subunit the modification of the PSII donor side can affect the protein environment around both the Mn cluster and Y_D, which can make $Y_D^{+\bullet}$ more accessible to exogenous reductants. This effect

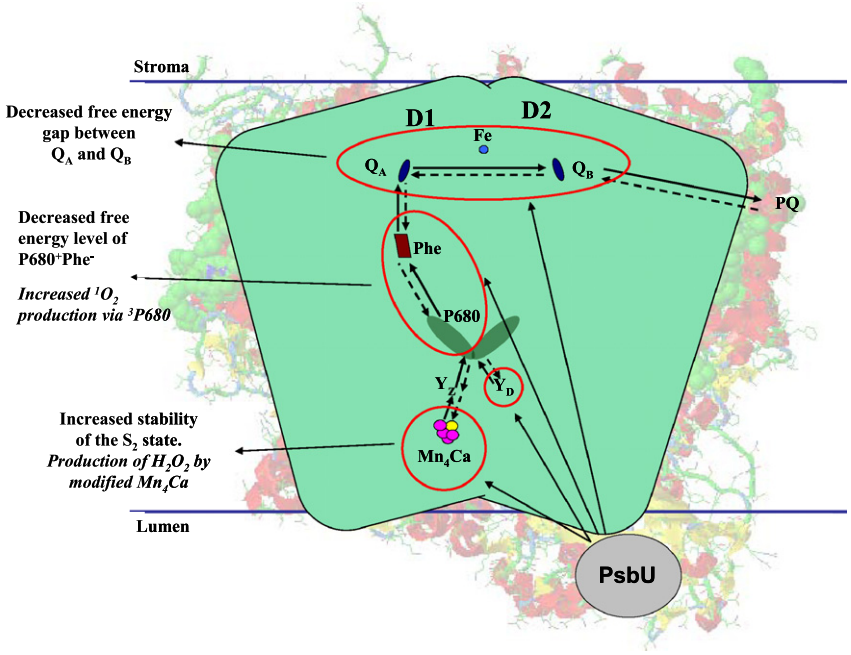

Fig. 4. Consequences of lacking PsbU in the PSII complex of *Synechococcus* 7942. The figure shows the main sites where the lack of PsbU affects PSII electron transport characteristics and thus may lead to high light sensitivity. The main sites of interest are: (i) The vicinity of the Mn cluster of water oxidation, whose disturbance may lead to H_2O_2 production, and decreased stability of the alternative Tyrosine-D electron donor (Y_D). (ii) The vicinity of P680 and/or Phe, whose disturbance may lead to decreased free energy level of the $P^+_{680}Phe^-$ primary radical pair, which can initiate increased singlet oxygen production. (iii) The vicinity of the Q_A and/or Q_B quinone electron acceptors, which results in decreased free energy gap between Q_A and Q_B.

can lead to partial reduction of $Y_D^{+\bullet}$ during dark adaptation before the fluorescence measurement and the produced Y_D could act as stable electron donor to P^+_{680} instead of the Mn cluster. An alternative explanation for the non-decaying fluorescence could be an increased proportion of the PSII centers in the S_0 state in the dark, which also yields the non-recombining $S_1Q_A^-$ state after one-flash illumination in the presence of DCMU. However, the S_1Y_D state behaves as an apparent $S_0Y_D^{+\cdot}$ state [32], therefore it is difficult to distinguish between the two options.

Besides flash induced chlorophyll fluorescence TL measurements are also very useful to obtain information about the energetic characteristics of the PSII electron transport components. Previous TL studies, which were performed with the same PsbU mutant as used here showed an upshift of the Q band, which arises from the $S_2Q_A^-$ recombination, relative to the WT. However, the B band, which arises from the $S_2Q_B^-$ recombination, could not be detected in this mutant earlier [12]. Here we used a TL measurement protocol, which avoids sample freezing and could detect the B band in the PsbU mutant, which showed an upshifted peak position relative to the WT (Fig. 2). We observed also the increased peak temperature of the Q band in the PsbU mutant, in agreement with the previous findings [12]. These data are in agreement with the flash fluorescence results and confirm the energetic stabilization of both the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs. The larger peak temperature shift for the Q band than for the B band reflects larger extent of stabilization of $S_2Q_A^-$ recombination as compared with that of $S_2Q_B^-$, which is in agreement with the above discussed fluorescence data. Upshift of both the Q and B bands has also been observed in *Synechocystis* 6803 mutants, which lack the PsbU subunit [10]. However, in this case the temperature shift of the bands was practically the same, indicating that *Synechocystis* 6803 and *Synechococcus* 7942 may not behave in the same way in response to the lack of PsbU.

Recent studies have shown that TL intensity is moderated by the free energy level of the $P^+_{680}Phe^-$ primary charge separated state, which changes the free energy gap between P^*_{680} and $P^+_{680}Phe^-$ [17]. Targeted modification of the redox potential of Phe and P_{680} by amino acid replacements in the D1 protein in *Synechocystis* 6803 resulted in significant intensity changes of the B and Q TL bands, as well as in the rates of the $S_2Q_A^-$ and $S_2Q_B^-$ recombination [17]. The replacements of D1-Gln130Leu or D1-His198Lys resulted in the increase of TL intensity concomitant with the slow down of $S_2Q_A^-$ and $S_2Q_B^-$ recombination. This effect has been explained by the decrease of E_m(Phe/Phe$^-$) or $E_m(P^+_{680}/P_{680})$ [17], which is caused by the modification of hydrogen bonding interactions in the vicinity of Phe and the PD1 component of the P_{680} assembly, respectively [33,34]. The opposite effect, i.e. decrease of TL intensity concomitant with the acceleration of $S_2Q_A^-$ and $S_2Q_B^-$ was observed in the mutants with D1-Gln130Glu or D1-His198Ala [17] due to the increase of E_m(Phe/Phe$^-$) or $E_m(P^+_{680}/P_{680})$, respectively [33].

Based on the above discussed data the increased TL amplitude, which is accompanied by slower $S_2Q_A^-$ and $S_2Q_B^-$ recombination rate, is indicative of a decreased free energy level of the $P^+_{680}Phe^-$ primary charge separated state (Fig. 4). The intensity of the B band, which is observed in the *Synechococcus* 7942 PsbU mutant in the absence of DCMU is increased 1.7 -fold relative to the WT. Although the amplitude of the Q band (measured in the presence of DCMU) was also significantly higher in the PsbU mutant than in the WT, the extent of the Q band intensity increase could not be estimated reliably due to the possible destabilization of the $S_2Q_A^-$ in the initial phase of the TL measurement as discussed above. Therefore, we based our calculation on the increase of TL intensity in the absence of DCMU (see Materials and Methods), which resulted in 14 meV decrease of the free energy level of $P^+_{680}Phe^-$ relative to P^*_{680}.

6.2. Light sensitivity

Our current results show that in the *Synechococcus* 7942 PsbU mutant PSII has increased susceptibility to light in the presence

of the protein synthesis inhibitor lincomycin. Similar results have been obtained earlier in the PsbU-less mutant of *Synechocystis* 6803 [11]. However, in the *Synechocystis* 6803 mutant the increased light sensitivity disappeared in the absence of protein synthesis inhibitor. This result was interpreted as showing that PSII repair can fully compensate the increased susceptibility to light, which is induced by the lack of PsbU [11]. In contrast to these findings our data also demonstrate that the light sensitivity of the *Synechococcus* 7942 PsbU mutant is increased not only in the presence, but also in the absence of lincomycin, in agreement with the earlier observed decrease of F_v/F_m values upon high light illumination [12]. The large difference between the light induced loss of PSII activity in the WT and the PsbU mutant cells in the absence of lincomycin (Fig. 3) shows that the repair capacity of PSII can not keep up with the rate of damage during high light exposure in the absence of the PsbU subunit in *Synechococcus* 7942. However, the PsbU mutant is capable of efficient PSII repair when the cells are transferred to growth light intensity after the high light treatment. Indeed, the D1 protein level has been shown to be similar in the WT and the PsbU mutant under standard growth conditions in the absence of protein synthesis inhibitor [12].

Recent studies on photoinhibition show that light induced damage of PSII most likely proceeds via multiple mechanisms, which include the formation of reactive oxygen species as a consequence of electron transport events (see [35]), as well as inactivation of the Mn cluster of water oxidation [36–38]. The contribution of the two pathways to the overall photodamage depends on the illumination conditions, and possibly on the PSII structure. It would be straightforward to argue that in the absence of the PsbU subunit, which stabilizes the PSII donor side, the increased rate of photodamage is due to the enhanced photoinactivation of the Mn cluster. However, the *Synechococcus* 7942 PsbU mutant has a functional donor side, with only a small modification of the charge stabilization energetics. Therefore, it is not at all trivial that the overall increase of light sensitivity could be related to an increased susceptibility of the Mn cluster to light. On the other hand, it has been shown that the *Synechococcus* 7942 mutant possesses a largely enhanced antioxidant capacity, which suggests that the modified PSII structure in the absence of PsbU leads to enhanced reactive oxygen formation [12].

It has been shown earlier that the disturbed structure of PSII in the absence of lumenal protein subunits leads to the production H_2O_2 instead of O_2 [39]. H_2O_2 is a long lived reactive oxygen species, which can cause protein damage directly, or after interacting with metal ions which produce $OH^{-\cdot}$ radicals. Inactivation of *psbU* in *Synechococcus* 7942 resulted in elevated activity of KatG, an enzyme belonging to the group of prokaryotic catalase-peroxidases, as well as higher activity of cellular peroxidase(s) [12]. This behavior indicates that increased H_2O_2 production probably occurs in the PsbU mutant, which might be involved in its light sensitivity (Fig. 4). However, the enhanced antioxidant capacity of the PsbU mutant does not necessarily help to cope with all the consequences of H_2O_2 production as evident by the rapid turnover of the D1 protein [12]. The increased level of light dependent reactive oxygen production in the absence of PsbU is supported further by the finding that a *Synechocystis* 6803 mutant, which lacks both PsbU and PsbO can regain the ability of photoautotrophic growth via spontaneous reversion, which enhances the expression of stress related and photoprotective genes [40].

A particular feature of the PsbU mutant is that the increased stability of the $S_2Q_B^-$ and $S_2Q_A^-$ recombination is accompanied by the significantly increased intensity of the B and Q bands of TL. It is very important to note that a *Synechocystis* 6803 mutant (D1-Gln130Leu) with similar electron transport characteristics and increased TL intensity as the PsbU mutant studied here shows increased light sensitivity [41]. Based on the similar charge recombination characteristics of the D1-Gln130Leu mutant of *Synechocystis* 6803 and the PsbU mutant studied here we can argue that the increased light sensitivity of the PsbU mutant has at least partly similar background as that of the D1-Gln130Leu mutant. The moderation of photoprotection by the free energy level of the primary radical pair has been explained by the modification of the rate of a non-radiative charge recombination pathway from the singlet state of the primary radical pair, $^1[P_{680}^+Phe^-]$, which is retarded when the Phe redox potential is increased (D1-Gln130Leu) and accelerated when the Phe redox potential is decreased (D1-Gln130Glu) [17,42]. The non-radiative recombination from $^1[P_{680}^+Phe^-]$ competes with the recombination pathway that proceeds from $^3[P_{680}^+Phe^-]$ and produces $^3P680$ whose interaction with ground state molecular oxygen leads to the production of highly reactive singlet oxygen. It has also been shown that the D1-Gln130Glu replacement plays a critical role in photoprotection of various cyanobacteria, including *Synechococcus* 7942 [42,43], which exchange two different forms of the D1 protein under low and high light conditions.

In accordance with the above-mentioned information, it may be suggested that the lack of the PsbU subunit at the donor side of PSII induces a structural change of PSII, which affects not only the vicinity of the Mn cluster, but transmitted also into the middle and stromal part of the complex (Fig. 4). We can speculate that the resulting structural change modifies not only the environment of the Q_A and Q_B acceptors, but weakens also the hydrogen bonding interactions around Phe, or the PD1 Chl of P_{680}, which leads to the shift of the free energy level of $P_{680}^+Phe^-$ to more negative values. This in turn enhances the probability of $^3P_{680}$ forming charge recombination (see [35,42]), which facilitates the production of harmful singlet oxygen that causes photo-oxidative damage of PSII.

It has to be noted that the lack of the PsbU subunit seem to induce partly different effects in *Synechocystis* 6803 and *Synechococcus* 7942. This notion is supported by the observations that in the absence of PsbU the B and Q bands of TL are upshifted to the same extent in *Synechocystis* 6803 [10] in contrast to the differential effect on the peak temperatures observed here for *Synechococcus* 7942. Increased light sensitivity in the absence of PsbU has been observed both in *Synechocystis* 6803 [11] and in *Synechococcus* 7942 [12]. However, in the *Synechocystis* 6803 PsbU mutant this effect could be observed only in the presence of protein synthesis inhibitor, whereas in the case of *Synechococcus* 7942 increased light intensity appears both in the presence and absence of PSII repair, which indicates differential effect of the PsbU subunit on the repair capacity of PSII. In addition, the increased antioxidant capacity has been observed only in the *Synechococcus* 7942 PsbU mutant [12], while the disrupted energy transfer from the phycobilisomes to PSII has been observed only in *Synechocystis* 6803 [15], but not in *Synechococcus* 7942 in the present study. These effects may be due to partly different fine structure of the PSII complex, which lead to partly different consequences of the lack of the PsbU subunit, and point to the necessity of caution with generalizing the conclusions abut the role of PsbU obtained with a particular species.

7. Concluding remarks

The lack of the PsbU subunit in *Synechococcus* 7942 affects the energetic stability of the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs primarily by decreasing the free energy level of the $P_{680}^+Phe^-$ primary radical pair and partly by modifying the Q_A and Q_B acceptors. This effect is most likely caused by a structural change in the inner and stromal part of the PSII complex, which is induced by the lack of the PsbU subunit from the lumenal part of the complex (Fig. 4). The light sensitivity of Photosystem II in *Synechococcus* 7942 in the absence of PsbU is assigned to the presence of reactive oxygen species, which are produced as a consequence of disturbed donor side

structure and/or due to the modified energetic properties of the primary radical pair.

8. Abbreviations

DCMU	(3-(3′,4′-dichlorphenyl)-1,1-dimethylurea)
DMBQ	(2,5-dimethyl-*p*-benzoquinone)
PSII	photosystem II
Q_A	primary quinone electron acceptor of PSII
Q_B	secondary quinone electron acceptor of PSII
ROS	reactive oxygen species
S_2 state	oxidation state of the water oxidizing complex
TL	thermoluminescence

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