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Net light-induced oxygen evolution in photosystem I deletion mutants of the cyanobacterium *Synechocystis* sp. PCC 6803 ☆

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ABSTRACT

Oxygenic photosynthesis in cyanobacteria, algae, and plants requires photosystem II (PSII) to extract electrons from H_2O and depends on photosystem I (PSI) to reduce $NADP^+$. Here we demonstrate that mixotrophically-grown mutants of the cyanobacterium *Synechocystis* sp. PCC 6803 that lack PSI (ΔPSI) are capable of net light-induced O_2 evolution *in vivo*. The net light-induced O_2 evolution requires glucose and can be sustained for more than 30 min. Utilizing electron transport inhibitors and chlorophyll *a* fluorescence measurements, we show that in these mutants PSII is the source of the light-induced O_2 evolution, and that the plastoquinone pool is reduced by PSII and subsequently oxidized by an unidentified electron acceptor that does not involve the plastoquinol oxidase site of the cytochrome b_6f complex. Moreover, both O_2 evolution and chlorophyll *a* fluorescence kinetics of the ΔPSI mutants are highly sensitive to KCN, indicating the involvement of a KCN-sensitive enzyme(s). Experiments using ^{14}C-labeled bicarbonate show that the ΔPSI mutants assimilate more CO_2 in the light compared to the dark. However, the rate of the light-minus-dark CO_2 assimilation accounts for just over half of the net light-induced O_2 evolution rate, indicating the involvement of unidentified terminal electron acceptors. Based on these results we suggest that O_2 evolution in ΔPSI cells can be sustained by an alternative electron transport pathway that results in CO_2 assimilation and that includes PSII, the platoquinone pool, and a KCN-sensitive enzyme.

1. Introduction

The light-driven reactions of photosynthesis in cyanobacteria, algae, and plants are described by a Z-scheme, the linear electron transport pathway from H_2O to ferredoxin (Fd) and $NADP^+$ that generates O_2 as a byproduct and drives the assimilation of CO_2 [1–3]. This linear electron transport pathway depends on photosystem II (PSII) [4], photosystem I (PSI) [5] and the cytochrome b_6f (Cyt bf) complex [6]. Although the Z-scheme serves as the dominant pathway in oxygenic photosynthesis, PSII-driven electron transport from H_2O without the involvement of PSI has been suggested [7–10]. Govindjee et al. [7] proposed an alternative electron transport pathway in algal cells in which PSII could reduce $NADP^+$ independent of PSI — a pathway similar to that in photosynthetic bacteria. Arnon ([9] and the references therein) proposed a more complicated pathway for the reduction of Fd by PSII — a pathway that required plastocyanin, but not PSI. Several PSI deletion mutants of *Chlamydomonas reinhardtii* have been shown to evolve O_2 in the light [11–13]. However, the rate of light-induced O_2 evolution in these mutants was lower than

Abbreviations: Chl, chlorophyll; PSII, photosystem II; Cyt bf, cytochrome b_6f; Cyt c_6, cytochrome c_6; PSI, photosystem I; Fd, ferredoxin; FNR, ferredoxin-$NADP^+$-oxidoreductase; Pheo, pheophytin; FQR, ferredoxin-plastoquinone-oxidoreductase; PQ, plastoquinone; PQH_2, plastoquinol; P680, special pair of Chl *a* molecules for primary photochemistry in PSII; P700, special pair of Chl *a* molecules for primary photochemistry in PSI; Q_A, first plastoquinone electron acceptor of PSII; Q_B, second plastoquinone electron acceptor of PSII; Q_o, the plastoquinol oxidase site of the Cyt bf complex; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; KCN, potassium cyanide; FeCN, potassium ferricyanide; DCBQ, 2,6-dichloro-*p*-benzoquinone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCPIP, 2,6-dichlorophenolindophenol; MV, methyl viologen; WT, wild type; ΔPSI, PSI deletion; PEP, phosphoenol pyruvate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase

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that of respiration, resulting in net O_2 uptake. In addition, neither CO_2 assimilation nor H_2 production was observed in these mutants.

The observation of net light-induced O_2 evolution in the absence of PSI was first reported in two PSI deletion mutants (ΔPSI) of the cyanobacterium *Synechocystis* sp. PCC 6803 ([8] and L. B. Smart, personal communication). Smart et al. [8]. speculated that the plastoquinone (PQ) pool, $NADP^+$, H^+ and/or O_2 may serve as the terminal electron acceptor(s) for the PSI-independent O_2 evolution. Additional ΔPSI mutants of *Synechocystis* 6803 have been generated in the laboratories of Himadri Pakrasi (Washington University, St. Louis, MO) and Wim F. J. Vermaas (Arizona State University, Tempe, AZ) [14–17]. Vermaas and co-workers (see e.g. [18–20].) proposed PSII-driven electron transfer from H_2O to O_2 *via* a pathway that involved PQ and potassium cyanide (KCN)-sensitive respiratory terminal oxidases. However, Vermaas and co-workers did not observe net light-induced O_2 evolution in the absence of PSI.

In the present work, we report that in several mixotrophically-grown ΔPSI mutants of *Synechocystis* 6803, the rate of PSII water oxidation in the light in the presence of glucose and $NaHCO_3$ is significantly faster than the respiration rate, resulting in net O_2 evolution. Inhibitor studies and chlorophyll (Chl) *a* fluorescence measurements demonstrate the presence of electron transport from H_2O to the PQ pool and the involvement of KCN-sensitive enzyme(s) in the ΔPSI mutants. We show that although the ΔPSI mutants are capable of light-minus-dark CO_2 assimilation, the rate is about half of that required to account for the rate of net O_2 evolution.

2. Materials and methods

2.1. Mutants and growth conditions

The glucose tolerant wild type (WT) strain of *Synechocystis* sp. PCC 6803 and six PSI deletion mutant strains were gifts from Himadri Pakrasi (E. Zak and H. Pakrasi, personal communication) and W. F. J. Vermaas [14–17]. Details of these mutants are summarized in Table 1. These cyanobacterial strains were maintained according to the methods provided by the laboratories donating the strains. The antibiotics and glucose concentrations used for the BG-11 plates were as follows: 50 μg/ml kanamycin and 10 mM glucose for ΔAB; 25 μg/ml spectinomycin and 10 mM glucose for ΔVIII–XI; 25 μg/ml chloramphenicol and 15 mM glucose for ΔPSI/WV; 10 μg/ml erythromycin, 25 μg/ml chloramphenicol and 15 mM glucose for ΔPSIΔApcE; 15 μg/ml zeocin, 25 μg/ml erythromycin, 35 μg/ml chloramphenicol, 25 μg/ml spectinomycin and 15 mM glucose for ΔPSIΔNdbABC; 25 μg/ml erythromycin, 35 μg/ml chloramphenicol, 25 μg/ml spectinomycin and 15 mM glucose for ΔPSIΔCtaDIIEIIΔCydAB.

The WT cells were cultured in the BG-11 medium in the presence or the absence of 5 mM glucose at 30 °C under 65 μmol photons·m^{-2}·s^{-1} fluorescent light. All of the ΔPSI mutant strains were grown in the presence of 5 mM glucose at 30 °C without antibiotics. All of the ΔPSI mutant strains except for the ΔPSIΔApcE strain were grown under ca. 1.5 μmol photons·m^{-2}·s^{-1} fluorescent light. The ΔPSIΔApcE strain was grown under 65 μmol photons·m^{-2}·s^{-1} fluorescent light. The cell cultures were bubbled with water-saturated air through a sterilized filter (Gelman #4210, 0.3 μm pore size). Cells were grown in the BG-11 medium either with or without the supplement of 1 μM Cu^{2+} (in the form of $CuSO_4$), which has been previously reported to be sufficient for these cells to express either plastocyanin or Cyt c_6, respectively [21]. The absence of plastocyanin in the cells grown without the Cu^{2+} supplement[3] was indirectly examined by the presence of the light-minus-dark absorption peak of Cyt c_6 at 553 nm. The cell density was monitored by measuring the optical density of the cell cultures at 730 nm [22].

Table 1
The ΔPSI mutants used in this study.

Name	Mutation	Reference
ΔAB	A significant portion of the *psaAB* operon was deleted	E. Zak and H. Pakrasi, personal communication
ΔVIII–XI	A part of the *psaB* gene that encoded the VIIIth to XIth helices of the PsaB protein was deleted	E. Zak and H. Pakrasi, personal communication
ΔPSI/WV	PSI was deleted	[14]
ΔPSIΔApcE	Both PSI and the phycobilisome linker protein ApcE were deleted	[15]
ΔPSIΔNdbABC	Both PSI and the type 2 NADH dehydrogenase Ndb were deleted	[16]
ΔPSIΔCtaDIIEIIΔCydAB	Both PSI and the alternative terminal oxidases CtaII and Cyd were deleted	[17]

2.2. Measuring the net light-induced O_2 evolution and dark respiration rates and the PSII activity

Cells were harvested during exponential growth phase by centrifugation at 5000 *g* for 5 min at room temperature and kept in fresh BG-11 medium in the presence of 5 mM glucose (unless noted otherwise) prior to the measurements. This glucose concentration was sufficient for maintaining the activities during the measurements. Based on the growth curves, it was also sufficient to sustain cell growth until stationary phase. The rates of the net light-induced O_2 evolution and the dark respiration of whole cells were monitored at 30 °C by a Clark-type electrode (YSI 5331 oxygen probe, Yellow Springs, OH) with a polarizing voltage of −0.7 V [23,24]. The actinic light was provided by a pair of Tungsten-halogen lamps (EG&G, 250 W) illuminating from both sides of the reaction chamber after being filtered through pairs of heat-reflecting filters (Melles Griot 03MHG007), heat absorbing blocking filters (Corning CS I-75, Rochester, NY), red blocking filters (Corning CS 2-63, Rochester, NY) and appropriate neutral density filters. The light intensity for reaching maximal net O_2 evolution rate was measured to be 500 μmol photons·m^{-2}·s^{-1} (same on each side) by a Licor Quantum Photometer LI-185B (Lincoln, NE). Sodium dithionite was used to calibrate the electrode. $NaHCO_3$ was added at 10 mM concentration to the cell suspensions in the reaction chamber right before the measurements.

For testing 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) adaptation, the WT cells were also grown in the presence of micromolar DBMIB and 5 mM glucose under ca. 1.5 μmol photons·m^{-2}·s^{-1} light intensity for a total of two inoculation cycles: the concentration of DBMIB on the day of each inoculation was 10 μM; an extra dose of DBMIB (equivalent to 2 μM final concentration) was supplied everyday afterwards to compensate for the light-sensitive loss of DBMIB. For titrating the net O_2 evolution rates of these DBMIB-adapted cells with DBMIB, the cells were washed with fresh BG-11 three times to remove DBMIB that was present during the cultivation.

The PSII activity was measured as the saturated light-induced O_2 evolution rate in the presence of artificial PSII electron acceptors (i.e., 1.2 mM potassium ferricyanide (FeCN) and 600 μM 2,6-dichloro-*p*-benzoquinone (DCBQ)) and with the saturating actinic light intensity as high as 3600 μmol photons·m^{-2}·s^{-1}.

2.3. Membrane preparation

Membranes from the *Synechocystis* 6803 cells were prepared as previously described [25] with minor modifications. All steps were carried out at 4 °C. The cell pellet from 50 ml cell culture was re-suspended in 1 ml breakage buffer containing 50 mM HEPES/NaOH (pH 7), 0.5 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, 0.1% (v/v) Protease

[3] No special procedure was employed to remove trace amounts of Cu^{2+}.

Inhibitor Cocktail (Sigma), and 4 μg/mL DNase I. These cells were broken by glass beads (size 150–212 μm, acid-washed, Sigma) with 5 cycles of 2 min of vortexing followed by 2 min of cooling on ice. After the final cycle, the glass beads were allowed to settle and the homogenate was collected. The glass beads were washed several times and the homogenates were pooled. Debris spin was carried out at 8160 *g* (Eppendorf, model Centrifuge 5415 C) for 5 min. The supernatant was collected and centrifuged again at 16,000 *g* for 30 min. The membrane pellet was collected and re-suspended in the breakage buffer.

2.4. Chl concentration, Chl-to-PSII ratio and PSII concentration determination

Concentration of Chl in *Synechocystis* 6803 cells was spectrophotometrically determined [26]. The Chl-to-PSII ratios of the membrane preparations were determined by measuring their O_2 evolution activity driven by single-turnover flashes [23]. The actinic light was provided by 600 flashes (frequency 10 Hz, width 6 μs) of a Xe-flash lamp (FX-200; EG&G, Salem, MA). The actinic flashes were filtered by a yellow filter (Corning CS 3-71, Rochester, NY) and 1 cm of water. The Xe-flash lamp was driven by 700 V to yield saturating light intensity. The O_2 evolution rate of the membranes was measured at 30 °C using a Clark-type oxygen electrode, as described in Section 2.2. The reaction buffer contained 50 mM MES-NaOH (pH 6.0), 10 mM $MgCl_2$, 5 mM $CaCl_2$, 5 mM NaCl and 0.5 M sucrose. The optimal O_2 evolution rate was reached when the reaction mixture contained 250 μM FeCN and 100 μM DCBQ. Varying the saturating single-turnover flash number between 400 and 800 or the flash frequency between 5 and 20 Hz had no effect on the O_2 evolution rate. When calculating the Chl-to-PSII ratios, we assumed that one O_2 molecule was evolved per PSII per four flashes. The PSII concentration of each sample was determined by converting the Chl concentration using corresponding Chl-to-PSII ratio. In our work, the net light-induced O_2 evolution rate and light-minus-dark CO_2 assimilation were calculated on a PSII basis, because the PSI-less mutants of *Synechocystis* 6803 had a much lower Chl-to-PSII ratio than WT. In WT, 80–85% of all Chl is associated with the PSI core complex, which is lost in the PSI-less mutants [15]. Because of the low Chl-to-PSII ratio, most PSI-less mutants could be grown only under low light, except for the ΔPSIΔApcE strain in which the light harvesting complex was impaired in addition to PSI.

2.5. The kinetics of PSI reaction center, P700

Flash-induced P700 oxidation and re-reduction kinetics of the membrane preparations were measured at room temperature using a laboratory-built single-beam spectrophotometer as described earlier [27]. The saturating single-turnover actinic flashes (width, 6 μs) were provided by a pair of Xe-flash lamps (FX-200; EG&G, Salem, MA) from both sides of the sample cuvette to induce a rapid oxidation of the PSI primary donor P700. The actinic flashes were filtered by two broad-band interference filters (DT-Blau, Balzers) and two blocking filters (Corning CS 4-96, Rochester, NY). The flash-induced P700 oxidation and re-reduction kinetics were recorded at discrete wavelengths ranging from 665 nm to 830 nm. Typically, at each wavelength, data from 8 runs were averaged. The reaction buffer for P700 measurements contained 50 mM Tris–HCl (pH 8.3), 33 μM 2,6-dichlorophenolindophenol (DCPIP), and 1.7 mM sodium ascorbate. Chl concentration was adjusted to be between 20 and 30 μM for best signal-to-noise ratio and to get desirable PSII concentration. The samples were stirred after measuring absorbance at each wavelength. The experiments were carried out at 24 ± 1 °C.

2.6. Chl a fluorescence kinetics

Chl *a* fluorescence kinetics was monitored using a dual-modulation kinetic fluorometer (PSI, Brno, Czech Republic). The single-turnover, saturating flashes were provided by diodes (LEDs) emitting at 660 nm. The measuring flashes were provided by LEDs emitting at 620 nm [28]. Cells at exponential growth phase were resuspended in fresh BG-11 medium in the presence of 5 mM glucose. The cell density was adjusted so that Chl concentration was ca. 1 μM for best signal-to-noise ratio. Cells were dark adapted for 10 min before each experiment. For measuring the Chl *a* fluorescence induction kinetics, three data points (1 ms apart) were collected in darkness for measuring the F_0 level. Afterwards, a train of 5000 saturating, single-turnover flashes were fired at 1 kHz for 5 s. For measuring the Chl *a* fluorescence dark relaxation kinetics, the actinic light was turned off at 370 ms when the Chl *a* fluorescence reached the maximum, and subsequently the redox states of Q_A^- and the PQ pool were probed for ca. 20 s by logarithmically spaced measuring flashes. The duration and intensity of the measuring flashes were adjusted to minimize their actinic effect. The experiments were carried out at 24 ± 1 °C.

2.7. Measurements of the CO_2 assimilation rates

The CO_2 assimilation rates were measured using ^{14}C-labeled $NaHCO_3$ [29]. Cells at the exponential growth phase were harvested. The cell pellet was re-suspended in freshly prepared HCO_3^--less BG-11 medium. $NaH^{14}CO_3$ was diluted with cold $NaHCO_3$. The final $NaHCO_3$ concentration in the reaction mixtures was either 5 mM or 10 mM for several independent experiments. An aliquot of 0.85 ml cell suspension combined with 0.85 ml diluted $NaH^{14}CO_3$ was used for O_2 evolution measurement and another aliquot was kept in the dark as the control. After the O_2 evolution measurement, both the sample and the control were quickly treated with a strong acidic solution containing 0.7 ml of 4 M formic acid and 1 M HCl to break the cells in order to release un-incorporated inorganic carbon. Both the sample and the control were dried overnight in the hood at 65 °C with air blowing over them. Then 0.5 ml of 0.1 M HCl was added to each vial to dissolve the organic forms of carbon. After addition of the scintillation cocktail to the sample, radioactivity of the sample was counted in a liquid scintillation counter (liquid scintillation analyzer, Packard, model tri-carb 1600tr). The specific activity was calculated from three replicas of the diluted $NaH^{14}CO_3$ aliquots. The calculated specific activities for these experiments ranged from 200 to 1000 cpm/nmol $NaH^{14}CO_3$.

3. Results

3.1. Spectroscopic measurements confirm the absence of PSI activity in Synechocystis 6803 mutant strains lacking PSI

We characterized six ΔPSI mutants, which were generated in the laboratories of Himadri Pakrasi and Wim F. J. Vermaas (Table 1). In each of these mutants, a significant portion of either the *psaB* gene (encoding for the PSI core protein PsaB) or the *psaAB* operon (encoding for the PSI core proteins PsaA and PsaB) was replaced by an antibiotic resistance marker. These ΔPSI mutant strains were completely segregated and the absence of PSI was confirmed in their original laboratories by Southern and Western blots (E. Zak and H. Pakraski, personal communication and [14–17]) (see discussion in Supplementary Material S1). We further examined the absence of PSI in these mutant strains by measuring their P700 oxidation and re-reduction kinetics. When a saturating single-turnover flash was applied to the WT membranes, a large absorbance change reflecting a rapid light-induced oxidation of P700 (peak 703 nm), followed by its re-reduction in the dark, was observed. In contrast, such P700 oxidation transient was not detected in the membranes prepared from the ΔPSI (ΔAB)

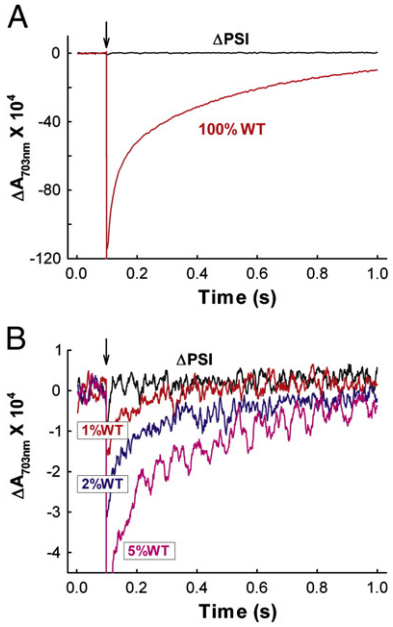

Fig. 1. The absence of PSI in the PSI deleted ΔAB mutant strain of *Synechocystis* 6803 is confirmed by the lack of P700 oxidation-induced absorbance change at 703 nm in the ΔAB membrane preparation. (A) Absorption transient at 703 nm reflecting the P700 oxidation and re-reduction kinetics of the WT *Synechocystis* 6803 membranes and lack of it in the ΔAB membranes. (B) Absorption transient at 703 nm (on an expanded scale) demonstrating the lack of the P700 response in the ΔAB membranes as compared to that of the ΔAB membranes with 1%, 2% and 5% WT admixtures. In both (A) and (B), the arrows indicate the times when the actinic flashes were fired. The reaction buffer contained 50 mM Tris–HCl (pH 8.3), 33 μM DCPIP, 1.7 mM sodium ascorbate and 50 nM PSII. The experiments were carried out at 24 ± 1 °C.

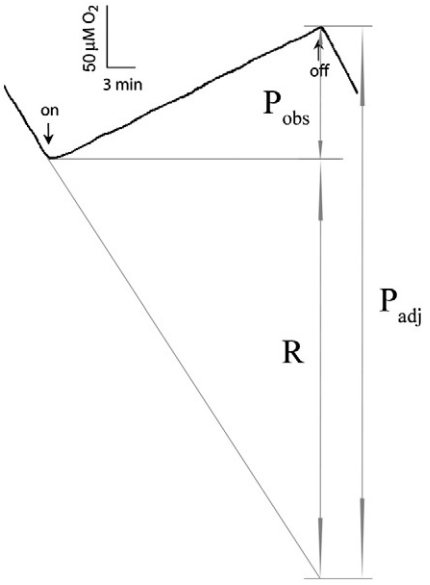

Fig. 2. A typical chart recorder trace of the light-induced O_2 evolution of the ΔPSI (ΔAB) cells in the presence of 5 mM glucose and 10 mM $NaHCO_3$. The rates of the dark respiration (R) and of the light-induced O_2 evolution either with (P_{adj}) or without (P_{obs}) adjustment for dark respiration are labeled. The down- and up-arrows indicate the times when the actinic light was turned on and off, respectively. This figure shows that the ΔAB cells were able to maintain substantial and constant rates of net light-induced O_2 evolution P_{obs} for 30 min. The Chl concentration of this sample was 1.3 μM. The experiment was carried out at 30 °C.

mutant (Fig. 1A). To determine the detection limit of our instrument, we 'titrated' the ΔAB membranes with small amounts of the WT membranes. As shown in Fig. 1B, the light-induced absorbance change resulting from as little as 1% WT P700 oxidation (on a PSII basis) can be detected with our instrument. Therefore, within a maximal experimental error of 1%, there was no detectable PSI activity in the ΔAB mutant. Similar conclusions were reached with the other ΔPSI strains, where there was no detectable PSI activity within maximal experimental errors of 1–5% (data not shown).

3.2. ΔPSI mutants show net light-induced O_2 evolution in the presence of glucose and $NaHCO_3$

Despite the lack of PSI, all the ΔPSI cells showed a net light-induced O_2 evolution *in vivo* in the presence of 5 mM glucose and 10 mM $NaHCO_3$.[4] For example, Fig. 2 shows a representative chart recorder trace of net light-induced O_2 evolution in the ΔAB cells that was sustained for 30 min. The trace, which is similar to that for the other mutants, shows a net O_2 uptake in the dark due to respiration, followed by a net oxygen evolution in the light, indicating that the rate of light-induced O_2 evolution exceeds the rate of respiration in the light.

Here we define the net light-induced O_2 evolution rate (i.e., the observed O_2 evolution rate, not adjusted for respiration) as P_{obs}, the dark respiration rate (i.e., O_2 consumption rate, of positive values) as R, and the O_2 evolution rate that is adjusted for dark respiration as P_{adj} (Fig. 2). Thus $P_{adj} = R + P_{obs}$, assuming the respiration rate in the light was the same as that in the darkness.[5] Classically, O_2 evolution rate is calculated as P_{adj} with the above assumption. We note that O_2 uptake in the light could also be inhibited (e.g., "Kok effect") or stimulated (e.g., as reported in another cyanobacterium *Synechococcus elongatus* at medium and high light intensities [30]) in the ΔPSI cells. Therefore, in this work, we primarily used P_{obs} as the readout for O_2 evolution. As summarized in Table 2, on a PSII basis, the net light-induced O_2 evolution rate P_{obs} was 4 ± 1 mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$ in the PSI deleted ΔAB cells, amounting to about 13% of that in the WT

Table 2
Comparison of the O_2 evolution and CO_2 assimilation rates of WT *Synechocystis* 6803 (grown either in the absence or in the presence of 5 mM glucose, Glc) and the PSI deleted ΔAB (grown in the presence of 5 mM glucose) cells. Both the O_2 evolution and the CO_2 assimilation rates were measured in fresh BG-11 growth medium supplemented with 10 mM $NaHCO_3$ and the corresponding glucose content. As shown in Fig. 2, P_{obs} is the net light-induced O_2 evolution rate obtained from the above-zero portion of the O_2 evolution traces and P_{adj} is the O_2 evolution rate adjusted for respiratory O_2 consumption, assuming that the respiration rate in the light is the same as in the dark (positive values for O_2 consumption). Since the ΔAB cells differed vastly from the WT cells in their Chl-to-PSII ratios, both the O_2 evolution and the CO_2 assimilation rates are normalized to the PSII content, utilizing the measured Chl-to-PSII ratios. The rates of corresponding electron fluxes can be obtained by multiplying the O_2 evolution rates by 4. The numbers of independent experiments preformed are indicated in parentheses. Detailed experimental conditions are described in Materials and methods. Despite the high variation in the dark CO_2 assimilation rates, the light-minus-dark CO_2 assimilation rate of the deltaPSI cells is highly statistically significant, as examined by the paired *t*-test (one-tailed, $p = 0.0003$) for 7 independent measurements.

Parameters measured	WT + Glc	WT-Glc	ΔPSI + Glc
Net O_2 evolution rate P_{obj} (mmol $O_2 \cdot$ mol $Chl^{-1} \cdot s^{-1}$)	68 ± 10 (6)	82 ± 15 (7)	56 ± 14 (7)
Chl-to-PSII ratio	490 ± 25 (2)	518 (1)	78 ± 4 (2)
Net O_2 evolution rate P_{obj} (mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$)	34 ± 5 (6)	42 ± 8 (7)	4 ± 1 (7)
Dark respiration rate R (mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$)	5 ± 1 (6)	4 ± 2 (7)	6 ± 2 (7)
Adjusted O_2 evolution rate P_{adj} (mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$)	39 ± 5 (6)	46 ± 8 (7)	10 ± 3 (7)
PSII activity (mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$)	66 (1)	–	110 (1)
Dark CO_2 assimilation rate (mol $CO_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$)	1.4 ± 0.7 (3)	1.4 ± 0.8 (5)	5 ± 2 (7)
Light-minus-dark CO_2 assimilation rate (mol $CO_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$)	16 ± 7 (3)	34 ± 6 (5)	2.2 ± 0.9 (7)

[4] In the present work, both O_2 and CO_2 were monitored in the presence of 5 mM glucose and 10 mM $NaHCO_3$ unless stated otherwise.
[5] In the formula $P_{adj} = R + P_{obs}$, respiration (R) is positive.

cells (34 ± 5 mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$); the dark respiration rate R was 6 ± 2 mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$ in the ΔAB cells, similar to that in the WT cells (5 ± 1 mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$); and P_{adj} was 10 ± 3 mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$ in the ΔAB cells, amounting to about 26% of that in the WT cells (39 ± 5 mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$). Similarly, significant net light-induced O_2 evolution was also observed in all other ΔPSI strains tested (e.g., the net light-induced O_2 evolution rates were 4 ± 1 mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$ in ΔPSIΔNdbABC and 2.2 ± 0.4 mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$ in ΔPSIΔCtaDIIEIIΔCydAB). Our data are consistent with the previous observation of net light-induced O_2 evolution in the absence of supplemental electron acceptors for either the *psaA*- or the *psaB*-deletion mutant of *Synechocystis* 6803 — the ADK9 [8] or BDK8 (L. B. Smart 2006, personal communication) strains.

The significant amount of net light-induced O_2 evolution of the ΔPSI mutants suggests the existence of alternative electron transport pathway(s) downstream of oxygenic PSII water oxidation in the absence of PSI. This net light-induced O_2 evolution in the ΔPSI mutants also indicated that the electron sinks of the alternative electron transport pathways in the absence of PSI are not limited to O_2, which has previously been suggested to be a sink for the PSII-generated electrons *via* a respiratory terminal oxidase in the ΔPSI/WV mutant [19,20].

3.3. O_2 evolution in the absence of PSI requires glucose

Synechocystis sp. PCC 6803 is known to be a facultative photoheterotroph in the presence of glucose [31,32]. Consistent with this observation, we found that the PSI deleted ΔAB mutant did not grow either photoautotrophically or mixotrophically with non-glucose carbon sources such as sucrose, fructose, glycerol or mannose. We next examined whether glucose was required to sustain O_2 evolution in the ΔAB cells. We found that upon depleting glucose from the medium, P_{obs} (Fig. 3A), R (Fig. 3B) and P_{adj} (Fig. 3C) dropped gradually to basal levels with similar time constants (ca. 8 h). After 42 h of glucose depletion, we added 5 mM glucose back to the ΔAB cell culture. While respiration recovered almost instantly, the net light-induced O_2 evolution P_{obs} was restored slowly and partially (Fig. 3D). In contrast, adding back non-metabolic glucose analogs, including 3-*O*-methyl glucose and 2-deoxy glucose, was not effective in restoring either the net light-induced O_2 evolution or the dark respiration of the glucose-depleted ΔAB cells. These results suggest the requirement of glucose and/or its metabolite(s) for sustaining the net light-induced O_2 evolution in the absence of PSI. In addition, after incubating the ΔAB cells with added glucose for ca. 30 min, we re-deprived these cells of glucose. Again, P_{obs}, R and P_{adj} dropped gradually to basal levels. However, the decay of these rates during the second glucose deprivation was much faster (time constant ca. 10 min) than that during the first glucose deprivation (time constant ca. 8 h), suggesting depletion of the unidentified electron sinks as a result of the prior prolonged glucose deprivation. To further characterize the net light-induced O_2 evolution of the mixotrophically-grown ΔPSI mutants of *Synechocystis* 6803, we examined whether several known linear electron transport pathway components were involved and whether the ΔPSI mutants were capable of CO_2 assimilation, as will be described in Sections 3.4 to 3.8.

3.4. PSII is the source of O_2 evolution in the absence of PSI

To examine the involvement of PSII in O_2 evolution in the absence of PSI, we titrated the ΔPSI cells with the PSII inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). We found that oxygen evolution was completely inhibited in the ΔPSI cells by 10 μM DCMU, while respiration was insensitive. This observation is consistent with the reported DCMU sensitivity of O_2 evolution in the ΔPSI mutant strain ADK9 [8]. The observed DCMU sensitivity of O_2 evolution in the ΔPSI cells was similar to that in the WT cells (I_{50} ~100 nM for both), suggesting that O_2 evolution of the ΔPSI cells originated from oxygenic PSII water oxidation.

To directly assess the PSII activity in the absence of PSI, we measured the light-induced O_2 evolution rate of the ΔAB cells in the presence of the artificial PSII electron acceptors DCBQ and FeCN. The PSII

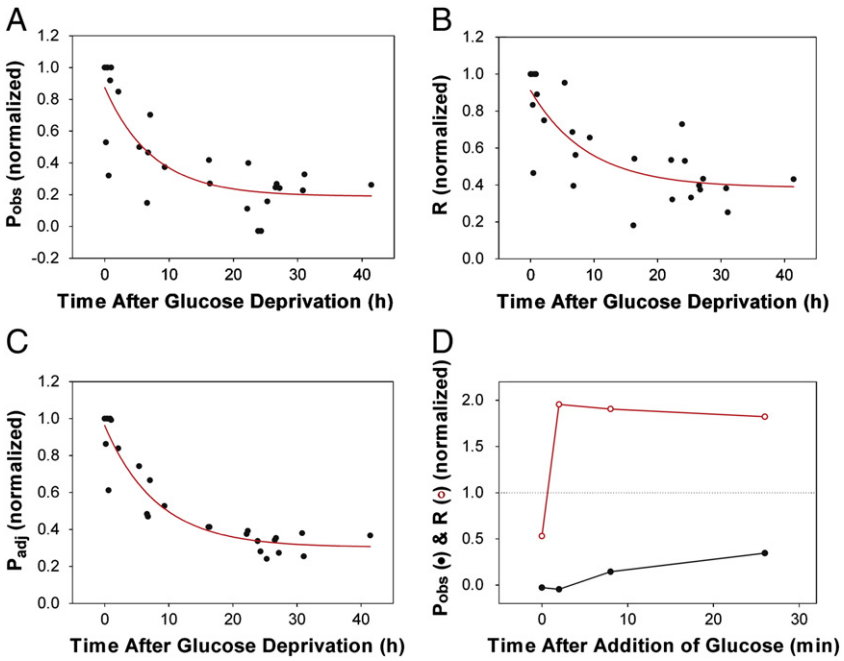

Fig. 3. Requirement of glucose for both O_2 evolution and dark respiration in the ΔPSI (ΔAB) mutant. The net light-induced O_2 evolution rate P_{obs} (A), dark respiration rate R (B) and the adjusted O_2 evolution rate P_{adj} (C) of the ΔAB cells dropped gradually after glucose deprivation (time constant ca. 8 h). The exponentially grown cells were harvested, washed and re-suspended in fresh BG-11 medium without glucose and kept at 30°C and under 1 μmol photons $\cdot m^{-2} s^{-1}$ light prior to experiments. A final concentration of 10 mM $NaHCO_3$ was added before each measurement. For each panel, data from 3 independent experiments were normalized to the maximal rate (right before glucose deprivation) and combined. For each rate, 100% activity is the activity before the sample was deprived of glucose. (D) Recovery of the net light-induced O_2 evolution rate (P_{obs}) (closed circles) and the dark respiration rate (R) (open circles) after glucose (5 mM) was supplied to the 42 h glucose-depleted ΔAB cells. Since 5 mM glucose was enough to maintain cell growth until stationary phase, the remaining glucose concentration during/after the measurements was assumed to be sufficient.

activity of the ΔAB cells (110 mol $O_2 \cdot$mol $PSII^{-1} \cdot s^{-1}$) was comparable to that of the WT cells (66 mol $O_2 \cdot$mol $PSII^{-1} \cdot s^{-1}$) (Table 2). This result is consistent with the reported similarity of the light-induced O_2 evolution rates (on a per cell basis) in the presence of 1 mM DCBQ between the WT and the ΔPSI mutant (ADK9) strains [8], suggesting un-impaired PSII in the ΔPSI cells.

To characterize PSII activity in the ΔPSI cells, we monitored Chl *a* fluorescence induction kinetics (the Kautsky effect) [33,34]. Chl *a* fluorescence emission yield reflects the redox state of Q_A, the first PQ electron acceptor in PSII: It increases with the reduction of Q_A and decreases with the oxidation of Q_A^- [35,36]. In the absence of DCMU, hundreds of milliseconds of illumination was required to reduce Q_A and the PQ pool to reach the fluorescence maxima F_P ($\approx F_M$) (Fig. 4A, closed symbols; ca. 170 ms in WT, ca. 320 ms in the ΔAB cells, and ca. 560 ms in the ΔPSIΔApcE cells). In contrast, in the presence of DCMU, Q_A was fully reduced to give maximal Chl *a* fluorescence emission yield (F_M) within 2 ms of illumination by actinic flashes (Fig. 4A, open symbols). These results show that DCMU has the same effect on Chl *a* fluorescence induction kinetics in the WT cells (Fig. 4A, circles) and ΔPSI cells (Fig. 4A, squares and triangles), demonstrating the PSII-origin of O_2 evolution in the ΔPSI cells. Furthermore, these results indicate that the lower net light-induced O_2 evolution observed the ΔPSI cells compared to that in the WT cells, is the result of a limitation in electron transport downstream of PSII, rather than an impaired PSII capacity.

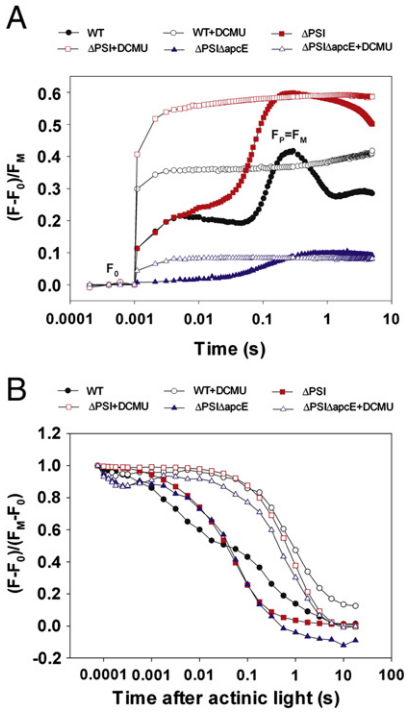

Fig. 4. DCMU effects on Chl *a* fluorescence kinetics of the WT and the ΔPSI cells. (A) Chl *a* fluorescence induction curves of the WT (circles), ΔAB (squares, labeled as ΔPSI) and ΔPSIΔApcE (triangles) cells show the same effect of DCMU on the WT and ΔPSI cells: The illumination time needed to fully reduce Q_A decreased from hundreds of milliseconds (WT: 170 ms; ΔAB: 320 ms; ΔPSIΔApcE: 560 ms) in the absence of DCMU to 2 milliseconds or less when DCMU is present. (B) Chl *a* fluorescence dark relaxation curves of the WT (circles), ΔAB (squares, labeled as ΔPSI) and ΔPSIΔApcE (triangles) cells show the same slow kinetics in both the WT and ΔPSI cells in the presence of DCMU (open symbols, $T^{DCMU} \approx 1$ s) and yet there is drastically different kinetics between the WT (black closed circles, bi-phasic, $T^{WT}_{slow} = 0.1–1$ s and $T^{WT}_{fast} \approx 1–10$ ms) and ΔPSI (red closed squares for ΔAB and blue closed triangles for ΔPSIΔApcE, monophasic, $T^{\Delta PSI} \approx 10–100$ ms) cells in the absence of DCMU. A total of 370 saturating actinic flashes were given at the frequency of 1 kHz to maximally reduce Q_A and the PQ pool prior to recording the dark relaxation kinetics. The time when the actinic flashes were turned off was set as zero. DCMU was applied at a final concentration of 10 μM.

3.5. The PQ pool is involved in O_2 evolution of the ΔPSI cells

To investigate the involvement of the PQ pool in O_2 evolution, we monitored the oxidation kinetics of Q_A^- and the plastoquinol (PQH_2) pool in the WT and ΔPSI (ΔAB and ΔPSIΔApcE) cells by recording their Chl *a* fluorescence dark relaxation kinetics after their Q_A and the PQ pool were reduced (Fig. 4B). In the presence of 10 μM DCMU, Chl *a* fluorescence of the WT and the ΔPSI cells relaxed slowly in the darkness with the same time constant $T^{DCMU} \approx 1$ s (Fig. 4B, open symbols). This slow Chl *a* fluorescence dark relaxation kinetics in the presence of DCMU was also reported previously in the ΔPSI/WV cells and is attributed to the Q_A^- oxidation through the back reaction of PSII [20].

In contrast, in the absence of DCMU, the Chl *a* fluorescence dark relaxation curves of the WT and ΔPSI mutants were significantly different (Fig. 4B, closed symbols): The relaxation kinetics were bi-phasic in the WT cells (Fig. 4B, closed circles) with time constants of $T^{WT}_{slow} = 0.1–1$ s and $T^{WT}_{fast} \approx 1–10$ ms, respectively, whereas the relaxation kinetics of both the ΔAB (Fig. 4B, closed squares) and the ΔPSIΔApcE (Fig. 4B, closed triangles) cells were monophasic with an intermediate time constant of $T^{\Delta PSI} \approx 10–100$ ms. The slow phase of the Chl *a* fluorescence dark relaxation kinetics in the WT cells in the absence of DCMU is attributed to the Q_A^- oxidation through the back reaction of PSII [20]. Alternatively, it may reflect the cyclic electron transport around PSI which reduces the PQ pool [37,38]. The fast phase of Chl *a* fluorescence dark relaxation kinetics in the WT cells in the absence of DCMU indicates a more oxidized PQ pool as a result of the PSI activity before the actinic light is switched off. In contrast, the intermediately-fast time constant of the monophasic Chl *a* fluorescence dark relaxation kinetics in the ΔPSI cells in the absence of DCMU reflects a more reduced PQ pool, possibly as a result of less effective PQH_2 oxidation pathway(s) than PSI.

These observations show that the PQ pool is involved in O_2 evolution in the ΔPSI cells, and that PQH_2-oxidizing pathway(s) other than PSI must exist to sustain the prolonged O_2 evolution in the absence of PSI.

3.6. PQH_2 oxidation in the ΔPSI cells does not involve the Q_o site of the Cyt bf complex

At low concentrations (≤ 1 μM), DBMIB inhibits PQH_2 oxidation at the plastoquinol oxidase (Q_o) site of the Cyt bf complex [39], whereas at higher concentrations (≥ 10 μM), DBMIB can inhibit Q_A^- oxidation in a DCMU-like manner (reviewed in [40]). To examine whether the Cyt bf complex could effectively oxidize PQH_2 in the absence of PSI, we titrated O_2 evolution of the ΔPSI cells with DBMIB. Our data show that O_2 evolution of the ΔPSI cells was much less sensitive to DBMIB than that of the WT cells. The DBMIB concentration leading to 50% inhibition (I_{50}) of the net O_2 evolution rate P_{obs} was 0.5 μM for the WT cells (Fig. 5A, closed circles) and 4.2 μM for the ΔPSI cells (Fig. 5A, open circles), respectively. Significantly different I_{50} values for the DBMIB inhibition of the adjusted O_2 evolution rate P_{adj} were also observed for the WT (0.6 μM; Fig. 5C, closed circles) and ΔPSI cells (23.7 μM; Fig. 5C, open circles). In contrast, the dark respiration rate R was not inhibited by DBMIB (50 μM) in either WT (Fig. 5B, closed circles) or ΔPSI (Fig. 5B, open circles) cells. These results indicate that the Q_o site of the Cyt bf complex is unlikely to be involved in the PQH_2-oxidizing pathway(s) that contributes to the net light-induced O_2 evolution in the ΔPSI cells.

3.7. Oxygen evolution in the ΔPSI cells is highly sensitive to KCN

Cyanide is a potent inhibitor of the respiratory cytochrome oxidase and other metalloproteins including plastocyanin [41] and Rubisco [42,43]. To examine the possible involvement of KCN-sensitive redox components in the PQH_2-oxidizing pathway(s) in

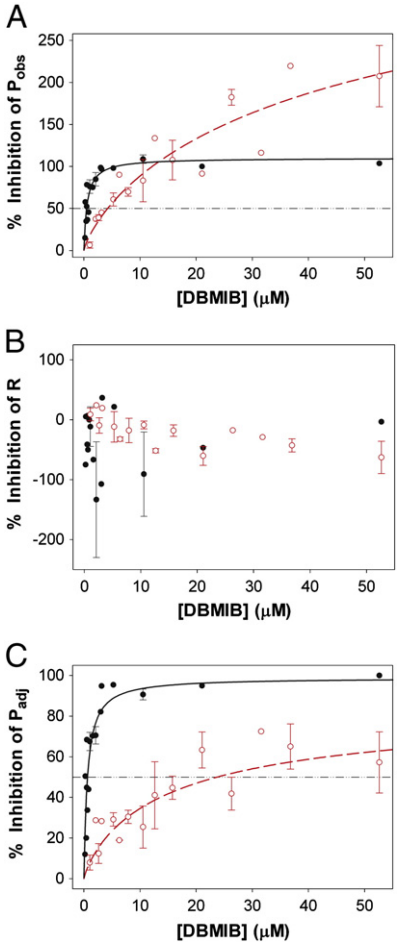

Fig. 5. DBMIB titration of (A) P_{obs}, (B) R and (C) P_{adj} of the WT and the ΔPSI cells. P_{obs} and P_{adj} of the ΔPSI cells were significantly less sensitive to DBMIB than those of the WT cells, whereas the rates of R in both the WT and the ΔPSI cells were not sensitive to DBMIB. The I_{50} values for DBMIB inhibition were 0.5 μM for P_{obs} of the WT cells (Panel A, black closed circles), 4.2 μM for P_{obs} of the ΔPSI cells (Panel A, red open circles), 0.6 μM for P_{adj} of the WT cells (Panel C, black closed circles), and 23.7 μM for P_{adj} of the ΔPSI cells (Panel C, red open circles). P_{obs}, R and P_{adj} of both the WT and ΔPSI cells were measured in BG-11 medium in the presence of 5 mM glucose and 10 mM $NaHCO_3$. The titration curves of the WT cells (black closed circles) are plotted from 3 data sets. The titration curves of the ΔPSI cells (red open circles, for ΔAB, ΔVIII–XI, and ΔPSI/WV strains) are plotted from 4 data sets. Standard errors are indicated. In addition, we grew the ΔPSI cells either in the presence or in the absence of 1 μM Cu^{2+} supplement so that they expressed either plastocyanin or Cyt c_6, respectively [21]. Both plastocyanin- and Cyt c_6-expressing ΔPSI cells exhibited indistinguishable DBMIB titration curves: The data shown include those from cells grown both with and without Cu^{2+} supplement. For each rate, 100% inhibition by DBMIB means the rate is reduced from the control value (with no DBMIB) to zero. An inhibition that is greater than 100% in the case of P_{obs} means that in the presence of DBMIB, the cells show net O_2 consumption instead of displaying net O_2 evolution.

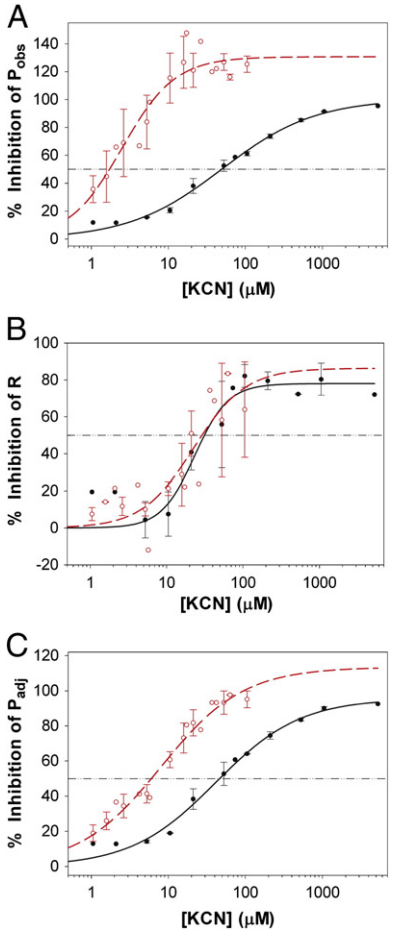

Fig. 6. KCN titration of (A) P_{obs}, (B) R and (C) P_{adj} of the WT and the ΔPSI cells. P_{obs} and P_{adj} of the ΔPSI cells were much more sensitive to KCN than those of the WT cells, whereas R of the ΔPSI cells had the same sensitivity to KCN as that of the WT cells. The I_{50} values for KCN inhibition were 49 μM for P_{obs} of the WT cells (Panel A, black closed circles), 1.7 μM for P_{obs} of the ΔPSI cells (Panel A, red open circles), 46 μM for P_{adj} of the WT cells (Panel C, black closed circles), 6 μM for P_{adj} of the ΔPSI cells (Panel C, red open circles) and 30 μM for R of both the WT (Panel B, black closed circles) and ΔPSI (Panel B, red open circles) cells. P_{obs}, R and P_{adj} of both the WT and the ΔPSI cells were measured in BG-11 medium in the presence of 5 mM glucose and 10 mM $NaHCO_3$. The titration curves of the WT cells (black closed circles) are plotted from 2 data sets. The titration curves of the ΔPSI cells (red open circles, for ΔAB, ΔVIII–XI, and ΔPSI/WV strains) are plotted from 5 data sets. Standard errors are indicated. In addition, the KCN sensitivities of both the WT and ΔPSI cells were not affected by the supplemental Cu^{2+} in the growth medium, as the data shown are for cells grown both with and without Cu^{2+} supplement. For each rate, 100% inhibition by KCN means the rate is reduced from the control value (with no KCN) to zero. An inhibition that is greater than 100% in the case of P_{obs} means that in the presence of KCN, the cells showed net O_2 consumption instead of displaying net O_2 evolution.

the ΔPSI cells, we titrated O_2 evolution and dark respiration with KCN. Our data showed that P_{obs} of the ΔPSI cells was highly KCN-sensitive (I_{50} ~1.7 μM, Fig. 6A, open circles), as compared to that of the WT cells (I_{50} ~49 μM, Fig. 6A, closed circles). Similarly, P_{adj} of the ΔPSI cells was also highly KCN-sensitive (I_{50} ~6 μM, Fig. 6C, open circles), as compared to that of the WT cells (I_{50} ~46 μM, Fig. 6C, closed circles). In contrast, dark respiration of the ΔPSI cells was much less sensitive to KCN, with the I_{50} values of 30 μM in both the WT (Fig. 6B, closed circles) and ΔPSI (Fig. 6B, open circles) cells.

To further investigate the KCN-sensitive PQH_2-oxidizing pathway(s) in the ΔPSI cells, we examined the possible involvement of PSII, plastocyanin and respiratory terminal oxidases. By monitoring O_2 evolution in the presence of the artificial PSII electron acceptors FeCN and DCBQ, we found that the PSII activities of both the WT cells and the PSI deleted ΔAB cells were not inhibited by 500 μM KCN. Therefore, the KCN inhibition of both P_{obs} and P_{adj} of the WT and ΔPSI cells does not result from the KCN inhibition of PSII.

Plastocyanin has been previously shown to react readily with high concentrations of KCN (>10 mM) [41]. We grew the ΔPSI cells either in the presence or in the absence of 1 μM Cu^{2+} supplement so that they expressed either plastocyanin or Cyt c_6, respectively [21]. Both plastocyanin- and Cyt c_6-expressing cells exhibited indistinguishable KCN titration curves (Fig. 6), further supporting that the PQH_2-oxidizing pathway(s) in the absence of PSI does not involve plastocyanin.

Lastly, we monitored the KCN effect on Chl *a* fluorescence dark relaxation kinetics of the ΔPSI cells (Fig. 7A). Chl *a* fluorescence dark relaxation in the ΔPSI mutants was severely inhibited by 1.5 mM KCN (Fig. 7A, cf. closed squares with closed circles). We note that, Chl *a* fluorescence dark relaxation kinetics of the ΔPSI mutants in the presence of KCN (Fig. 7A, closed squares) was similar to that in the

presence of DCMU (Fig. 7A, open circles), suggesting effective inhibition of the oxidation of Q_A^- and the PQH_2 pool by KCN in the ΔPSI cells. In contrast, in the WT cells, KCN did not cause significant inhibition of the PQH_2 pool oxidation (Fig. 7B, closed squares); rather, it induced a small light-to-dark transient increase of the Chl *a* fluorescence yield which was previously attributed to the delayed reduction of the PQ pool by the KCN-independent cyclic electron transport around PSI [37,38]. In spite of the fact that KCN inhibits the oxidation of Q_A^- and the PQH_2 pool, which was previously attributed to the KCN-sensitive PQH_2-oxidizing activity of respiratory cytochrome oxidases in the ΔPSI/WV cells [20], we suggest that the KCN-sensitivity of the net light-induced O_2 evolution in the ΔPSI cells is independent of direct involvement of respiratory terminal oxidases (see the Discussion and the Supplementary Material S2). In addition, the observed KCN-sensitivity of the net light-induced O_2 evolution in the WT cells has an I_{50} value close to the reported I_{50} value for the cyanide inhibition of Rubisco [42,43]; thus, it may be attributed to the KCN inhibition of the Calvin–Benson cycle.

3.8. The ΔPSI cells are capable of light-minus-dark CO_2 assimilation in the presence of glucose

We examined CO_2 assimilation in the ΔPSI cells by performing *in vivo* radioisotope-labeling studies using $NaH^{14}CO_3$. Our data show that the ΔPSI cells take up CO_2 both in the darkness and upon illumination, resulting in significant amount of light-minus-dark CO_2 uptake (Table 2 and Fig. 8A). Since acid-stable intermediates instead of acid-labile carbamates of proteins or bicarbonate of carbon concentrating mechanism (CCM) [44–47] were monitored in these experiments, the observed CO_2 uptake in the ΔPSI cells was in fact CO_2 assimilation. The light-minus-dark CO_2 assimilation rate was measured to be 2.2 ± 0.9 mol $CO_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$ in the PSI deleted ΔAB cells, when the CO_2 assimilation rate in the darkness was surprisingly as high as 5 ± 2 mol $CO_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$. The light-minus-dark CO_2 assimilation rate in the ΔAB cells was likely under-estimated because the dilution of $^{14}CO_2$ as a result of the release of non-labeled CO_2 by respiration was ignored in the analysis. This notion was supported by the increase of the measured light-minus-dark CO_2 assimilation rate of the WT cells from 16 ± 7 mol $CO_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$ to 34 ± 6 mol $CO_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$ when the reaction media were changed from those containing 5 mM glucose to those without glucose. Therefore, we conclude that the PSI deleted ΔAB cells assimilate CO_2 at a significant rate as compared to the O_2 evolution rates: The light-

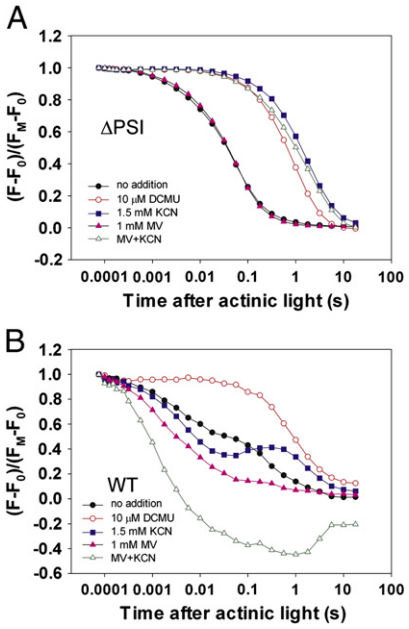

Fig. 7. KCN effects on Chl *a* fluorescence dark relaxation kinetics of the (A) ΔPSI (ΔAB) and (B) WT cells. Like DCMU (Panel A, red open circles), KCN severely inhibited Chl *a* fluorescence dark relaxation in the ΔPSI cells (Panel A, blue closed squares), as compared to without KCN (Panel A, black closed circles), suggesting that KCN may effectively slow down the oxidation of Q_A^- and the PQH_2 pool in the ΔPSI cells. In contrast, in the WT cells, KCN did not cause inhibition of the PQH_2 pool oxidation; rather, it induced a small light-to-dark transient increase of the Chl *a* fluorescence yield (Panel B, blue closed squares) which was absent in the ΔPSI cells. This result is consistent with the lack of PSI and the cyclic electron transport around PSI in the ΔPSI cells. This result was further supported by the capability of methylviologen (MV), an electron acceptor for PSI [56], to reverse the KCN inhibition of the PQH_2 pool oxidation in the WT cells (Panel B, green open triangles), but not in the ΔPSI cells (Panel A, green open triangles). A total of 370 saturating actinic flashes were given at the frequency of 1 kHz to maximally reduce Q_A and the PQ pool prior to recording the dark relaxation kinetics. The time when the actinic flashes were turned off was set as zero. The concentrations of the inhibitors and electron acceptor used were as follows: 10 μM DCMU, 1.5 mM KCN, and 1 mM MV.

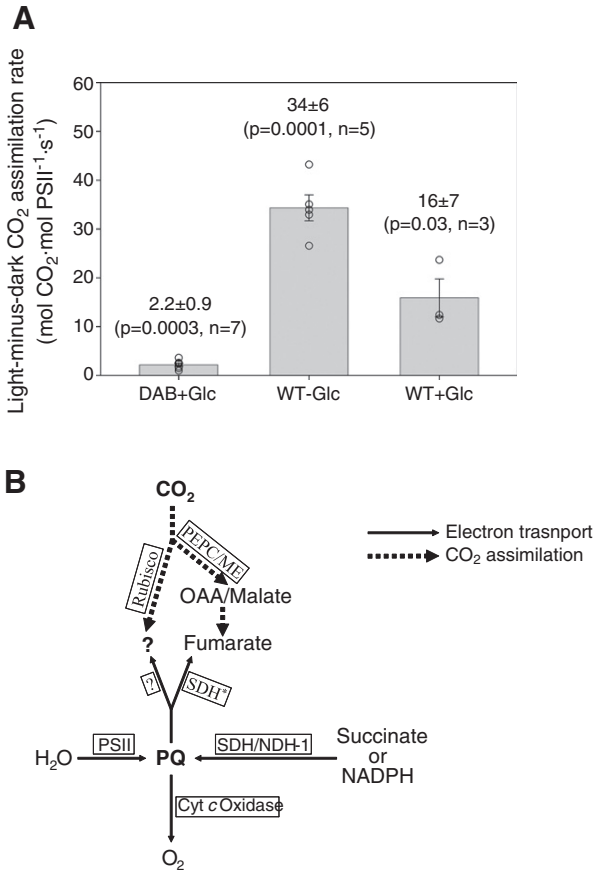

Fig. 8. (A) The light-minus-dark CO_2 assimilation rates in the PSI deleted ΔAB and the WT cells in the presence of glucose and in the WT cells in the absence of glucose. Each experiment is represented by one open circle. The mean, the standard error, the p-value, and the number of independent experiments are indicated. The light-minus-dark CO_2 assimilation rates were calculated as the difference between the light and the dark CO_2 assimilation rates. Despite the high variation in the dark CO_2 assimilation rates, the light-minus-dark CO_2 assimilation rates are statistically highly significant, as evaluated by the paired *t*-test. (B) A working model for the O_2 evolving electron transport (thin solid-line arrows) and light-minus-dark CO_2 assimilation (thick dotted-line arrows) in the ΔPSI cells. The PQ pool serves as the "hub" of the electron flow with the incoming electrons from H_2O (*via* PSII) as well as succinate (*via* succinate dehydrogenase) and/or NADPH (*via* type 1 NAD(P)H dehydrogenase) and with the outgoing electrons to O_2 (*via* cytochrome *c* oxidases) and CO_2 (*via* Rubisco-dependent mechanisms and/or a glycolysis-dependent mechanism). The glycolysis-dependent CO_2 assimilation may involve PEP carboxylase and/or malic enzyme, which generate malate and/or oxaloacetate *via* carboxylation reactions, followed by a succinate dehydrogenase working in reverse as a fumarate reductase to reduce fumarate to succinate. Abbreviations used in this model are: PSII — photosystem II, PQ — the plastoquinol pool, SDH — succinate dehydrogenase, SDH* — succinate dehydrogenase working in reverse as fumerate reductase, NDH-1 — type 1 NAD(P)H dehydrogenase, Cyt c oxidase — cytochrome c oxidase, OAA — oxaloacetate, PEPC — PEP carboxylase, ME — malic enzyme.

minus-dark CO_2 assimilation rate (2.2 ± 0.9 mol $CO_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$) is at least 55% of the light-minus-dark O_2 evolution rate (4 ± 1 mol $O_2 \cdot$ mol $PSII^{-1} \cdot s^{-1}$).

We tested the effects of the electron transport inhibitors and glucose deprivation on CO_2 assimilation of the ΔPSI cells. Our data show that the light-minus-dark CO_2 assimilation rate was inhibited by 10 μM DCMU (90% inhibition) or 1 mM KCN (100% inhibition). In addition, the light-minus-dark CO_2 assimilation rate dropped upon glucose deprivation and recovered upon adding back glucose (e.g., in one experiment, the light-minus-dark CO_2 assimilation rate dropped to 29% after 24 h glucose deprivation and recovered to 66% after adding back glucose for 3.5 h). The similarity between the light-minus-dark CO_2 assimilation and O_2 evolution in their responses to the electron transport inhibitors and glucose deprivation indicates that the observed light-minus-dark CO_2 assimilation of the ΔPSI cells is a key PQH_2-oxidizing pathway enabling the net light-induced O_2 evolution in the ΔPSI cells.

4. Discussion

In this paper, we have demonstrated the following: (1) a net light-induced O_2 evolution is present in *Synechocystis* 6803 cells lacking PSI; (2) this net light-induced O_2 evolution of the ΔPSI cells requires glucose and can be sustained for over 30 min; (3) the rate of net light-induced O_2 evolution (P_{obs}) of the ΔPSI cells is ca. 13% of the WT rate on a PSII basis; (4) this net light-induced O_2 evolution of the ΔPSI cells is driven by PSII and the pathway of electrons includes the PQ pool but not the Q_o site of the Cyt bf complex; and (5) this net light-induced O_2 evolution of the ΔPSI cells is highly sensitive to KCN addition.

We also looked for the terminal electron acceptor pools of this PSI-independent, net light-induced O_2 evolution. As will be discussed below, we suggest that CO_2, but not the PQ pool or O_2, is a key terminal electron acceptor. A working model depicting O_2 evolving electron transport and CO_2 assimilation in the ΔPSI cells is shown in Fig. 8B.

First, with the limited size of the PQ pool (ca. 10 molecules $PSII^{-1}$) [34], all PQ molecules can be reduced within 2 s. Therefore, our observation that the ΔPSI cells could sustain the net light-induced O_2 evolution for 30 min at a rate as high as 4 ± 1 $O_2 \cdot PSII^{-1} \cdot s^{-1}$ strongly argues against the hypothesis of Smart et al. [8] that the PQ pool serves as the main terminal electron acceptor for the net light-induced O_2 evolution in the ΔPSI cells.

Second, O_2 is a logical candidate to be an electron sink for the PQH_2-oxidizing pathway(s) in the PSI deletion mutants of *Synechocystis* 6803, as respiration and photosynthesis in cyanobacteria share common components such as the Cyt bf complex, the PQ pool and NADPH [48]. Vermaas et al. [20] and Vermaas [19] suggested that a respiratory terminal oxidase may direct the electrons generated by PSII to O_2 in the ΔPSI/WV cells. Notably different from our results, Vermaas [19] showed that the ΔPSI/WV cells did not display net O_2 evolution in the light. However, we observed net light-induced O_2 evolution in the ΔPSI (including ΔPSI/WV) cells; if all the PSII-generated electrons were directed to O_2, there would have been no net O_2 evolution [49–52]. Thus, to explain our data, other electron acceptors must also exist (see the Supplementary Material S2 for preliminary data and a detailed discussion). We speculate that this discrepancy may be due to the differences in growth conditions, including light intensities and glucose concentrations.[6] Of note, in this work, we primarily use the net light-induced O_2 evolution rate P_{obs} as the readout. However, because the O_2 evolution rate is classically calculated with adjustment for dark respiration (i.e., P_{adj}), we also report in this work P_{adj}, assuming that respiration does not change in light. As respiration and photosynthesis are intertwined in cyanobacteria, O_2 uptake possibly varies during the light phase in the ΔPSI cells. For instance, as a result of enhanced oxidase activity, which is likely in the absence of PSI, O_2 uptake in the light could be stimulated. Further, mass spectroscopic data exist on another cyanobacterium *S. elongatus* (previously known as *Anacystis nidulans*) that at medium and high light intensities, O_2 uptake is enhanced in the light [30]. If O_2 uptake in the light is indeed enhanced in the ΔPSI cells, true O_2 evolution in these cells would be much higher than reported by P_{obs}.

Third, we have further demonstrated that CO_2 may serve as a key terminal electron acceptor of the PQH_2-oxidizing pathway(s) in the ΔPSI cells: Upon illumination, the ΔPSI cells can assimilate CO_2 at a rate that accounts for ca. 55% of the net light-induced O_2 evolution rate, and this light-minus-dark CO_2 assimilation is DCMU- and KCN-sensitive. These observations were made in the presence of 5 mM glucose and 10 mM $NaHCO_3$. We also found that the O_2 evolution rate in the ΔPSI cells decreased when CO_2 was depleted by bubbling $NaHCO_3$-less BG-11 medium with air filtered through Soda Lime (containing >75% $Ca(OH)_2$ and <3.5% NaOH). This finding is consistent with CO_2 being a key terminal electron acceptor in the ΔPSI O_2 evolution pathway. In *Synechocystis*, it is known that CO_2 assimilation is intertwined with a network of carbon metabolism. In this network, glycolysis and the tricarboxylic acid (TCA) cycle are connected not only to the Calvin–Benson cycle, but also to the oxidative pentose phosphate pathway, the glyoxylate pathway and nitrogen storage. It is also known that TCA cycle is incomplete in *Synechocystis*, lacking 2-ketoglutarate dehydrogenase or 2-ketoglutarate ferredoxin oxidoreductase [53]. Based on our pilot studies (see Supplementary Material S3) that attempted to address the mechanism responsible for the net light-induced O_2 evolution and the light-minus-dark CO_2 assimilation in the ΔPSI cells and the reported metabolic flux analysis showing the operation of a C_4-like pathway involving PEP carboxylase and malic enzyme in the mixotrophically-grown *Synechocystis* 6803 for the delivery of substantial carbon flow from the TCA cycle to the glycolysis pathway [54], we speculate that (1) the CO_2-assimilating PQH_2-oxidizing pathway in the ΔPSI cells may involve a glycolysis-dependent pathway with a glucose metabolite serving as the immediate electron acceptor after the PQ pool, but not the Calvin–Benson cycle or the cyanophycin synthesis pathway that assimilates both carbon and nitrogen at the same time; and (2) the alternative glycolysis-dependent CO_2-assimilating pathway in the ΔPSI cells may involve fumarate generation *via* PEP carboxylase/malic enzyme and subsequent fumarate reduction *via* succinate dehydrogenase working in reverse (Fig. 8B) (see Supplementary Material S3 for preliminary data and an elaborate discussion).

Finally, our efforts to identify additional terminal electron acceptor pools (e.g., H^+) (see Supplementary Material S4) were unsuccessful. Due to the complex nature of this newly discovered alternative PQH_2-oxidizing pathways in the ΔPSI cells, we suggest that techniques such as mass spectrometry-based metabolic flux analysis utilizing isotopically labeled O_2/CO_2 and GC/MS [54,55] as well as genomic and/or proteomic studies will be particularly valuable to dissect the intercalated network of the metabolite pools and enzymes for the final solution of the mechanism of the net light-induced O_2 evolution in PSI deletion mutants of the cyanobacterium *Synechocystis* sp. PCC 6803.

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[6] Light intensities: 5 μmol photons $\cdot m^{-2} \cdot s^{-1}$ for ΔPSI/WV and 1.5 μmol photons $\cdot m^{-2} \cdot s^{-1}$ in the present work. Glucose concentrations: 15 mM for ΔPSI/WV and 5 mM in the present work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2012.01.004.

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Supplementary Material

S1. Molecular biology evidence for the loss of PSI in the ΔAB mutant strain

The absence of PSI expression in the ΔAB mutant strain was confirmed in its original laboratory (H. Pakrasi at the Washington University in St. Louis, Missouri) by Southern and Western blots (E. Zak and H. Pakraski, personal communication). We also independently confirmed the deletion of *psaAB* mRNA using Northern blot and microarray studies as well as the loss of PSI protein using Western blot.

S2. Is O_2 a terminal electron acceptor for the net light-induced O_2 evolution in the ΔPSI cells?

Oxygen is a logical candidate to be an electron sink for the PQH_2-oxidizing pathway(s) in the PSI deletion mutants of *Synechocystis* 6803, as respiration and photosynthesis in cyanobacteria share common components such as the Cyt bf complex, the PQ pool and NADPH [1]. Vermaas et al. [2] and Vermaas [3] suggested that a respiratory terminal oxidase may direct the electrons generated by PSII to O_2 in the ΔPSI/WV cells. Vermaas [3] showed that the ΔPSI/WV cells did not display net O_2 evolution in the light. However, we observed net light-induced O_2 evolution in the ΔPSI cells (see Fig. 2 in the main paper), including the ΔPSI/WV cells; if all the PSII-generated electrons were directed to O_2, there would have been no net O_2 evolution [4-7]. Thus, we suggest that the net light-induced O_2 evolution in the ΔPSI cells cannot depend on the direct involvement of respiratory terminal oxidases for O_2 consumption.

However, the net light-induced O_2 evolution in the ΔPSI cells may indirectly depend on respiratory terminal oxidases. For example, respiratory terminal oxidases may build up proton gradient or ATP that is utilized in the net light-induced O_2 evolution in the ΔPSI cells, which might be one of the explanations for KCN-sensitivity of the process. Thus, to test the involvement of proton gradient or ATP, we investigated the effects of uncouplers* (e.g. 500 μM phlorizin, 100 μM CCCP, 3.3 mM CROWN-18, 10 μM nigericin, 20 μM DCCD, 5 μM

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* Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CROWN-18, dicyclohexano-18-crown-6; DCCD, dicyclohexylcarbodi-imide.

gramicidin D and 0.8 μM valinomycin) on the alternative PQH_2-oxidizing pathway in the ΔPSI cells. Among all the uncouplers that we tested, only CROWN-18 and CCCP had some inhibitory effects on the net light-induced O_2 evolution of both WT and the ΔPSI cells (data not shown). Because CCCP is known to inhibit PSII when used at high concentration [8] and the uncoupler property of CROWN-18 is not well established, our uncoupler studies are not conclusive.

The respiratory terminal oxidase in the ΔPSI cells can be either a cytochrome aa_3-type cytochrome *c* oxidase (the major respiratory oxidase CtaI) or a DBMIB-insensitive quinol oxidase of the cytochrome *bd*-type (Cyd) [9-10]. In addition, O_2 may also serve as the sink for the PSII-generated electrons in the ΔPSI cells through a chlororespiration-like pathway. The involvement of cytochrome *c* oxidase CtaI cannot be tested directly because of the lethality of PSI and CtaI double deletion [10], although this lethality might indicate the importance of CtaI in PSI mutant O_2 production. We observed net light-induced O_2 evolution in both ΔPSIΔCtaDIIEIIΔCydAB cells (in which both the alternative oxidase CtaII and the quinol oxidase Cyd were deleted [10]) and ΔPSIΔNdbABC cells (in which the type 2 NADH dehydrogenase Ndb was deleted [11]), excluding alternative oxidase CtaII, quinol oxidase Cyd, or type 2 NADH dehydrogenase from being the major contributor of the net light-induced O_2 evolution in the absence of PSI.

We do not rule out the involvement of respiratory terminal oxidases in the net light-induced O_2 evolution of the ΔPSI cells, but we suggest that there must exist respiratory terminal oxidase-independent PQH_2-oxidizing pathway(s) to funnel the PSII-generated electrons to terminal acceptors other than O_2 for the net light-induced O_2 evolution of the ΔPSI cells.

S3. What is the mechanism for CO_2 assimilation in the ΔPSI cells?

Despite the identification of CO_2 as a key terminal electron acceptor of the PQH_2-oxidizing pathway(s) in the ΔPSI cells, our attempts to elucidate the mechanism for CO_2 assimilation in the ΔPSI cells yielded intriguing but inconclusive results, presumably due to the intertwining of CO_2 assimilation with the network of carbon metabolism. In this network, glycolysis and the tricarboxylic acid (TCA) cycle are connected not only to the Calvin-Benson cycle, but also to the oxidative pentose phosphate pathway, the glyoxylate pathway, and nitrogen storage. We know

that the TCA cycle is incomplete in *Synechocystis*, lacking 2-ketoglutarate dehydrogenase or 2-ketoglutarate ferredoxin oxidoreductase [12]. As a result of the complexity of this network, and due to the lack of specific inhibitors for the enzymes in this network, it is difficult to identify the enzymes involved in, as well as the intermediates of the ΔPSI O_2 evolution pathway.

First, we tested if electrons from PSII water oxidation in the ΔPSI cells were used to reduce nitrate (because BG11 medium was used) to cyanophycin, a nitrogen- and carbon-storage compound specific to cyanobacteria. Cyanophycin synthesis utilizes glucose as the carbon source and cyanophycin is known to be present in *Synechocystis* 6803 [13-16]. Due to the large size of the electron sinks in the alternative PQH_2-oxidizing pathway in the ΔPSI cells, it is possible that cyanophycin is the terminal electron sink. To test this idea, we grew the ΔPSI cells in the BG11 medium omitting the addition of nitrogen (NO_3^- and/or ferric ammonium citrate)**. We observed that these cells still showed net light-induced O_2 evolution, suggesting that the nitrogen assimilation was not related to the alternative PQH_2-oxidizing pathway in the ΔPSI cells.

Second, we examined the effects of a number of inhibitors of carbon metabolism, including inhibitors of the Calvin-Benson cycle, glycolysis, the TCA cycle and the oxidative pentose phosphate pathway, on the light-minus-dark CO_2 assimilation of the ΔPSI cells. We found that 5 mM iodoacetamide, an irreversible inhibitor of glyceraldehyde-3-phosphate dehydrogenase [17], abolished more than 90% of the light-minus-dark CO_2 assimilation in both the WT and ΔPSI (ΔAB) cells. Since glyceraldehyde-3-phosphate dehydrogenase functions in both the Calvin-Benson cycle and glycolysis, we speculate that the CO_2-assimilating PQH_2-oxidizing pathway in the ΔPSI cells may involve the Calvin-Benson cycle and/or a glycolysis-dependent pathway, with a glucose metabolite serving as the immediate electron acceptor after the PQ pool. We also found that rotenone (~200 μM), which has previously been reported to inhibit NADH dehydrogenase and the Calvin-Benson cycle [18-21], did not inhibit the net O_2 evolution of the ΔPSI/WV cells, compromising the possibility of a functioning Calvin-Benson cycle in the ΔPSI cells. In contrast, we found that the light-minus-dark CO_2 assimilation of the ΔPSI cells was partially inhibited by 100 μM shikimic acid, 10 mM malate, 10 mM aspartate and 10 mM citrate,

** No special procedure was employed to remove trace amount of nitrogen.

all of which are inhibitiors of phosphoenolpyruvate (PEP) carboxylase [22-23]. Based on these observations and the reported metabolic flux analysis showing the operation of a C_4-like pathway involving PEP carboxylase and malic enzyme in the mixotrophically grown *Synechocystis* 6803 for the delivery of substantial carbon flow from the TCA cycle to the glycolysis pathway [24], we speculate that the alternative glycolysis-dependent CO_2-assimilating pathway in the ΔPSI cells may involve fumarate generation via PEP carboxylase/malic enzyme and subsequent fumarate reduction via succinate dehydrogenase working in reverse (see Fig. 8B in the main paper). This hypothesis is further supported by (1) the similarity between the estimated rate of respiratory electron flux flowing into the PQ pool via succinate dehydrogenase (11-23 mol electrons·mol $PSII^{-1}\cdot s^{-1}$)*** [25-26] and the net O_2 evolution rate in the ΔPSI cells (equivalent to 16 mol electrons·mol $PSII^{-1}\cdot s^{-1}$), (2) the similarity between the $E_{m,7}$ value of PQ/PQH_2 (+10 mV) and that of succinate/fumarate (0 mV) [26], and (3) the reported high flux of CO_2 assimilation through the PEP carboxylase (25% of the total) in the mixotrophically-grown WT *Synechocystis* 6803 cells in the light [24]. To test this hypothesis, we monitored the responses of the net light-induced O_2 evolution rate (P_{obs}) of the PSI deletion mutant ΔAB to the succinate dehydrogenase inhibitor malonic acid (200 μM) or the TCA cycle intermediates malate, fumarate and succinate. However, these experiments were inconclusive, possibly due to insufficient concentration of inhibitor in the case of malonic acid and slow uptake in the case of malate, fumarate and succinate. We were also unable to grow PSI-succinate dehydrogenase double deletion mutant to test the involvement of succinate dehydrogenase in the net light-induced O_2 evolution rate and to find whether the light-minus-dark CO_2 assimilation in the ΔPSI cells is directly dependent on succinate dehydrogenase.

We note that in addition to the above succinate dehydrogenase hypothesis, CO_2 may also be assimilated by Rubisco without the Calvin-Benson cycle [27] (see Fig. 8B in the main paper). Despite the fact that the ΔPSI cells are capable of net light-induced O_2 evolution and light-minus-dark CO_2 assimilation, and since the ΔPSI cells must be grown in the presence of 5 mM glucose, we speculate that the light-minus-dark CO_2 assimilation in the ΔPSI cells would be insufficient to make all the carbohydrates needed for growth.

*** For this calculation, the Chl-to-PSII ratio was assumed to be 490, the value that was obtained in this work for the WT cells.

S4. Are there additional terminal electron acceptor pools for the net light-induced O_2 evolution in the ΔPSI cells?

Since the DBMIB- and KCN- sensitivities of O_2 evolution in the absence of PSI were plastocyanin-independent, the alternative PQH_2-oxidizing pathway(s) in the ΔPSI cells were not related to the plastocyanin-dependent, alternative Fd reduction pathway *via* PSII as had previously been proposed [28-29].

We also found that other growth medium components, including citrate, trace mineral or SO_4^{2-}, could not restore O_2 evolution in the glucose starved ΔPSI cells and, therefore, did not act as the terminal electron sink.

Synechocystis 6803 has only a reversible hydrogenase HoxEFUYH, and H_2 production has been observed only under anoxygenic conditions [30-31]. Using a Clark-type electrode [32], we did not detect H_2 evolution in the ΔPSI cells under our experimental conditions (i.e., in the presence of O_2, glucose and $NaHCO_3$, and either in the light or in the darkness). Therefore, H^+ is unlikely to be the terminal electron acceptor of the PQH_2-oxidizing pathway(s) in the ΔPSI cells.

References for Supplemental Materials

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