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Spectrally decomposed dark-to-light transitions in *Synechocystis* sp. PCC6803

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**Abstract**

Photosynthetic activity and respiration share the thylakoid membrane in cyanobacteria. We present a series of spectrally resolved fluorescence experiments where whole cells of cyanobacterium *Synechocystis* sp. PCC6803 and mutants thereof underwent a dark-to-light transition after different dark-adaptation (DA) periods. The following mutants were used: i) a PSI-lacking mutant (ΔPSI) and ii) M55, a mutant without NDH-1. For comparison, measurements of the wild-type were also carried out. The study consists of spectrally resolved fluorescence traces that were recorded over several minutes with 100 ms time resolution. The excitation light was 590 nm so as to specifically excite the phycobilisomes. In ΔPSI, DA time has no influence. In ΔPSI and dichlorophenyl-dimethylurea (DCMU)-treated samples we identify three main fluorescent components: PB-PSII complexes with closed (saturated) RCs, a quenched or open PB-PSII complex and a PB-PSII ‘not fully closed’. We postulate that the flavodiiron proteins Flv2/4 might form a pool of electron acceptors. For the PSI-containing organisms without DCMU, we conclude that mainly three species contribute to the signal: a PB-PSII-PSI megacomplex with closed PSII RCs and i) slow PB→PSI energy transfer, or ii) fast PB→PSI energy transfer and iii) complexes with open (photochemically quenched) PSII RCs. Furthermore, their time profiles demonstrate a light-adaptive response that we identify as a state transition. Our results suggest that deceleration of the PB→PSI energy transfer rate is the molecular mechanism underlying a state 2 to 1 transition.

This chapter has been submitted for publication.
4.1 Introduction

Cyanobacteria possess a remarkable thylakoid membrane that contains both photosynthetic and respiratory subunits. Photosynthetic activity is best described by the so-called Z scheme (167, 191, 196). Input light interacts with pigment–protein complexes, the phycobilisomes (PB), that absorb and transfer excitation energy downhill to a series of cofactors until it reaches a particular pair of chlorophylls (Chls), the special pair, located in the core pigment-protein complexes of photosynthesis: the photosystems (PS) I and II. The special pair is excited to a charge transfer state (197) from which an electron is released and, after being itself transferred through a series of cofactors, including primary electron acceptor Q_, it is expelled from PSII in the form of the twice reduced secondary electron acceptor Q_B (PQH_2) that physically wanders to the cytochrome b6f (cyt b6f). From there electrons are shuttled to a small copper containing protein, the plastocyanin (PC), that can deliver the electron to PSI. This, so-called, linear electron flow (LEF) finalizes with the electron transfer from PSI to ferredoxin (Fd) and to the ferredoxin–NADP+ reductase (FNR) which in turn catalyzes the reduction of NADP+ to NADPH (198).

Remarkable in cyanobacteria is that respiratory processes share some of the same components: the plastoquinone (PQ) pool, the PC pool, the cyt b6f and the cytochrome oxidase participate both in photosynthetic and respiratory activities (161, 168, 169, 199, 200). The respiratory subunits providing the electron input to the PQ pool are the type-I and –II NADPH dehydrogenase, NDH-I and NDH II, and the succinate dehydrogenase (SDH). NDH-I and SDH have been reported to be the main electron donors (165, 169). As for LEF, PQH_2 delivers the electrons to cyt b6f from where they either continue their way to the cytochrome oxidase via the PC pool and accomplish respiration or, alternatively, get shuttled to PSI and participate in cyclic electron flow (CEF) around PSI, which is a mechanism by means of which the cell adjusts the NADPH/ATP production ratio. Thus, as light input drops and photosynthetic electron flow stops, the PQ pool still serves as a vehicle for electron transfer related to ongoing respiratory activity.

It has been reported that the flavodiiron proteins (FDPs) provide alternative electron transfer pathways (201, 202) and are involved in photoprotection (195, 203, 204) although the specific function of these heterodimers remains a controversial topic. The cyanobacterium Synechocystis sp. PCC6803 (Synechocystis hereafter) has four FDPs: Flv1–4. While the pair Flv1/3 is involved in photoreduction of O_2 (194) after accepting electrons from PSI, Zhang et al. (2009) reported plummeting levels of evolved oxygen in a mutant of Synechocystis lacking Flv2/4 when exposed to high-light, making them more susceptible to photoinhibition, and therefore, suggesting that Flv2/4 can supply PSII with an additional channel for electron transfer to alleviate excitation pressure.
Crucially, Bersanini et al. (2014) (205) found that overexpression of the *flv4-2* operon in *Synechocystis* led to improved PSII photochemistry and kept the PQ pool in a predominantly oxidized redox state, providing unequivocal evidence that Flv2/4 acts as an important electron sink at the PSII acceptor side. The authors also report that a fully-assembled phycobilisome is required for the stable expression of the electron sink mechanism.

Extended periods (several minutes) of darkness are known to drive the cyanobacterial cell towards a low fluorescence state denominated state 2 (178, 206-208). This state is characterized by an increase of the effective antenna size of PSI. Exposing dark-adapted cells to light drives a state 2 to 1 transition, typically within 10-30 s (51, 186, 209, 210). State 1, is characterized by an increase in the effective antenna size of PSII, hence state 1 has a higher fluorescence yield (174). The cell is able to reversibly transition between state 1 and 2 (state transitions) depending on the light conditions in an effort to re-distribute excitation energy between PSI and PSII. See (49) for an in-depth review on state transitions.

This fluorescence study is an effort in disentangling these heavily intertwined energy and electron transfer pathways. After exciting PB with 590 nm light, spectrally resolved fluorescence traces were recorded over several minutes with 100 ms time resolution. The following mutants of *Synechocystis* were used: *i*) a PSI-lacking mutant (ΔPSI hereafter) and *ii*) a mutant without NDH-1 (M55 hereafter). For comparison measurements of the wild
type (WT) were also carried out. In absence of PSI, the cell lacks linear electron flow and, consequently, cannot produce NADPH, and it needs glucose to enable growth (180); also, it presumably possesses additional photoprotective mechanisms related to overexpression of the high-light inducible protein (Hlip) HliD (184, 185). By definition, state transitions do not take place in ΔPSI. The M55 mutant, lacking the ndhB gene, does not assemble the NDH-1 complexes (211). This is a highly disruptive mutation since it has been reported to be involved in both respiratory and photosynthetic activities (200, 212). Since NDH-1 serves as an electron acceptor for ferredoxin (Fd), it is involved in CEF, which helps increase the PMF, leading to increased synthesis of ATP. The M55 mutant retains the capacity to undergo state transitions (38). Furthermore, this mutant reportedly has impaired CO₂ uptake (213). In addition to the genetic disruptions, some samples have been treated with dichlorophenyl-dimethylurea (DCMU), a common inhibitor known for closing the PSII RCs as it blocks electron transfer from Qₐ to Qₐ.

Postulating what these fluorescence experiments might bring, Fig. 4.1 displays a cartoon overview of putative fluorescent species that may play role in the experiments reported in section 3. Beyond the evident species, i.e. the PB-PSII complex, a functional PB-PSII-PSI megacomplex (Fig. 4.1A/E/G) and the CpcG2-type of PB (Fig. 4.1B) are presented. The PB-PSII-PSI megacomplex has been isolated by Liu et al. (2013) (214) and Kondo et al. (2009) (215), and Gao et al. (2016) have reported on the CpcG2 type of PB (216). Chukhutsina et al. (2015) claim to observe such antenna in ultra-fast time-resolved fluorescence experiments (217). Additionally, we explore the possibility of a PB-PSII dimer complex with a single closed RC while the other remains open (Fig. 4.1D and F).

4.2 Materials and Methods

4.2.1 Cell cultures

Wild type *Synechocystis* was obtained from D. Bhaya (Stanford). Wild-type cells were grown in a photobioreactor in turbidostat mode (growth rate ca. 0.05 h⁻¹) at an optical density at 730 nm (OD₇₃₀) of 0.40 ±0.01, inoculated in modified BG-11 supplemented with 10 mM NaHCO₃ and a 636 nm light intensity of 80 µmol photons m⁻² s⁻¹ as measured outside the reactor, opposite and in the middle of the light panel.

The PSI-deficient mutant of *Synechocystis* (16) was a gift from C. Funk (Umeå University, Sweden) and was stored at -80 °C in 15 % glycerol. Prior to preparing a liquid culture, cells were streaked on BG-11 agar plates as described before (Chapter 3). The plates were incubated in an incubator (Versatile Environmental Test Chamber MLR-350H, Sanyo) with a humidified atmosphere of elevated CO₂ (2 % v/v) kept at 30 °C. Incident light intensity was reduced to below 5 µmol photons m⁻² s⁻¹ by covering the plates with layers of paper. Liquid cultures were prepared by inoculating 25 mL modified BG-11 (BG-11-PC, Chapter 2) supplemented with 10 mM glucose, 25 mM 1,4-Piperazinedipropanesulfonic acid (PIPPS)-KOH buffered at pH 8.0, 5 µg/mL chloramphenicol in a 100-mL flask (FB33131, Fisherbrand) and NH₄Cl as nitrogen source (10 mM). The flasks were covered in multiple layers of paper to reduce the incident light intensity to below 5 µmol photons m⁻² s⁻¹ and
were placed in a shaking incubator (Innova 44, New Brunswick Scientific), equipped with a custom LED panel containing LEDs of 632 nm (orange-red) and 451 nm (blue, both 8 nm full width at half maximum) at 120 rpm and 30 °C.

The M55 mutant was a gift from T. Ogawa (Nagoya University, Japan) and was grown in a chemostat kept at 30 °C, with a dilution rate of \( D = 0.027 \) h\(^{-1}\), 635 nm light intensity of 40 µmol photons m\(^{-2}\) s\(^{-1}\) and bubbled with 1 % (v/v) CO\(_2\) in N\(_2\).

4.2.2 Spectrally resolved fluorescence induction and data analysis

The multiple LED set-up described in Acuña et al. (2016), Lambrev et al. (2010) and Chapter 3 was used as described in Chapter 3. Data analysis was performed as detailed in Acuña et al. (2016) and expanded on in Chapter 3.

4.3 Results and discussion

First, we show results of the photosynthetically ‘simpler’ system, ΔPSI (which does not contain any of the species depicted in Fig. 4.1A, D, E and G) after a series of DA periods. Then, we move on to the PSI−containing samples of the strains M55 and WT, first showing results of DCMU-treated samples with two different DA periods, then, a series of several DA periods with intact cells in the absence of DCMU.

4.3.1 ΔPSI cells

The rank of the data matrices for all DA periods is at least three, which have been used to perform a decomposition analysis (Fig. 4.2). Based on visual inspection of the F660/F680 ratio and the relative amplitudes (fluorescence quantum yield) of the SASs, they are interpreted as follows: SAS\(_3\) (green) as a PB-PSII complex with closed RCs (Fig. 4.1H); SAS\(_2\) (red) could be a PB-PSII with a single PSII RC closed (Fig. 4.1F) or, alternatively, a PB-PSII complex whose PSII RCs are ‘closed’ in the sense that the primary quinone is unable to accept electrons but still transfers electrons to a Flv2/4 pool; and, SAS\(_1\) (black) as a PB-PSII complex with PSII RCs open or quenched by HliP (50). The rationale behind the interpretation of SAS\(_2\) lies in the decreasing \( c_2 \) (red) during saturation pulses, as opposed to the rising \( c_3 \) (green) which is formed as more light is shone onto the sample.

Thus, despite the strong Chl \( a \) signature of SAS\(_2\), the organism demonstrates the ability to close more RCs as \( c_2 \) decreases. Indeed, \( c_2 \) behaves as a partially ‘open’ species. The capacity to keep PSII RCs open, we suggest, stems from the additional Flv2/4 pool. Consequently, the very definition of ‘open’ would have to be extended; while hitherto ‘open’ exclusively referred to the PQ-pool ability to accept electrons from the PSII special pair, we will hereafter refer to two types of mechanisms that contribute to alleviating the excitation pressure on PSII RCs: \( pq \)-open when related to the capacity of the PQ-pool and \( flv \)-open when related to that of the Flv2/4 heterodimer. The fluorescence is directly proportional to the fraction of \( Q_{A'} \), and here we propose an additional route for the electron to escape from the RC.
Fig. 4.2. Spectral decomposition of six independent experiments with varying dark-adaptation (DA) periods carried out on ΔPSI cells. For each condition, a zoom view of the first (last) pulse is depicted in panels A, B, C, D, E and F (H, I, J, K, L and M). The full time profiles and corresponding SASs (shown as right insets) are depicted on panel G. The colored bar on top illustrates the light regime: 450 µmol photons m⁻² s⁻¹ of 590 nm light (orange) or darkness (black). Left insets depict the deviation in the sum of concentrations from unity.
Fig. 4.3. Spectral decomposition of fluorescence spectra of M55 cells previously treated with DCMU and no dark-adaptation. (A) The SAS (Red: SAS$_2$; green: SAS$_3$) obtained after transformation of the singular vectors. (B) Time profiles. Key: orange: 450 μmol photons m$^{-2}$ s$^{-1}$; black: darkness. The colored bar on top illustrates the light regime with arrows indicating the beginning of a saturation pulse. Inset: The deviation in the sum of concentrations from unity.

Therefore, SAS$_2$ (red) would correspond to a $pq$-closed but flv-open PB-PSII complex, whereas SAS$_3$ (green) would correspond to a $pq$-closed and flv-closed PB-PSII complex. Most probably the SAS of a PB-PSII complex with PSII RCs open or quenched by HliP differ slightly, which result in a data matrix of rank 4. Since the analysis is performed using the main three components, it is not possible to describe the data matrix fully. This manifests in the sum of concentrations shown in gray on the insets of Fig. 4.2. For short DA periods, the sum of concentrations is close to flat. As DA increases, however, it becomes increasingly difficult to obtain a fully flat pattern, especially during initial phases. Also, while SAS$_2$ and SAS$_3$ give robust results, SAS$_1$ because of its low $\phi_f$ provides the highest uncertainty. Even though a quenched component is consistently required to minimize the residuals, determining the exact shape and the F660/F680 ratio becomes non-trivial due to only subtle changes in the sum of concentrations profile. Certainly, the optimization routine may be compensating for the lack of the fourth component, in which case SAS$_1$ would be a mixture of the two quenched components.

Assuming so, this would explain that the $c_1$ (black) decreases during pulses, as expected for open complexes (Fig. 4.1C), while tending towards a baseline level of around 40 % (Fig. 4.2). These results suggest the existence of two quenched components of similar SAS, one of which is photochemically, the other non-photochemically quenched. Judging from the deviations in the sum of concentrations, the fourth putative quenched component plays a role predominantly during the first 30 s.

The fluorescence levels in ΔPSI show no systematic changes related to the DA periods as opposed to the observations in rank 2 analyses (Chapter 3). This is probably due to a crucial difference in the growth conditions of the different batches: while in previously NaNO$_3$ was used as the nitrogen source in the medium, here it was NH$_4$Cl. The former may result in a deficiency in nitrogen assimilation: reduction of NO$_3^-$ to NH$_3$ requires the direct output of PSI, i.e. reduced ferredoxin.
Fig. 4.4. Spectral decomposition of two independent experiments with two distinct dark-adaptation (DA) periods carried out on M55 (left) and WT (right) cells. Panels A and B depict a zoom view into the first and last pulse of M55 with DA = none. Panels C and D: M55, DA = 8 min; E and F: WT, DA = none; G and H: WT, DA = 8 min. The panels I and J show the full time profiles. The corresponding SASs and the deviation in the sum of concentrations from unity are shown as insets. The colored bar on top illustrates the light regime: 450 µmol photons m\(^{-2}\) s\(^{-1}\) of 590 nm light (orange) or darkness (black).

Thus, in the absence of PSI, this reaction is hampered. Additionally, NH\(_4\)Cl has a lower ‘electron cost’ than NaNO\(_3\) since NH\(_4\)\(^+\) can be directly used for metabolic reactions unlike NO\(_3\) which has to be first reduced to NH\(_3\) (a total of 8 electrons required). This may lead to less of an overload in the electron transfer pathways of this mutant and, as a result, the cell may need to undertake less regulatory mechanisms during darkness explaining why different DA periods do not result in the systematic patterns observed in Chapter 3. Nevertheless, the response is highly dynamic with none of the three concentration profiles standing out for being the predominant one. The black component is interpreted as a mixture of open PB-PSII complexes and a quenched species, presumably by HliPs, and it shows surprisingly high levels of 20-30%. The fact that the red component decreases during saturation pulses indicates that complexes are closing. A possible explanation could be, as introduced above, the presence of Flv2/4 pool that accepts electrons from QA. An alternative interpretation involves a PB-PSII complex with a single RC closed, as depicted in Fig. 4.1F.
Fig. 4.5. Spectral decomposition of six independent experiments with varying dark-adaptation (DA) periods carried out on M55 cells. For each condition, a zoom view of the first (last) pulse is depicted in panels A, B, C, D, E and F (H, I, J, K, L and M). The full time profiles and corresponding SASs (shown as right insets) are depicted on panel G. The colored bar on top illustrates the light regime: 450 µmol photons m\(^{-2}\) s\(^{-1}\) of 590 nm light (orange) or darkness (black). Left insets depict the deviation in the sum of concentrations from unity.
4.3.2 M55 and WT cells treated with DCMU

Reduced data matrix

We inspect the first 15 s of the data (rank 2) matrix. After transformation of the singular vectors we obtain the SASs shown in Fig. 4.3A (for consistency with other figures, referred to as SAS2 and SAS3 in red and green, respectively). The corresponding concentrations are shown in Fig. 4.3B. Therein, the fluorescence signal is dominated by SAS3 as evidenced by the high $c_3$ (green in Fig. 4.3B) levels. As the measurement was carried out using the inhibitor DCMU, we infer that this must be a species with closed PSII RCs. However, as argued by Acuña et al. (2016) before, the F660/F680 is larger in PSI–containing systems presumably due to EET supplied to PSI which is arranged in a PB-PSII-PSI megacomplex conformation (214). Thus, SAS3 is interpreted as a PB-PSII-PSI megacomplex with closed PSII RCs. The second species (SAS2, in red) is also a highly-fluorescent one with a somewhat lower fluorescence yield and compared to SAS3, lacking some 660 nm (PB) emission.

The two concentrations show correlated increase/decrease during the saturation pulses, after which the F$_{S}$ level of $c_2$ (red) is fully restored (ca. 15 %). This opening/closing behavior may be counter-intuitive since DCMU inhibits $Q_A \rightarrow Q_B$ electron transfer leading to the assumption that all the RCs are closed and therefore, no open species should be observable. We hypothesize that DCMU ensures that all RCs are pq-closed, but that alternative electron transport via the Flv2/4 pathway would remain accessible. In other words: the complexes are pq-closed but flv-open. Furthermore, this mechanism is likely to bypass $Q_B$ as DCMU treatment does not lead to flv-closure, but further experiments are required to further test this hypothesis. Independently from the proposed mechanism, this experiment reveals that even when cells have been treated with DCMU, a certain number of PSII RCs can still be fully (i.e. pq + flv) closed when light intensity increases. Consequently, this number of closable RCs was flv-open before the saturation pulse was applied. The two SASs obtained in this section are used as a guide for the analyses of rank 3 systems presented in the following sections.

Full data matrices of DCMU-treated samples

Full matrices are all of rank 3. The full analyses of WT and M55 cells pre-treated with DCMU and after two different DA periods are shown in Fig. 4.4I and J. The three SASs are shown as the insets and two of them, SAS2 and SAS3, are in good agreement with the ones shown in Fig. 4.3A. Additionally, a quenched species (black) is present in all samples. The interpretation of SAS3 (green) is a PB-PSII-PSI complex with fully (pq+flv) closed RCs. Here again, SAS2 (red) has a quenched 660 nm emission relative to SAS3 and this is interpreted as a PB-PSII-PSI complex that is pq-closed but flv-open. SAS1 (black) is interpreted as a species quenched by HliP, which may be flv-open or flv-closed. Saturation pulses are still able to close a certain fraction of PSII RCs as manifested in the decrease of both $c_1$ and $c_2$. 
Fig. 4.6. Spectral decomposition of six independent experiments with varying dark-adaptation (DA) periods carried out on WT cells. For each condition, a zoom view of the first (last) pulse is depicted in panels A, B, C, D, E and F (H, I, J, K, L and M). The full time profiles and corresponding SASs (shown as right insets) are depicted on panel G. The colored bar on top illustrates the light regime: 450 µmol photons m⁻² s⁻¹ of 590 nm light (orange) or darkness (black). Left insets depict the deviation in the sum of concentrations from unity.
Interestingly, the fraction that can be closed in M55 is smaller than in WT, suggesting that a fully assembled NDH-I unit (in the presence of DCMU) contributes to efficiently alleviating excitation pressure on PSII. As for the time profiles, we read the following: for 8 min dark-adapted samples, \( c_1 \) shows a slight increase suggesting that formation of the quenched species (black) is favored in darkness. This is valid for both M55 (cf. Fig. 4.4A and C) and WT (cf. Fig. 4.4E and G). Also visible on these panels is the fact that WT forms more of the quenched species as compared to M55. Note that \( c_1 < c_2 \) systematically for M55 as background light sets in. This ratio is reversed for the WT. Moreover, while M55 very quickly finds a steady-state level that varies little over time, WT shows an equilibration phase of ca. 10 s during which the quenched species decreases and then levels off.

![Fig. 4.7. Zoom-view into the first minute of the light protocol for WT and the M55 mutant. The profile \( c_2 \), corresponding to megacomplexes with slow PB to PSI energy transfer is shown for all DA periods. Background illumination sets in at \( t = 7 \) s. Immediately afterwards, three intervals are indicated: A: first 300 ms; B: 300 ms < \( t < 13 \) s and C: 13 s < \( t \). A is characterized by a rapid population increase and decrease presumably due to closure of a single RC followed by a population of \( c_3 \) (Fig. 4.9), B is characterized by a steady increase reaching a maximum and during C the profiles decline steadily again. In the case of M55 cells, long DA times display a longer period of steady increase, \( B_2 \), reaching a maximum value at \( t = 20 \) s.](image)

### 4.3.3 M55 cells without DCMU

The full analyses of M55 cells that have undergone different dark-adaptation periods are shown in Fig. 4.5. The SASs are interpreted as follows: SASs (green) as a PB-PSII-PSI complex with moderate EET from PB to PSI and fully \((pq + flv)\) closed PSII RCs (see Fig. 4.1G and Fig. 4.4I), SASs (red) as a PB-PSII-PSI megacomplex with fast energy transfer
to PSI and with $pq$ closed PSII RCs (see Fig. 4.1E), and, SAS$_1$ (black) as a PB-PSII(-PSI) complex with fully open PSII RCs (see Fig. 4.1A). EET from PB to PSI most probably occurs via ApcD (218) and depending on whether it is slow or fast, more or less 680 nm light is able to escape. This would explain the strong decrease in the F660/F680 ratio which serves as a proxy for how much excitation light was ultimately trapped by PSI. The corresponding concentration profiles also show interesting dynamics: unlike the DCMU-treated samples, the shape of the pulses resembles much less that of a step function (cf. Fig. 4.4C and Fig. 4.5A); take for instance $c_3$ which after reaching a maximum within the first 200-300 ms, decreases as much as 10 % within the duration of the pulse. This suggests efficient electron transfer from PSII to the PQ pool and subsequent re-opening of PSII RCs. The behavior of $c_2$ is of particular interest: while for short DA periods, during the first pulse, $c_2$ tends towards zero. For DA = 8 min, $c_2$ averages to 5 %, and it increases to 14 % and 19 % for DA = 21 and 34 min, respectively. Hence, darkness favors the formation of the species SAS$_2$. Seemingly, this occurs at the expense of the species SAS$_3$ only. Thus, for longer DA periods, $c_2$ ($c_3$) has a larger (smaller) initial concentration and, as background light sets in, it continues to gradually increase (decrease) during the first 10–20 s resulting, for long DA periods, in a momentarily larger concentration of SAS$_2$ than SAS$_3$. Then, $c_3$ tends to a $F_3$ level of ca. 40 % and it restores that level quite consistently regardless of the initial conditions.

Fig. 4.8. Zoom-view into the first minute of the light protocol for WT and the M55 mutant. The profile $c_3$, corresponding to megacomplexes with fast PB to PSI energy transfer is shown for all DA periods. The decrease in the initial concentration values correlates with increasing DA periods. This is interpreted as a state 1 to 2 transition. Background illumination trigger a state 2 to 1 transition which results in a relative increase of ca. 20 %.
We attribute this behavior to a state 2 to state 1 transition. Furthermore, the $c_2$ $F_S$ value is also ca. 40% in all experiments, thus the background light is only able to close 60% of the PSII RCs. In both WT and M55, DA favors the formation of a megacomplex in which fast energy transfer from PB to PSI (see Fig. 4.1E) is ensured (214), consequently increasing its antenna size and ultimately affecting the ratio CEF/LEF.

In DCMU–treated samples (Fig. 4.3), the reversibility of the signal induction suggests that no photoinhibition occurs. Rather, we interpret the additional closure to be that due to the Flv2/4 pool (flv-closure). Although the exact binding site and rate are still unknown for Flv2/4, the assumption in this case would have to be that $Q_b$ is not involved in the electron transfer. Instead, we observe a quenched 660 nm emission which would align well with the observations by Bersanini et al. (2014) that point towards Flv2/4-related activity with concomitant phycobilisome co-operation. Indeed, even the properties of a PB “super core” as recently proposed by Zlenko et al. (2017) might be required (219). Furthermore, as manifested in the lower $F_S$ levels of $c_3$ in WT, the amount of flv-closure is ca. 20% larger (for both DA periods) in the WT than it is M55. This directly points at a role of NDH-I in keeping the PQ-pool reduced (220). Certainly, there must be additional acceptors to Flv2/4 that explain why, despite NDH-I dysfunction, the RCs are not fully flv-closed in M55. Spectrally resolving the fluorescence signal of DCMU pre-treated cells of a Δflv2/4 mutant would help solidifying this hypothesis.

4.3.4 WT cells without DCMU

The full analyses of WT cells that have undergone different dark-adaptation periods are shown in Fig. 4.6. The SASs are interpreted as in the previous section. Also in this series of experiments, the initial $c_2$ increases with longer DA periods, and, as visible from the first pulse of every experiment (see Fig. 4.6A–F), it reaches up to 40% for DA=34 min. As background illumination sets in, the levels of open and closed complexes (black and green) are similar. For all experiments, the concentration $c_1$ (open complexes) also shows a relatively steady level throughout the whole period of background illumination only decreasing during saturation pulses. Similar to the M55 mutant, the initial $c_2$ and $c_3$, depend on DA.

4.3.5 Time dependence in M55 and WT cells without DCMU

Without DCMU, i.e. with unblocked quinone acceptors, the F660/F680 of SAS2 greatly increases evidencing the efficient $pq$-reopening of RCs. Also, the time profiles, especially $c_2$ and $c_3$, are much more dynamic. Fig. 4.7 and Fig. 4.8 show $c_2$ and $c_3$, respectively, during the first minute of the light protocol. In Fig. 4.7, the zoom view of $c_2$, which corresponds to the concentration of a PB-PSII-PSI complex with fast energy transfer to PSI, reveals roughly three different phases: as background illumination sets in, there is a quick equilibration that happens within 300 ms and that we refer to as phase A. During this phase, the $c_2$ profile quickly decreases until a minimum value is reached while $c_3$ shows a correlated increase, as observed during the first data points of the saturation pulse applied in darkness.
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Fig. 4.9. Left: Selection of SASs from experiments with DA = 34 min. The spectra have been normalized relative to the PB emission at 640 nm. Key: green: SAS 3 in WT; light green: SAS 3 in M55; red: SAS 2 in WT; orange: SAS 2 in M55; light green dashed: SAS 3 in ΔPSI. Right: Zoom-in of the first minute of the experiment. The inferred fluorescent species are shown as an inset and correspond to Fig. 4.1E and G.

We conclude that this is due to opening/closing of RCs which means that SAS 2 has a hybrid nature. A possible explanation could be that the PB-PSII-PSI megacomplex with fast energy transfer to PSI and *pq*-closed ([Fig. 4.1E](#)) goes from *flv*-open to *flv*-closed within these first 300 ms of illumination.

The phase B (7.5 < *t* < 13 s) is characterized by a progressive increase of *c* 2 meaning that the cell first works on decreasing the excitation pressure on PSII by increasing the energy transfer to PSI. This may be interpreted as an effort to increase CEF around PSI. In the WT, this phase consistently lasts ca. 5 s for all DA periods. After reaching a maximum, *c* 2 enters a phase C of steady decline attributed to state 2 to 1 transition and which leads to an increase in PB-PSII-PSI complexes with slow energy transfer to PSI (see [Fig. 4.1G](#)). In M55, however, long DA periods result in a delayed state 2 to 1 transition (see [Fig. 4.7](#)). For DA of 1, 3, 5 and 8 min the phase B1 is as long as the phase B observed in WT, but for DA = 21 and 34 min (orange and red in [Fig. 4.7](#)), the phase B2 extends until *t* = 20 s. Seemingly, once a certain number of megacomplexes with fast PB→PSI energy transfer has been formed and with a lacking NDH-I unit, which plays a role in CEF, the cell needs a longer time to reach the convenient CEF/LEF ratio before triggering a state 2 to 1 transition.

In [Fig. 4.8](#), the concentration profiles of PB-PSII-PSI complexes with slow energy transfer to PSI (*c* 3) are shown. The first peak illustrates their decreasing concentration (state 1 to 2 transition) assumed to be due to the membrane’s re-arrangement during darkness related to respiratory activity. Based on the stoichiometry published by Moal and Lagoutte (2012) that suggests a highly dense packing of pigment-protein complexes in *Synechocystis*, we speculate that the membrane’s conformational change during darkness could be a ‘ruffling’ of the membrane which would yield an even denser packing favoring fast energy transferring PBs. Physical re-distribution of the complexes could result in slowing down the EET rate to PSI.

A summary illustration is given in [Fig. 4.9](#), which shows traces for DA periods of 34 min exclusively. The spectra shown have been normalized relative to the 640 nm phycocyanin
emission which is expected to be the same in all samples. For comparison, SAS\textsubscript{3} from ΔPSI is also shown, where the small F\textsubscript{660}/F\textsubscript{680} ratio comes to be through the intense Chl \textit{a} emission that peaks at 680 nm. This result confirms the analyses of independent measurements in the ΔPSI carried out in a set-up in Třeboň, CZ (see Fig. 7 in Acuña et al. (2016)). Moreover, the F\textsubscript{660}/F\textsubscript{680} ratio is consistently lower (cf. SAS\textsubscript{2} and SAS\textsubscript{3}) in the M55 mutant than in the WT, which points at a larger amount of energy back-transferred to PB in M55, which may be due to the hampered CEF in M55. While the F\textsubscript{5} levels of \textit{c\textsubscript{2}} and \textit{c\textsubscript{3}} are similar in both WT and M55, clearly, \textit{c\textsubscript{3}} (\textit{c\textsubscript{2}}) reaches higher (lower) F\textsubscript{M} values in the M55 mutant. This is consistent with the idea that a fully assembled NDH-I enhances the ability of the cell to cope with a high number of excitations. While in the DCMU (\textit{pq}-closure) results discussed in section 4.3.2, this expresses in the form of lower F\textsubscript{5} levels in the WT, in Fig. 4.9, the F\textsubscript{V} values are systematically lower in the WT, i.e. the saturation pulses are able to (\textit{pq+flv}) close less efficiently the RCs of the WT than those of the M55, thus evidencing that, under the same illumination conditions, the WT manages to keep more RCs (\textit{pq+flv}) open than M55 does, in agreement with the conclusions of DCMU-treated cells.

4.4 Concluding remarks
We have presented a series of experiments during which WT and mutants thereof undergo a dark-to-light transition after being prepared under different conditions. The data matrices have been analyzed as rank 3 systems. Two major findings are presented: first, from the DCMU experiments we conclude the existence of a pool of PSII electron acceptors additional to the PQ pool. We postulate that the Flv 2/4 proteins build such a pool. The presence of such a pool implies that a PSII RC is fully open when it is both \textit{pq}- and \textit{flv}-open and the reverse. In the DCMU experiments, the F\textsubscript{5} levels suggest PSII RCs that are in the state ’\textit{pq}-closed/\textit{flv}-open’ (Fig. 4.4). Moreover, the PSII→Flv2/4 electron transfer mechanism \textit{i}) may require a high energy back transfer rate to the PB and \textit{ii}) increases its efficiency with a well-functioning NDH-I complex which points to a possible (so far unknown) electron acceptor to the Flv2/4 heterodimer.

A fundamental assumption in this reasoning implies that electrons transferred to the Flv2/4 pool bypass the secondary quinone Q\textsubscript{B}. Second, we postulate that SAS\textsubscript{2} in Fig. 4.6 is a PB-PSII-PSI megacomplex with fast EET to PSI and \textit{pq}-closed. A slow EET to PSI results in SAS\textsubscript{3} in Fig. 4.6. During darkness, the thylakoid membrane may ‘ruffle’ leading to a more tightly packed ensemble of pigment-protein complexes favoring fast EET of PB to PSI. This would be the state 1 to 2 transition. With the onset of photosynthetic activity, the membrane ‘stretches’ and the pigment-protein complexes re-distribute over the membrane leading to the loosening of the PB-PSI coupling and ultimately slowing down EET. This, in turn, would be the state 2 to 1 transition. This interpretation aligns well with the generally accepted idea that state transitions are a mechanism by means of which the energy inputs to PSI and PSII are optimized resulting in a low (state 2) and in a high (state 1) fluorescent state. With the current spectral and time resolution of the set-up, we do
not see any evidence for CpcG2-type of PB nor uncoupled PB. Furthermore, it is also not possible to resolve PB-PSII-PSI megacomplexes and PB-PSII complexes when PSII RCs are (either \( pq \)- or \( flv \)-)open. Though this study may provide important hints of the molecular mechanism of state transitions, further studies involving multiple excitation wavelengths could help establishing the precise relationship between the LEF/CEF ratio and the molecular triggers of state transitions.

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