Functional flexibility of photosystem I in cyanobacteria

Yeremenko, N.

Publication date
2004

Citation for published version (APA):
Mechanism and function of Photosystem I cyclic electron transport studied by deletion of two hypothetical proteins of Synechocystis sp. PCC 6803
Mechanism and function of Photosystem I cyclic electron transport studied by deletion of two hypothetical proteins of *Synechocystis* sp. PCC 6803

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**keywords:** antimycin A, ferredoxin quinone oxidoreductase, photosystem I cyclic electron flow, ssr2016, slr1208, *Synechocystis* sp. PCC 6803
Summary

A series of mutants has been designed to clarify pathways and to evaluate the physiological significance of cyclic electron flow around photosystem I in the cyanobacterium *Synechocystis* sp. PCC 6803. Results of P700 reduction kinetics, photoacoustic spectroscopy and fluorescence quenching assays suggest that two hypothetical proteins, products of the neighbouring ORFs *slr1208* and *ssr2016*, are involved in cyclic electron flow and participate independently of the established NADH-dehydrogenase 1, succinate dehydrogenase and ferredoxin:NADP\(^+\) reductase mediated pathways. The *ssr2016* knock-out mutant exhibits a phenotype insensitive to the inhibitor antimycin A suggesting functional involvement of its product in the still poorly defined antimycin A sensitive ferredoxin:quinone reductase pathway. Absence of the *slr1208* product, a hypothetical protein with a putative oxidoreductase function, renders high sensitivity to inhibition by N-ethylmaleimide and decreased sensitivity to antimycin A. The data establish the existence of at least four independent pathways that operate in parallel in the reduction of plastoquinone-cytochrome *b\(_6\)f* with electrons from stromal donors. The physiologically diverse functions and essential implications of these pathways emerged from determination of the relative growth rates of selected mutants in a range of light intensities. It is concluded that photosystem I cyclic electron flow supports growth at low light and is essential in protection against high light.

Abbreviations: NDH-1, type I NADH-dehydrogenase; FNR, ferredoxin:NADP\(^+\) reductase; FQR, ferredoxin:quinone reductase; PSI, photosystem I; PSII, photosystem II; PQ, plastoquinone; NEM, N-ethylmaleimide; WT, wild type; M55, mutant of *Synechocystis* sp. PCC 6803 with interrupted *ndhB*

Introduction

Conversion of solar energy into chemical energy makes photosynthesis one of the fundamental processes of life on Earth. Photosystem II (PSII) and Photosystem I (PSI) located in the thylakoid membrane operate in tandem to produce a linear flow of electrons from water to NADP\(^+\) rendering NADPH. At the same time, electron transfer through the PQ-cytochrome *b\(_6\)f* complex, an intermediate between the two photosystems, generates a proton gradient across the thylakoid that is utilized in ATP synthesis. Linear electron transfer can switch to cyclic electron flow that relies on transmembrane charge separation by PSI only. In cyclic flow electrons from the stromal side of PSI are not used for NADP\(^+\) reduction but instead return to the stromal side of the PQ-cytochrome *b\(_6\)f* complex. Transmembrane passage of electrons through the latter complex to the luminal side of the thylakoid membrane goes...
hand in hand with formation of additional proton motive force that boosts ATP synthesis. In green plants, the generation of a ΔpH also functions as a trigger for the down-regulation of photosystem II photochemistry (Heber and Walker, 1992). This process is mediated by PsbS, an intrinsic subunit of PSII (CP22), which is essential to dispose of excess energy at high light intensities by thermal or non-radiative decay, and correlates with decreased pH in the thylakoid lumen established by PSI cyclic electron flow (Li et al., 2000). Excessive light energy is safely dissipated as heat by this regulatory process and suppresses the generation of toxic reactive oxygen species (Allakhverdiev et al., 1997; Endo et al., 1999). Despite the possible physiological importance and the long history of interest, the functional identity of essential elements in cyclic electron flow around PSI has remained unclear. In particular the full molecular identity and mechanism of action of the enzymes that take part in the electron transport from stromal donors to PQ and/or cytochrome $b_{\text{f}}$, and the functional interplay between those pathways has largely remained unresolved (Ravenel et al., 1994; Bendall and Manasse, 1995; Jeanjean et al., 1999; Allen, 2003).

Several lines of evidence suggested the existence of different and independent pathways for PSI mediated cyclic electron flow. According to one of them, reducing equivalents would be supplied from the stromal pool of NADP(H) to PQ via a NDH-1 complex. A clear phenotype of chloroplast ndh disruptants is a lack of post-illumination increase of chlorophyll fluorescence ($F_{0}'$), which is attributed to electron flow from the stromal pool to PQ (Burrows et al., 1998; Shikanai et al., 1998). In M55, a mutant of Synechocystis without NHD-1 activity (Ogawa, 1991), the rate of respiration and cyclic electron flow around PSI dramatically decreased, which was taken at that time to indicate that NDH-1 dominantly mediates these electron flow pathways in cyanobacteria (Mi et al., 1992, 1994, 1995). However, the prominent role of NDH-1 in electron flow from the stroma has been disputed from the observation that succinate dehydrogenase (SDH) contributes importantly to electron flow in Synechocystis sp. PCC 6803 and from in vitro assays in which the oxidation of NADPH and reduction of PQ appeared insufficient to cover the electron consumption in respiration, whereas added succinate would (Cooley et al., 2000). Absence of turnover of NADPH in M55 restrains the in vivo function of SDH in the tricarboxylic acid cycle and inhibits the SDH-mediated reduction of PQ pool (Cooley and Vermaas, 2001). The M55 mutant requires a high level of CO$_2$ for growth, which was related to a requirement of the inorganic carbon concentrating mechanism for PSI cyclic flow (Tchernov et al., 2001; Matthijs et al., 2002). It was shown that salt stress restored growth of M55 on low inorganic carbon supply (Jeanjean et al., 1998) by triggering of another PSI cyclic electron transfer.
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pathway that proceeds via ferredoxin-NADP\(^+\) reductase (FNR) in configuration when it is attached to the thylakoid membrane (Jeanjean et al., 1999; van Thor et al., 2000). Evidence for direct interaction of FNR with PQ (Bojko et al., 2003) and the cytochrome \(b_5f\) complex (Zhang et al., 2001) has recently been published. A mutant SM8, in which the FNR enzyme was truncated by a 9.7 kDa amino-terminal extension that typically exists in cyanobacteria only, lacked salt-stress inducible PSI cyclic electron flow (van Thor et al., 2000). In this FNR pathway the soluble electron carrier flavodoxin, of which expression is also induced with salt, may play a role (Hagemann et al., 1999).

By far the first known pathway for PSI cyclic flow was revealed by the inhibition of electron transfer from ferredoxin to PQ by antimycin A in chloroplasts (Tagawa et al., 1963). Mediation of this pathway by a putative ferredoxin-quinone reductase (FQR) has been proposed (Cleland and Bendall, 1992; Bendall and Manasse, 1995). The existence of antimycin A sensitive PSI cyclic flow has also been advocated for cyanobacteria (Yu et al., 1993; Cleland and Bendall, 1992; Mi et al., 1995; Jeanjean et al., 1999). However, in marked contrast to the SDH, NDH-1 and FNR catalysed pathways, FQR is still poorly characterized at the biochemical and molecular level.

Recent determination of the crystal structure of the PQ-cytochrome \(b_{5f}\) complex from the thermophilic cyanobacterium \textit{Mastigocladus laminosus} at 3.0 Å revealed an additional, unique heme \(x\) (Kurisu et al., 2003). This new heme is covalently linked to the cytochrome \(b_6\) protein and is located in the same position as the antimycin A binding site in the analogous respiratory cytochrome \(bc_{1}\) complex (Kurisu et al., 2003; Stroebel et al., 2003). The heme can readily contact plastoquinone in the central cavity and therefore may be part of the elusive ferredoxin-plastoquinone reductase. The positive stromal-side surface potential of cytochrome \(b_{5f}\) would facilitate docking of anionic ferredoxin to the stromal side of the complex near heme \(x\) (Kurisu et al., 2003).

Another recent finding that has attributed to knowledge about the transfer of electrons from ferredoxin to plastoquinone was published by Munekage and co-workers (Munekage et al., 2002). A mutant of \textit{Arabidopsis thaliana} denoted as PGR 5 displayed high chlorophyll fluorescence at high light intensity that was insensitive to antimycin A suggesting impaired cyclic electron flow via FQR. The N-terminus of PGR5 from \textit{Arabidopsis} is highly homologous to a hypothetical protein in the \textit{Synechocystis} genome encoded by \textit{ssr2016}. About 200 bp downstream of \textit{ssr2016} a relatively big ORF, \textit{slr1208}, encoding a probable oxidoreductase is located. This ORF was judged to be related to the family of photosynthetic genes (Raymond et al., 2002). Global transcriptome analysis of \textit{Synechocystis} genes by DNA
array (this thesis, Chapter 3) revealed induction of expression of both of these ORFs during adaptation to 0.5 M NaCl, an energy demanding condition linked to the induction of cyclic electron flow (Jeanjean et al., 1993).

To investigate the potential role of srr2016 and slr1208 in cyclic electron flow, here we report on single and double knockout mutants created in *Synechocystis* sp. PCC 6803. Because stromal oxidation of succinate or NADPH constitutes a large contribution to PSI-dependent cyclic electron transfer in cyanobacteria, disrupted srr2016 and slr1208 genes were also transformed into the NDH-1 deficient M55 mutant. The phenotypes thus obtained were studied for PSI cyclic electron flow capacity in the absence or presence of NaCl and/or selected inhibitors. Analysis of various photosynthetic characteristics as well as the relative growth rates of these mutants in different light intensities highlights the mechanism and function of PSI cyclic electron flow.

**Materials and Methods**

**Growth conditions.** *Synechocystis* sp. PCC 6803 wild type and mutants cells (Table 1) were cultivated in liquid culture at 30°C in BG 11 medium (Rippka et al., 1979) or in mineral medium according to Allen (Allen, 1968) at a light intensity of 50 μmol photons m⁻² s⁻¹ in a rotary shaker incubator or on agar plates. Solid medium was supplemented with 1.5% agar, 0.3% sodium thiosulfate, 10 mM TES/NaOH buffer, pH 8.2 and 5 mM glucose. Liquid cultures of the M55 mutant and all mutants with the M55 background were bubbled with air supplemented with 3% CO₂, solid cultures of those mutants were placed in a transluminescent box aerated with 3% CO₂. For cultivation of the mutants appropriate antibiotics were added, dependent on the antibiotic resistance cassette used. Salt-stressed cultures were obtained by the addition of NaCl to 0.5 M to a photoautotrophically growing culture (van Thor et al., 1998). *Escherichia coli* strain XL1-Blue used for routine DNA manipulations was cultivated in Luria broth (LB) medium at 37°C.

**Chromosomal DNA isolation.** Isolation of genomic DNA of *Synechocystis* sp. PCC 6803 was performed according to Ermakova-Gerdes and Vermaas (1999). Briefly, cells were pelleted and incubated at 37°C for 20 min with 2 ml of saturated NaI solution/g (wet weight) of cells. After dilution of NaI with 5–10 volumes of water, cells were pelleted by centrifugation and resuspended in 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 5 mM EDTA; lysozyme was added to a final concentration of 7 mg/ml. After incubation at 37°C for 20 min, N-lauryl sarcosine was added to 1% (w/v) final concentration, and cells were incubated at 37°C for 20 min to induce cell lysis. DNA was extracted several times with phenol, and then with a 1:1 phenol:chloroform mix. During extraction, very gentle agitation was used to avoid extensive fragmentation. After precipitation with ethanol, DNA was resuspended in TE buffer and ammonium acetate was added to a final concentration of 2.5 M. The solution was incubated on ice for 1 h and cleared by centrifugation in a microcentrifuge at 4°C. Ethanol (1.5 volumes) was added to
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the supernatant to precipitate the DNA. DNA was pelleted by centrifugation, washed in 70% ethanol, and resuspended in TE (10 mM Tris-HCl, pH 7.6, and 2 mM EDTA) buffer.


Molecular cloning strategies and vector-DNA manipulation were performed according to standard protocols (Sambrook et al., 1989). All restriction enzymes were purchased from NEB (USA), routine PCR was performed using HotStart polymerase (Qiagen, Germany) and FastStart High Fidelity polymerase (Roche) was used for amplification of long fragments. All DNA fragment for cloning were purified from agarose gel using Gel Extraction Kit (Qiagen). PCR products were purified using a PCR Purification Mini Kit (QIAquick, Qiagen).

To amplify slr1208 and its flanking regions by polymerase chain reaction (PCR) the following two oligonucleotides were designed from the genomic sequence of Synechocystis (Kaneko et al., 1996) (NCBI access, NC_000911): dir1_ Xba (GGGTCTAGAGCCCAAGGAACGGGTA) as direct primer and rev Xho (GATCTCGAGCTTCTTTACAAATTATTA) as reverse primer (underlined face type indicates Xbal and Xhol recognition sites, introduced in the primers). PCR was performed on an Uno II Thermoblock (Biometra, Westburg) under standard conditions (30 cycles of: 92°C 30 sec, 60°C 40 sec, 72°C 1 min per kb, dependent on the length of the amplified fragment; Saiki et al., 1988), using the genomic DNA from Synechocystis as a template. A 3.25 kb fragment resulting from PCR was digested with Xbal and Xhol and cloned into the pBluescript SK+ commercial vector (Promega) resulting in plasmid pSM1. The EcoRI fragment (925 bp) from the pSM1 plasmid was replaced by the 2 kb EcoRI fragment of pHP45Q (Prentki and Krisch, 1984) carrying the streptomycin/spectinomycin resistance genes, resulting in plasmid pSM1Ω (Fig. 1A).

Similarly, to inactivate ssr2016, a DNA construct pSM2Ω was created in which the region of ssr2016 was substituted by the streptomycin/spectinomycin resistance cassette. For this purpose, a fragment of chromosomal DNA covering ssr2016 and its flanking regions was amplified with two pairs of primers. Amplification with the dir3_Xba (CAGGGTCTAGAAGTAGTGGCCAGG) as direct primer and rev_Bam (GGCATGGATCCCCCACCCCGTTACA) as reverse primer and with the dir_Bam (GGCATGGATCCCAACAGCGCCAAA) as direct primer and rev2_Xho (GGCATGGATCCCAAA CAGCGCCAAA) as reverse primer (underlined face type indicates Xbal and BamHI recognition sites for the first pair and BamHI and Xhol for the second one, introduced in the primers) resulted in 840 bp and 2410 bp PCR products. The PCR products obtained were digested with Xbal/BamHI and BamHI/Xhol and subsequently cloned into the Xbal/BamHI and BamHI/Xhol sites of pBluescript resulting in pSM2 plasmid. The 2 kb BamHI fragment of pHP45Q carrying the streptomycin/spectinomycin resistance genes was inserted into the unique BamHI site of pSM2, resulting in plasmid pSM2Ω (Fig. 1B).

Finally, to generate a double slr1208-ssr2016 inactivated mutant, a DNA construct pDM1Ω was created, in which the complete ssr2016 and the largest part of slr1208 was substituted by a streptomycin/spectinomycin resistance cassette. For this purpose, fragments of chromosomal DNA covering the upstream flanking region of ssr2016 and part of slr1208 with its downstream region were
amplified with two pairs of primers. Amplification with the dir3_Xba as direct primer and rev_Eco (CTCCTGAATTCCCTACGAGCAATCAAGAA) as reverse primer and with the dir_Eco (CTAGGA ATTCAATGTGACCTTACTG) as direct primer and rev_Xho as reverse primer (underlined face type indicates EcoRI recognition site, introduced in primer) resulted in 700 bp and 1200 bp PCR products. Obtained PCR products were digested with XbaI/EcoRI and EcoRI/XhoI and subsequently cloned into the XbaI/EcoRI and EcoRI/XhoI sites of pBluescript resulting in pDM1 plasmid. The 2 kb EcoRI fragment of pH45Ω carrying the streptomycin/spectinomycin resistance genes was inserted into the EcoRI site of pDM1, resulting in plasmid pDM1Ω (Fig. 1C).

Plasmids pSM1Ω, pSM2Ω and pDM1Ω served as integrative vectors for the genetic manipulation of Synechocystis wild type and the M55 mutant (the latter carrying a kanamycin resistance cassette). The cells of Synechocystis were transformed with the plasmid containing the modified DNA fragment according to Williams and Szalay (1983). The mixture of cells (400 μl, 4 x 10^9 cells per ml) and plasmids (5 μl, 1 mg of DNA per ml) was incubated in a sterile tube under growth conditions for 6 h and aliquots were plated on a sterile nylon filter (Nucleopore, Whatman) on solid medium. After 20 h of incubation under nonselective conditions, the filters were transferred onto solid medium containing 5 μg of appropriate antibiotics (dependent on the type of inserted antibiotic resistance cassette) per ml. Colonies of transformed cells, which are visible in 7 days, were streaked onto solid medium containing

Figure 1. Schematic representation of the strategy for knock-out of slr1208 (A), ssr2016 (B), and both slr1208 and ssr2016 (C) in Synechocystis. Solid segments denote the position of primers for cloning, open segments represent the position of primers for segregation control.
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Table 1. Strains of *Synechocystis* sp. PCC 6803 used and constructed in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Selective marker</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Glucose-tolerant strain</td>
<td>none</td>
<td>S. Shestakov</td>
</tr>
<tr>
<td>M55</td>
<td>Inactivation of <em>ndhB</em></td>
<td>kanamycin</td>
<td>Ogawa, 1991</td>
</tr>
<tr>
<td>SM8</td>
<td>5'-truncation of <em>petH</em></td>
<td>spectinomycin</td>
<td>van Thor <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>DM4(M55-SM8)</td>
<td>Inactivation of the <em>ndhB</em> and</td>
<td>kanamycin</td>
<td>van Thor <em>et al.</em>, 2000</td>
</tr>
<tr>
<td></td>
<td>5'-truncation of <em>petH</em></td>
<td>spectinomycin</td>
<td></td>
</tr>
<tr>
<td>SM1</td>
<td>Inactivation of <em>srl1208</em></td>
<td>spectinomycin</td>
<td>This study</td>
</tr>
<tr>
<td>SM2</td>
<td>Inactivation of <em>ssr2016</em></td>
<td>spectinomycin</td>
<td>This study</td>
</tr>
<tr>
<td>DM1</td>
<td>Inactivation of <em>srl1208</em> and</td>
<td>spectinomycin</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td><em>ssr2016</em></td>
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<tr>
<td>M55-SM1</td>
<td>Inactivation of <em>ndhB</em> and</td>
<td>kanamycin</td>
<td>This study</td>
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<tr>
<td></td>
<td><em>srl1208</em></td>
<td>spectinomycin</td>
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<tr>
<td>M55-SM2</td>
<td>Inactivation of <em>ndhB</em> and</td>
<td>kanamycin</td>
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<tr>
<td></td>
<td><em>ssr2016</em></td>
<td>spectinomycin</td>
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<tr>
<td>M55-DM1</td>
<td>Inactivation of <em>ndhB</em>, <em>srl1208</em></td>
<td>kanamycin</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>and <em>ssr2016</em></td>
<td>spectinomycin</td>
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</table>

15 µg of appropriate antibiotics. For segregation of transformants, the restriction step was repeated several times with subsequent increasing amounts of antibiotics to 100 µg per ml. The complete segregation of mutants was approved by PCR (See Results, Fig. 2). DNA for routine segregation control was isolated as follows: the colonies from plates were resuspended in 10 mM TE buffer pH 8.0 containing 5 mg per ml of lysozyme and incubated at 37°C for 1 h with gentle shaking followed by phenol-chloroform extraction and precipitation of DNA with 2 volumes of ethanol and 1/10 volume of 3 M NaAc pH 5.0. PCR for segregation control was performed under standard conditions with the following oligonucleotides: *diroxy* (GGACCATACCCACGCTGATGCA) and *rev oxy* (GCACTAGCATGCATGTCCAAACCGCCA) for SM1; *dir2 Xba* (GCATCTAGACCTAGGAGGAGGAGCCCA) and *rev pgr* (GCTGTTGCTACCCAGGCTATTA) for SM2; and *dir2 Xba* and *rev oxy* for DM1.

Oligonucleotides for PCR amplification of *ndhB* gene were as in Ogawa (1991).

**Measurement of P700 absorbance changes as a monitor for PSI cyclic electron flow rates.** Measurements were done with a PAM-101 chlorophyll fluorometer (Walz, Effeltrich Germany), equipped with the standard multi-channel glass fiber optics, and a dual wavelength accessory for P700 measurement at 820 nm (Schreiber *et al.*, 1988; Herbert *et al.*, 1995; Schreiber and Krieger, 1996). Excitation was with the standard far-red LED light source (Walz). Data were stored with WinPAM software. Samples corresponding to about 25 µg Chl *a* were deposited on water retaining filters (Whatman type GFC) by vacuum aspiration. The multi-channel glass fiber optics device with a light passage permitting protective cap was placed directly on top of the sample. Far-red light was switched on at maximum intensity (180 W.m⁻²) for 15 s to oxidize P700 maximally (about 100 W.m⁻² of far-red light proved saturating already, not shown). The absorbance change occurring directly after
switch-off of the far-red light was taken as a guide for the rate at which electrons flow back from the stroma to oxidized P700. The signals obtained with samples on filters were quite stable and permitted to work without noise damping thus making possible measurement of fast kinetic changes. The halftime of reduction of P700 was calculated from the difference between the time that the far-red light was switched off and the time at which the absorbance corresponding to \((A_{\text{max}} - A_{\text{min}})/2\) was reached.

**Treatment with inhibitors.** The stock solutions of inhibitors were freshly prepared. Antimycin A (Sigma) dissolved in ethanol was used at a final concentration of 20 μM, N-ethylmaleimide (NEM, Sigma), dissolved in water at a final concentration of 1 mM. After addition of the inhibitors cells were incubated in darkness for 15 min and concentrated on glass microfibre filters (Whatman) by vacuum filtration for PAM measurements or on nitrocellulose filters (Millipore) for PAS (see below). Antimycin A inhibits the putative FQR pathway and NEM fully inhibits the pathway that proceeds via thylakoid-bound FNR, it also shows some a-specific inhibition.

**Measurement of photochemical energy storage in far-red light.** Light obtained by cyanobacterial cells can be used for photochemistry (‘energy storage’) or is dissipated as fluorescence or heat. At room temperature PSI dissipates excess light energy only as heat. Hence, photoacoustic spectrometry is an ideal method to monitor functionality of PSI cyclic electron flow in situ. Cyanobacterial cells deposited on a nitrocellulose filter (Millipore SCWP01300, 8 μm) were placed in the hermetically closed cell of a photoacoustic spectrometer as described by Ravenel et al. (1994). Light from an Oriel 1000-W halogen lamp was filtered through a RG715 Schott filter and was chopped at 11 Hz with a mechanical chopper. The signal recorded by the microphone was fed into a two-phase lock-in amplifier and was analyzed as previously described (Ravenel et al., 1994; Malkin and Canaani, 1994). The far-red light fluence rate was measured with a Li-Cor radiometer (Li-185B / Li-200SB, Li-Cor, Lincoln, USA). PSI photochemistry was saturated with a strong (continuous) far-red light (>715 nm, 320 W.m⁻²), leading to maximal heat emission. From the amplitude of this light-saturated photothermal signal (\(A_{\text{pt}}^\text{max}\), measured when the strong background far-red light was added to the measuring light) and the actual photothermal signal (\(A_{\text{pt}}\), measured with the modulated exciting light alone), the fraction of absorbed light energy stored in photochemical products (photochemical energy storage, ES) was calculated as \((A_{\text{pt}}^\text{max} - A_{\text{pt}})/A_{\text{pt}}^\text{max}\). As far-red light is exclusively absorbed in PSI, the measured ES is specifically related to PSI function, reflecting ES in photochemical products associated with the cycling of electrons around PSI (Herbert et al., 1990; Malkin and Canaani 1994). Applications of the photoacoustic method to the study of *Synechocystis* sp. PCC 6803 and other cyanobacteria can be found in previous publications (Hagemann et al., 1999; Jeanjean et al., 1999; van Thor et al., 2000; Herbert et al., 2000).

**Measurement of ATP synthesis with acridine yellow. Investigation of cytosolic energization by PSI cyclic electron flow.** Time-resolved measurements of Acridine yellow (AY, Aldrich) fluorescence with cells at 30°C were done in a laboratory-built setup according to Teuber et al. (2001). The measurements were based on the Lock-In amplifier modulation technique, using a pulsed
blue LED light source for dye excitation (445 nm). The light source was equipped with band pass filters (Schott DAL). Excitation and detection of AY fluorescence was done by a branched fiber optics. The detector was a photomultiplier, protected by appropriate filters (Balzers DT Blue or DT Cyan.) The light intensity for dye excitation was sufficiently low to avoid actinic effects. An AY concentration of 5 μM and a chlorophyll a concentration of about 15 μM were used. The cell suspension was supplemented with 50 mM Tricine-NaOH/pH 8.0 before measurements. For the detection of light-induced changes of the fluorescence indicator cells were incubated with AY in darkness for 20 min prior to the measurement. To eliminate linear photo-phosphorylation the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to a final concentration of 10 μM. The source for actinic light was a halogen lamp with filters to provide for red light (‘RL’, 4 mm Schott RG 630 + a Balzers Calflex-3000 heat filter).

**Growth experiments.** Pre-grown cells of WT and mutants were adjusted to optical density 0.1 at 750 nm with growth medium. 200 μl aliquots were inoculated in slots of microtiter plates that were covered with different semi-transparent grey filters. The light passage rendered 182, 89, 43, 22, and 13 μmol photons m⁻²s⁻¹ of incident light. The plates were placed on a flat Rotomix table shaker (Thermolyne) at 100 rpm in a humidity and temperature controlled growth chamber (Sanyo) with an ambient gas phase enriched with 3% CO₂ in air. The changes in optical density at 750 nm were recorded with a Versamax tunable platereader (Molecular Devices, Sunnyvale, CA, USA). The linear slope of logarithmic plots of the growth curves served as a measure for the growth rate. Within each experiment all measurements were replicated sixfold.

**Results**

Two open reading frames, *srl1208* and *ssr2016* were cloned, and deletion constructs (pSM1Ω, pSM2Ω, pDM1Ω) were created with insertion of a cassette conferring resistance to spectinomycin. These plasmids were used for transformations of WT *Synechocystis* sp. strain PCC 6803 to create two single mutants (SM1 and SM2) and one double mutant (DM1). Furthermore, *Synechocystis* strain M55 (*ndhB*) was used to create M55-SM1, M55-SM2 and M55-DM1. The construction of mutants SM1, SM2 and DM1 is depicted in Figure 1. A list of mutants used in this study is shown in Table 1. Full segregation of the deletion mutants was confirmed by PCR (Figure 2).

Dependable photoautotrophic growth for all mutants was enabled by use of Allen medium and addition of 3% CO₂ to the ambient gas phase. Phenotypical characterization of the mutants involved estimation of PSI-driven cyclic electron flow capacity by kinetic spectrophotometry, photoacoustic spectroscopy and monitoring of the rate of ATP formation from cyclic photophosphorylation. The effect of two inhibitors, antimycin A and NEM, were monitored.
Figure 2. Control of segregation of mutants by PCR. Genomic DNA from the wild type, M55, SM1, SM2, DM1 and M55-SM1, M55-SM2 and M55-DM1 strains was used as a template for amplification of the slr1208, ssr2016 and ndhB genes to verify segregation in the mutants. First and last line: DNA ladder.

Cyclic electron transfer rates

The P700 reduction assay informs on rates of electron flow from electron donors in the stroma to the luminal side of P700 via a choice of pathways that all proceed via the PQ-cytochrome $b_6f$ complex and through plastocyanin or cytochrome $c_6$ onwards to P700$, resulting in formation of P700$^-$. Measurement of oxidation-reduction kinetics of P700$^-$ includes two separate steps: the oxidation of P700 to P700$^+$ by far-red light, and the reduction of P700$^+$ after switch-off of the light. The reduction of P700$^+$ can be observed as loss (actually shown as increase in Figure 3) of light absorbance at 820 nm. The method permits to judge the impact of functional knockout of one or more electron flow pathways. Typical examples of P700 oxidation-reduction experiments for WT, M55 and M55-DM1 are shown in Figure 3. At switch-on of the far-red light the signal shows a decrease towards a steady state that was reached within a few seconds. The differences in amplitude observed between strains
reflect the difference in completeness of oxidation of P700 with the actinic light on, and the degree of reduction realised after the actinic light had been switched off. The typical large amplitude for M55 follows from very complete oxidation in the light, and slow but sufficient reduction of P700* in darkness. In the WT the reduction of P700* is taking place while the light is still on, this makes full oxidation impossible and makes the amplitude of the signal smaller. In the M55-DM1 triple mutant the rate of PQ reduction from the stroma is so slow that P700* remains present even after appreciable time in darkness.

The focus in this work was on the rate of reduction of P700* after switch-off of the far-red actinic light. The differences in rates for full reduction of P700* after switch-off of the actinic light are evident. The full reduction of P700* in the M55-DM1 mutant was slower than in M55 and much slower than in the wild type.

The time for reduction of P700* to 50% of the maximum amplitude between the reduced state in darkness and the oxidised state in the light has been calculated for each strain from the average of three independent experiments. The data have been expressed as percentage of the 100% capacity in WT cells from standard Allen medium (Fig. 4A). The half time for P700* reduction in WT cells ranged from 280 ms to 340 ms, with an average of 320 ms. Slower rates extending to over several seconds have been shown in mutants tested in presence of the inhibitors antimycin A and/or NEM. In WT addition of antimycin A rendered only little inhibition of the electron transfer to oxidized P700, whereas combination of antimycin A with NEM resulted in inhibition by slightly over 50% (Fig. 4A). In WT cells kept in darkness for 24 h the inhibitory effect of antimycin in the absence of NEM was appreciable (data not shown). The largest impact of a single mutation was demonstrated by exclusion of electron transfer via NDH-1 and SDH in the M55 mutant. In the M55 mutant electron transfer was at least twice slower than in WT and was also substantially affected by addition of antimycin A. The remaining activity in that mutant after addition of antimycin A and NEM was about 5% of the wild type. In the single mutants SM1, SM2 and SM8, the
electron transfer to oxidized P700 with or without inhibitors was comparable to the WT (data not shown). The double mutant M55-SM2 exhibited electron transfer to P700\(^+\) that was much slower than in M55. Important to note is that this mutant, contrary to M55, showed complete insensitivity to antimycin A. NEM further decreased the low capacity in M55-SM2 like in M55. The analysis of mutant M55-SM1 showed that the sensitivity to antimycin A was less than in M55. The inhibition by NEM in M55-SM1 appeared stronger than in M55 and M55-SM2. The triple mutant M55-DM1 showed P700\(^+\) reduction rates comparable to the double mutant M55-SM2 with full resistance to inhibition by antimycin A. The relatively strong sensitivity to NEM compares well to that observed for M55-SM1. The double mutant M55-SM2...
SM8 behaved similar to M55, however after growth with NaCl a clear difference between M55 and the M55-SM8 double mutant was observed.

Presence of NaCl in the growth medium resulted in a stimulating effect on PSI cyclic flow capacity in all strains (Fig. 4B) with the exception of the SM8 (data not shown) and M55-SM8 mutants. The stimulation of electron flow activity in mutant M55-SM2 was similar as in WT. The sensitivity for NEM increased in all strains that increased PSI cyclic flow capacity with salt.

**Light energy conversion capacity**

A physiological role for PSI cyclic flow is to store the energy from light into ATP to support photoautotrophic anabolism. We studied the impact of mutations and the effect of inhibitors on the conversion of light energy via PSI cyclic electron flow by photoacoustic spectroscopy (PAS). This technique is an indicator for the percentage of the absorbed light energy that is actually used in photochemistry, and stored in photochemical products. Energy storage (ES) is a relative measure and depends on the actinic light intensity. The availability of cyclic electron flow pathways determines the photochemical energy storage that results from PSI cyclic electron flow. Representative traces for WT, M55, M55-SM1, and M55-DM1 are displayed in Fig. 5. WT shows a typical in vivo photothermal signal that is generated by cells with modulated far-red actinic light. Addition of strong non-modulated light to the modulated light beam saturates PSI photochemistry, causing a noticeable rise in the photoacoustic signal (from increased loss of heat). In the condition used, the ES in WT was 14% (Fig. 5, Table 2). ES in M55 was substantially reduced and only a small fraction (<3%) of the absorbed far-red light was used for photochemistry in M55 in the presence of antimycin A. Double mutant M55-SM1 and triple mutant M55-DM1 even in the absence of inhibitors completely resemble the effect of energy storage in M55 in the presence of antimycin A. The overall impact of mutations and inhibitors (NEM and antimycin A) on the efficiency of the storage of light energy is presented in Table 2. The PAS technique reveals complete insensitivity to antimycin A in M55-SM1 in addition to M55-SM2 and M55-DM1. The SM1 and SM2 single mutants exhibit complete insensitivity to antimycin A.

The higher energy storage for cells grown with salt (see the example of M55-DM1) clearly depicts the induction of PSI cyclic flow for the purpose of biochemical energy generation, and is in agreement with the extra energy demand in case of growth with salt. This salt inducible activity is strongly inhibited by NEM, and is absent in mutants based on SM8 with truncated FNR (data not shown).
Table 2. Energy storage efficiency.
The effect of electron transfer inhibitors added at saturation concentration has been monitored by photoacoustic spectrometry. The data is expressed as an average of 3 to 5 experiments ± SD.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Energy storage</th>
<th>+antimycin A</th>
<th>+NEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>14±2</td>
<td>12±0.6</td>
<td>10±1.2</td>
</tr>
<tr>
<td>M55</td>
<td>6±2</td>
<td>2.5±0.9</td>
<td>3.3±1.2</td>
</tr>
<tr>
<td>SM1</td>
<td>16±3</td>
<td>19±3</td>
<td>12±4</td>
</tr>
<tr>
<td>SM2</td>
<td>14.8±1.8</td>
<td>13.9±2</td>
<td>12.4±4.3</td>
</tr>
<tr>
<td>M55-SM1</td>
<td>2±0.2</td>
<td>2.4±0.6</td>
<td>1.8±0.5</td>
</tr>
<tr>
<td>M55-SM2</td>
<td>4±0.15</td>
<td>3.9±0.9</td>
<td>3±0.1</td>
</tr>
<tr>
<td>M55-DM1</td>
<td>2±0.2</td>
<td>2±0.1</td>
<td>1±0.1</td>
</tr>
<tr>
<td>M55-DM1+NaCl</td>
<td>5.4±1.8</td>
<td>5.6±2</td>
<td>1.4±0.2</td>
</tr>
</tbody>
</table>

Figure 5. Photoacoustic signals of WT, M55, M55-SM1 and M55-DM1 cells, with PSI measuring light (>715 nm; 10 Hz; 30 W m⁻²). Upward-pointing arrows indicate when saturating white light (320 W m⁻²) is switched on.

Kinetics of photophosphorylation activity

Direct cyclic photophosphorylation may be demonstrated by the actual measurement of ATP formation or by using an approach, which is based on the relaxation of fluorescence quenching from a dye upon energization of thylakoid membranes as we have used here.
Cyclic electron transfer

(Reuber et al., 2001). The increase in fluorescence reflects ATP formation by PSI cyclic photophosphorylation. The kinetic patterns observed for fluorescence increase demonstrated three different phenomena (Figure 6): 1) a variable delay after switch on of the actinic light, 2) a difference in the rate of formation of fluorescence as visible from the slope of the line, and 3) differences in the capacity for ATP production that are indicated by the final amplitude of the fluorescence increase. The steady state reflects an equilibrium between repulsion and rebinding of dye. Note that red actinic light is needed to realize the fluorescence increase, in its absence the fluorescence quenched again (shown for the WT only, Figure 6A). This non-invasive test tracing the kinetics of ATP formation in PSI cyclic photophosphorylation renders clear differences between the WT and mutants. Both M55-SM1 and M55-SM2 double mutants displayed a delay before the increase of fluorescence starts. WT reaches its steady state faster than M55 and much faster than M55-SM1 and M55-SM2 (Figure 6 B, C, D).

Growth and light acclimation

The actual role that PSI cyclic electron flow and cyclic photophosphorylation play in cellular physiology is largely unknown. To investigate the impact of PSI cyclic flow on the moderation of the redox equilibrium in the plastoquinone/plastoquinol pool, we determined growth rates for the various mutants in a range of light intensities. Growth rates in different ambient light intensities are shown for WT and the single mutants SM1, SM2, and M55 in Fig. 7A and for double mutants DM1, M55-SM2, M55-SM8 and triple one M55-DM1 in Fig. 7B. At low light intensities the growth rates of the single mutants were reduced by about 10%
Figure 7. Relative growth rate of WT and single mutants (A), and double or triple mutants (B). The growth rate of the WT at a light intensity of 43 µmol photons m\(^{-2}\) s\(^{-1}\) was 0.042 h\(^{-1}\) and served as 100%. The values shown are the average of 3 to 6 experiments with a standard deviation below 10%.

Discussion

Electron transfer into and out of the PQ pool in cyanobacterial thylakoid membranes is complex with many different pathways in existence that in part also feature inducibility. In cyanobacteria photosynthesis and respiration both take place in the thylakoid membranes. The PQ-cytochrome \(b_{6}f\) complex is located at a common crossroad for linear electron transfer from PSII to PSI, for electron transfer in PSI driven cyclic electron flow, and for electron transfer in dark respiration. Our findings show that electron donation from the stroma to the PQ-cytochrome \(b_{6}f\) complex proceeds along the pathways indicated in Fig. 8. The relative rate of electron transfer via each of these pathways depends on the availability and type of electron donors and oxidized compounds to accept electrons, the capacity of the pathways, and the required balance between energy generation (by ATP synthesis) and formation of reducing power for the single mutant and by 15 to 20% for the double mutants. At higher light intensities the differences became more pronounced. At the highest photon flux density of 180 µmol photons m\(^{-2}\) s\(^{-1}\) the growth rates of the single mutants and double mutant DM1 were about 30% lower than WT, the double mutant M55-SM2 showed a growth rate of about 50% of that of the single mutant M55; M55-SM8 and triple mutant M55-DM1 grew nearly 80% slower than WT.
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(by NADP⁺ reduction). Control of the balance between energy generation and reducing power is vital for living cells and is secured by the possibility to make extra ATP instead of reducing NADP⁺ via PSI driven cyclic electron flow. In this process several pathways operate in parallel. Those pathways are depicted in Figure 8 as either ‘direct’ (i.e., pathways in cyclic electron transport), or ‘indirect’ (i.e., pathways shared with respiration). The presentation in Fig.8 is limited and donation from flavodoxin instead of ferredoxin (Hagemann et al., 1999), the presence of NDH-2 (Howitt et al., 1999), and participation of various acceptors of electrons different from P700⁺ (Berry et al., 2002) are not indicated.

In view of the multiple pathways, the use of inhibitors yields limited information on the existence, function and capacity of electron transfer. Making functional knockout mutants is helpful, but also in that case complete answers may be hard to retrieve because of the multiplicity of pathways operating in parallel. Mutagenesis of only one pathway by a single knockout may not be informative enough. The indirect pathways, NDH-1 and SDH render such a high electron flux capacity from the stroma to the PQ-cytochrome b₆f complex in cyanobacteria, that this easily takes over a knocked-out alternative function with lesser capacity. For these reasons, we have used a combination of multiple inhibitors and mutants in this study. The rates of P700⁺ reduction obtained in this study indeed indicate that cyclic electron transfer is impaired more strongly in double and triple mutants than in M55 itself. This observation shows that not only the catabolism related SDH and NDH-1 pathways

![Figure 8. A scheme of plastoquinone/cytochrome b₆f reductase routes that catalyze indirect and direct PSI cyclic electron transfer in the cyanobacterium Synechocystis sp. PCC 6803.](image-url)
but also the two pathways via FQR and FNR contribute to the reduction of P700' from the stroma. The observation that the rate of reduction of P700' and ES in far-red light in M55-SM2 and M55-SM1 was reduced compared to M55 alone shows that the hypothetical proteins of the ORFs \textit{ssr}2016 and \textit{slr}1208 are not participating in the indirect NDH-1 pathway and may belong to the set of routes here denominated as 'direct'. The fact that inhibition by antimycin A was absent in mutants lacking the product of \textit{ssr}2016 implies a function of that hypothetical protein in the antimycin A sensitive FQR route. Slight retention of inhibition by antimycin A of the P700' reduction rates in double mutant M55-SM1 might suggest that products of \textit{slr}1208 and \textit{ssr}2016 operate independently. This conclusion contrasts with the observation in the photoacoustic assay where the M55-SM1 and M55-DM1 mutants appeared completely insensitive to antimycin A. However, ES was strongly reduced (<4%) in these double mutants so that a small effect of antimycin A was probably very difficult to measure and could have been missed. Salt addition to the cultures increased the rate of P700' reduction capacity in all strains, except for mutants SM8 (data not shown) and M55-SM8. Absence of inhibition by NEM in M55-SM8 confirms that the salt stress activated pathway via FNR is NEM sensitive and operates independently from the NDH-1 and FQR mediated pathways (van Thor \textit{et al.}, 2000). Whether the induction by salt stress observed for \textit{ssr}2016 and \textit{slr}1208 by global transcriptome analysis (this thesis, chapter 3) is connected to the salt inducible FNR mediated pathway, possibly together with flavodoxin, is presently unknown.

A central role in the distribution of electrons from the stroma to the PQ-cytochrome \textit{b}_{6f} complex in cyclic electron flow may be conferred to ferredoxin. This is the first stable acceptor of electrons that cross the thylakoid membrane from the lumen to the stroma by the trans-membrane charge separation realised at PSI. The redox state of ferredoxin would be highly reduced in the light, unless a drain of electrons is possible. The latter is linked to needs in intermediary metabolism (NADP\textsuperscript{+} reduction, nitrate and sulfate reduction), to direct efflux via the PQ-cytochrome \textit{b}_{6f} complex, or to undesired reduction of oxygen in pseudocyclic electron transfer via a Mehler type of reaction. The possibility of direct electron efflux from overly reduced ferredoxin without passage through the coenzyme couple NADP\textsuperscript{+}/NADPH matches with the requirement to avoid undesirable reactions with oxygen. Overreduction of ferredoxin likely occurs if insufficient demand for electrons exists in anabolism. One principal reason is an insufficient phosphate potential, which is resolved by the extra boost to ATP synthesis provided by PSI cyclic photophosphorylation. The direct route(s) thus offer the option of ferredoxin oxidation independent of nucleotide coenzyme pools that are connected to and may be occupied by a range of other reactions in metabolism. Lesser capacity for
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cyclic photophosphorylation in the mutants was elegantly shown by the slower rate at which fluorescence increased as a function of decreased ATP formation. The ultimate consequence of lower ATP synthesis capacity was monitored from actual differences in growth rates.

In principle, cells of all strains grow well with just linear electron transfer and linear photophosphorylation active, since cyclic photophosphorylation only contributes additional ATP. The slightly lower rates in the mutants reflect the contribution of PSI cyclic electron flow to optimal growth in low light. The contribution of PSI cyclic flow is relatively more important at low light than at normal (50 μmol m² s⁻¹) intensity, because the costs for cell maintenance require relatively more ATP than NADPH. Here, PSI cyclic electron transfer provides the essential extra ATP for optimal growth. It is interesting to note that the rate of reduction of P700⁺ is most affected in the M55-SM2 mutant, but that reduction of the growth rate is stronger in the M55-DM1 and the M55-SM8 mutants. The antimycin sensitive FQR pathway thus appears to provide fast electron transfer with low ATP yield. In the M55-DM1 mutant in the presence of NEM a rapid P700⁺ reduction of small amplitude (about 5% of the WT) was retained, whereas energy storage in the photoacoustic measurement showed no remaining activity. These observations support existence for a shortcut from the stroma to P700⁺. In our work with a psaFJ mutant we noticed a very fast intermediate P700⁺ reduction transient during ongoing oxidation with actinic far red light, together with derepression of the isiAB operon (Jeanjean et al., 2003). In Chapter 5 of this thesis the consequences of overreduction of stromal acceptors in the psaFJ mutant have been addressed. A fast re-reduction of P700⁺ was earlier reported in a cytochrome c₅ less mutant, in which a functional relation with the observed induction of flavodoxin was suggested (Ardelean et al., 2001). In the current study we observed that in mutant SM8 the rate of P700⁺ reduction was 30% faster than in WT (data not shown). As a hypothesis we suggest that in cases that ferredoxin is really overly reduced, flavodoxin provides a less oxygen sensitive store, which itself may act as electron donor in one of the pathways shown in Figure 8, or may otherwise function in a shortcut for return of electrons to P700⁺. In Chapter 5 of this thesis consequences of overreduction of stromal acceptors have been addressed. Detailed biochemical analysis including studies on protein-protein interaction might provide a higher level of resolution for further interpretation of the interesting observations in these mutants.

Lower performance of PSI cyclic electron flow would reduce the capacity for cyclic photophosphorylation, but would also restrain the build-up of a proton gradient in high light that is essential for heat dissipation from Photosystem II (Herber and Walker, 1992). Indeed, the fact that the SM1 and SM2 mutants are more sensitive to high light than M55, and the
observed extremely slow growth rates for M55-DM1 and M55-SM8 under high light, suggest that these strains may severely suffer from photoinhibition. The observation that this occurs already at light intensities that are still permissible for rapid growth of the WT and even M55 points to an important role for direct PSI cyclic electron flow in photoprotection.

In conclusion, the strong impact of mutations on cell growth presents evidence that PSI cyclic electron flow has a clear physiological role in photo-autotrophic growth. Photosystem I cyclic electron flow can operate along several parallel pathways, and supports two functions. In low light, it secures correction of an imbalanced NADPH/ATP ratio. In high light, it protects against photo-oxidation. The combined results of this study suggest that products of slr1208 and ssr2016 are involved in cyclic electron flow and participate independently from NDH-1 and SDH. However, the nature of the mutants and possible interference of inactivation of one ORF with transcription of another one make strict conclusion about the exact protein involved in the effects observed difficult. Absence of inhibition by antimycin A in the ssr2016 knockout mutant provides evidence for a relation of its product and the FQR mediated pathway in cyanobacteria. Though the function of the product of slr1208 in cyclic electron flow still needs to be disclosed, it is very likely that this probable oxidoreductase plays a central role as well.

Acknowledgements
The authors thank Dr. J. Huisman for discussion and critical reviewing the manuscript and V. Krasikov for help in mutant creation.

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*Biochemistry* **41**: 3422-3429.


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Gene 29: 303-313.


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