Role of Type 2 NAD(P)H Dehydrogenase NdbC in Redox Regulation of Carbon Allocation in Synechocystis

Tuomas Huokko, Dorota Muth-Pawlak, Natalia Battchikova, Yakut Allahverdiyeva, and Eva-Mari Aro

Laboratory of Molecular Plant Biology, Department of Biochemistry, University of Turku, Turku FI-20014, Finland

ORCID IDs: 0000-0001-5176-3639 (N.B.); 0000-0002-9262-1757 (Y.A.); 0000-0002-2922-1435 (E.-M.A.).

NAD(P)H dehydrogenases comprise type 1 (NDH-1) and type 2 (NDH-2s) enzymes. Even though the NDH-1 complex is a well-characterized protein complex in the thylakoid membrane of Synechocystis sp. PCC 6803 (hereafter Synechocystis), the exact roles of different NDH-2s remain poorly understood. To elucidate this question, we studied the function of NdbC, one of the three NDH-2s in Synechocystis, by constructing a deletion mutant (ΔndbC) for a corresponding protein and submitting the mutant to physiological and biochemical characterization as well as to comprehensive proteomics analysis. We demonstrate that the deletion of NdbC, localized to the plasma membrane, affects several metabolic pathways in Synechocystis in autotrophic growth conditions without prominent effects on photosynthesis. Foremost, the deletion of NdbC leads, directly or indirectly, to compromised sugar catabolism, to glycogen accumulation, and to distorted cell division. Deficiencies in several sugar catabolic routes were supported by severe retardation of growth of the ΔndbC mutant under light-activated heterotrophic growth conditions but not under mixotrophy. Thus, NdbC has a significant function in regulating carbon allocation between storage and the biosynthesis pathways. In addition, the deletion of NdbC increases the amount of cyclic electron transfer, possibly via the NDH-1 complex, and decreases the expression of several transporters in ambient CO2 growth conditions.
Besides heterotrophic organisms, where NDH-2s are usually located either in mitochondria (eukaryotes) or in the PM (prokaryotes; for review, see Melo et al., 2004), there are several photosynthetic organisms with genes encoding NDH-2s. Plant-type NDH-2s fall into three distinct groups: NDA and NDB relating to fungal sequences and NDC of cyanobacterial origin (Michalecka et al., 2003). Most of these NDH-2s are targeted to mitochondria or peroxisomes, but some also are targeted to plastids. Of the six NDH-2s in the unicellular green alga Chlamydomonas reinhardtii, the thylakoid-localized NDA2 has been produced as a recombinant protein and characterized for its enzymological properties (Desplats et al., 2009). NDA2 was shown to be able to reduce PQ by oxidizing both NADH and NADPH. However, NDA2 showed higher affinity to NADH than NADPH and had FMN as a cofactor instead of the more common FAD. Another NDH-2 enzyme, NDA3, has been localized to C. reinhardtii chloroplasts (Terashima et al., 2010). Physcomitrella patens has six NDH-2 genes, and the products of three of them are targeted to chloroplasts (Xu et al., 2013). The genome of Arabidopsis (Arabidopsis thaliana) contains seven open reading frames encoding NDH-2 homologs, of which at least five have a mitochondrial localization (Michalecka et al., 2003). NDCl, the only NDH-2 of cyanobacterial origin (Michalecka et al., 2003), has dual targeting to both mitochondria and chloroplasts (Carrie et al., 2008), being involved in prenylquinone and vitamin K1 metabolism in the chloroplast (Piller et al., 2011).

All sequenced cyanobacterial species encode at least one NDH-2 enzyme in their genomes (Marreiros et al., 2016). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014). The thylakoid localization has been shown for one NDH-2 enzyme in their genomes (Marreiros et al., 2014).

RESULTS

Construction of the ndbC Deletion Mutant (ΔnadbC)

The interrupted ndbC gene from the ΔnadbC mutant constructed previously (Howitt et al., 1999) was amplified using PCR, and the resulting product was used in transformation (for details, see “Materials and Methods”). Segregation was confirmed by PCR analysis (Supplemental Fig. S1). The absence of the NdbC protein in the constructed ΔnadbC mutant was verified by western-blot analysis using extracted total proteins and specific antibody raised against NdbC (Agrisera; Fig. 1). To verify that the slr1485 and slr1486 genes, which are located immediately downstream of ndbC in the Synechocystis genome, were not affected, their expression was studied with reverse transcription-quantitative PCR (Supplemental Fig. S2). No decline in the expression of either slr1485 or slr1486 was observed in ΔnadbC compared with the wild type.

Growth, Morphology, and Intracellular Glycogen Content of the ΔnadbC Mutant under Ambient and High-CO2 Conditions

The growth of ΔnadbC was monitored under ambient CO2 conditions (LC) and in the presence of 3% CO2 (HC) for several days. Based on optical density (OD750), ΔnadbC grew slightly faster in LC (Fig. 2A) but clearly faster in HC conditions compared with the respective wild type under the same conditions (Fig. 2B). Concomitant inspection of wild-type and ΔnadbC mutant cells with a light microscope (Fig. 3A) and a transmission electron microscope (Fig. 3B) revealed an increased cell size of ΔnadbC compared with the wild type in both
LC and HC. In addition, the cell number in ΔndbC cultures, calculated from equal OD\textsubscript{750} of wild-type and mutant cultures, was almost 53% lower when cells were grown in LC and 59% lower in HC cultures as compared with the wild type (Table I). Since the cell size significantly affects light scattering in addition to OD, the growth was followed by cell counting. Expressing the growth as an increase in cell number reinforced the slow growth of the ΔndbC mutant in both LC and HC (Fig. 2, C and D). Based on cell number, the growth of ΔndbC was about 50% slower compared with the wild type in both LC and HC after 100 h of inoculation. In accordance with the bigger cell size, the dry weight per cell of ΔndbC was about 52% higher in LC and 142% higher in HC compared with the wild type (Table I). In line with this, the chlorophyll \textit{a} (Chl) amount per cell was about 109% bigger in the ΔndbC mutant in LC and 127% bigger in HC compared with the wild type. Similarly, the amount of total proteins per cell in the ΔndbC mutant was 94% higher in LC and about 115% higher in HC compared with the wild type.

Investigation of the cell ultrastructure with the transmission electron microscope revealed an accumulation of dark granules, possibly glycogen, in the ΔndbC mutant cells (Fig. 3B). Determination of the intracellular glycogen content from the ΔndbC and wild-type cells revealed an accumulation of 50% more glycogen in ΔndbC in LC and 18% more in HC when glycogen amount was normalized to Chl concentration (Fig. 3C). Nevertheless, when the glycogen content was normalized to the cell number, one ΔndbC cell contained about 320% more glycogen in LC and 260% more in HC compared with the wild type (Fig. 3D).

Photosynthetic Characterization of the ΔndbC Mutant

The wild-type and ΔndbC cells were first subjected to membrane inlet mass spectrometry (MIMS) with \textsuperscript{18}O-enriched oxygen for gas-exchange investigations. The advantage of MIMS compared with traditional oxygen electrode is the possibility to differentiate between the gross oxygen produced by PSII and the oxygen uptake under illumination. In addition, the oxygen and CO\textsubscript{2} exchanges can be monitored concomitantly.

There was no statistically significant difference in dark respiration between the wild type and ΔndbC in LC conditions (Table II). In ΔndbC, the light-induced oxygen uptake rate, which is defined as the difference between the light and dark oxygen uptake rates, was about 17% higher, whereas the gross and net oxygen evolution rates were 8.5% and 11% lower, respectively, compared with the wild type. The amplitude of the fast phase of CO\textsubscript{2} uptake, which is related to the function of carbon-concentrating mechanisms, was almost 19% smaller in ΔndbC compared with the wild type, but no significant difference in the steady-state CO\textsubscript{2} uptake during illumination was detected.

Next, the Chl fluorescence and P700 oxidoreduction measurements were performed with the ΔndbC cells using the Dual-PAM fluorometer. The PSII yield, measured from dark-acclimated cells (\(F_v/F_m\)\textsuperscript{D}), after illumination with a short far-red (FR) light (\(F_v/F_m\)\textsuperscript{FR}), and the effective
PSII yield \([Y_{(II)}]\), measured after illumination with actinic light for 5 min, were each slightly lower in \(\Delta ndbC\) compared with the wild type both in LC and HC (Fig. 4A; Supplemental Table S1). Likewise, no difference was observed in the maximum quantum yield of PSII \((F_{v}/F_{m})\), measured with 20 \(\mu\text{M} 3-(3,4\text{-dichlorophenyl})-1,1\text{-dimethylurea (DCMU), between the wild-type and \(\Delta ndbC\) cells grown at LC or HC (Supplemental Table S1). When the reoxidation of \(Q_{A}\) in the dark was recorded after a single-turnover saturating flash, the \(\Delta ndbC\) mutant demonstrated slightly slower decay of fluorescence compared with the wild type, indicating modified electron transfer at the PSII acceptor side (Supplemental Fig. S3A). In the presence of DCMU, which blocks electron transfer at the \(Q_{B}\) site, forcing \(Q_{A}\) reoxidation to occur via charge recombination with the donor side components (Vass et al., 1999), no difference in the decay of fluorescence was observed between \(\Delta ndbC\) and the wild type (Supplemental Fig. S2B). There was no significant difference in the effective yield of PSI \([Y_{(I)}]\), in the maximal amount of oxidizable P700 \((P_{m})\), in the maximal fluorescence of dark-adapted cells \((F_{m}^{D})\), or in the maximal fluorescence after FR illumination \((F_{m}^{FR})\) between the wild type and the \(\Delta ndbC\) mutant in LC or HC conditions (Fig. 4B; Supplemental Table S1). These results along with the MIMS data indicate that the deletion of NdbC does not structurally alter PSII or PSI, yet the electron transfer downstream from PSII was slightly slower in the \(\Delta ndbC\) mutant compared with the wild type.

The fact that the slower electron transfer from PSII might be due to a more reduced electron transfer chain made us next monitor the redox state of P700 during dark-light-dark transitions by the application of strong FR light (Fig. 4C). The kinetics of P700 oxidation was clearly slower and rereduction was faster in the \(\Delta ndbC\) mutant, suggesting elevated CET in the mutant. To verify this, the transient increase in fluorescence after illumination \((F_{0}\) rise) was monitored as a postillumination rise in Chl fluorescence, which reflects the efficiency of the NDH-1-related CET (Mi et al., 1992a; Holland et al., 2015). In the \(\Delta ndbC\) mutant, the \(F_{0}\) rise

### Table 1. Comparison of cell number, dry weight, Chl, and protein content between wild-type and \(\Delta ndbC\) mutant cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cells (10^{7}/\text{mL})</th>
<th>Dry Weight (\mu\text{g per OD})</th>
<th>Chl (\mu\text{g per OD})</th>
<th>Protein (\mu\text{g per OD})</th>
<th>Dry Weight (\mu\text{g per 10}^{7}) cells</th>
<th>Chl (\mu\text{g per 10}^{7}) cells</th>
<th>Protein (\mu\text{g per 10}^{7}) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type LC</td>
<td>18.4 ± 1.21</td>
<td>5.4 ± 0.25</td>
<td>4.2 ± 0.08</td>
<td>0.30 ± 0.00</td>
<td>0.29</td>
<td>0.23</td>
<td>0.016</td>
</tr>
<tr>
<td>(\Delta ndbC) LC</td>
<td>8.5 ± 1.01*</td>
<td>3.7 ± 0.41*</td>
<td>4.1 ± 0.11</td>
<td>0.27 ± 0.01*</td>
<td>0.44</td>
<td>0.48</td>
<td>0.031</td>
</tr>
<tr>
<td>Wild type HC</td>
<td>17.6 ± 1.30</td>
<td>4.6 ± 0.30</td>
<td>3.9 ± 0.03</td>
<td>0.23 ± 0.02</td>
<td>0.26</td>
<td>0.22</td>
<td>0.013</td>
</tr>
<tr>
<td>(\Delta ndbC) HC</td>
<td>7.2 ± 1.19*</td>
<td>4.5 ± 0.16</td>
<td>3.6 ± 0.04*</td>
<td>0.20 ± 0.01</td>
<td>0.63</td>
<td>0.50</td>
<td>0.028</td>
</tr>
</tbody>
</table>

---

* Asterisks indicate statistically significant differences between the wild type and the \(\Delta ndbC\) mutant \((P \leq 0.05)\).
Characterization of the ΔndbC Mutant at the Protein Level

Since the characterization of the ΔndbC mutant by the methods described above did not provide any clear functional role for the NdbC protein, we next took a proteomics approach and asked whether the NdbC deletion induces any major changes in the proteome. First, we performed a global label-free tandem mass spectrometry (MS/MS) protein identification and quantitation based on a data-dependent analysis (DDA). We quantified (with \( P \leq 0.05 \)) 543 proteins in HC (Supplemental Tables S2 and S4) and 699 proteins in LC (Supplemental Tables S3 and S5) with at least two peptides. The fold change (FC) threshold of the practical significance was set to \(-1.3 \leq FC \leq 1.3\).

The deletion of NdbC resulted in the differential regulation of a significant amount of proteins. In the HC-grown ΔndbC cells, 128 proteins were up-regulated while 287 proteins were down-regulated in comparison with the wild type (Supplemental Table S4); in the LC-grown ΔndbC cells, the numbers were 211 and 293, respectively (Supplemental Table S5). Proteins were quantified by DDA from cells grown both in HC and LC, while selected reaction monitoring (SRM) was applied only for LC-grown cells.

Differential Protein Expression in the Wild Type and ΔndbC Revealed by a Global Proteomic Approach

In both LC and HC conditions, three enzymes, phosphofructokinase (PfkA), pyruvate kinase (Pyk1), and glyceraldehyde-3-phosphate dehydrogenase (Gap1), which are involved specifically in glycolysis, were down-regulated in the ΔndbC mutant (Table III). A similar response was observed for phosphoglycerate kinase (Pfk) functioning in glycolysis and the Calvin-Benson cycle and for two key enzymes of the oxidative pentose phosphate (OPP) pathway, Glc-6-P dehydrogenase and 6-phosphogluconolactonase, encoded by zwf and pgl, respectively. It is important to note that the deletion of NdbC did not have a significant effect on the expression of proteins functioning in glycogen biosynthesis (GlgA,C,D) or in glycogen degradation (GlgX,P; Supplemental Tables S4 and S5). Furthermore, proteins essential to cell division, including FtsZ, ZipN, MinC, MinD, and MinE, were down-regulated in both growth conditions, as were the components of the urea transporter (UrtAE; Table III). In addition, PII and PipX, participating in the regulation of nitrogen metabolism, were down-regulated (Table III), while the global nitrogen regulator NtcA was not affected (Supplemental Tables S4 and S5). The cyanobacteria-specific AbrB-like transcriptional regulator (cyAbrB) Sll0359 and also the DNA repair protein RecA were down-regulated in LC and HC (Table III). In addition, the deletion of NdbC decreased, both in LC and HC conditions, the expression of proteins encoded by genes located in extrachromosomal plasmids. They included, among others, Sll0653 to Sll0655 and Slr6012 to Slr6016 from pSYX and several proteins from pSYSA that have been annotated as CRISP-related proteins (Scholz et al., 2013). The latter comprised Csm6 and Cas7 from the CRISP2 group as well as Cmr6, Cmr4, and Cmr2 from CRISP3 (Table III). In contrast, the flavodiiron proteins Flv1 and Flv3 involved in the Mehler-like reaction and ChlD involved in Chl biosynthesis were up-regulated in the ΔndbC mutant in both growth conditions. In addition, the circadian clock proteins KaiB1 and KaiC1 were up-regulated in a coordinated manner in both growth conditions, but proteins involved in polysaccharide (RfbB) and amino acid (Sll0664) transport, together with a putative Mg\(^{2+}\) (MgtE) transporter, were down-regulated.

Growth conditions, however, also had an impact on the expression of several proteins in the ΔndbC mutant. In HC, the deletion of NdbC specifically included the down-regulation of GlgB participating in glycogen biosynthesis and the bicarbonate transporter SbtA/B as well as the differential regulation of some subunits belonging to NDH-I (Table III). In particular, the expression of NdhD3 increased in the ΔndbC mutant, while CusP and NdhF4 were down-regulated in HC conditions. Contrary to HC, more prominent differences between the ΔndbC mutant and the wild type were detected at the protein level when cells were...
grown in LC conditions. Subunits of the ATP-binding cassette (ABC)-type bicarbonate transporter, CmpA,B, C,D, and the carbon-concentrating mechanism regulator CcmR were up-regulated in a coordinated manner (Table III). In contrast, NrtC belonging to the nitrate/nitrate transporter (NRT) was down-regulated. Considerable down-regulation was observed for both phosphate transporters, the low-affinity phosphate transporter system Pst1 (PstC1, PstB1, PstB1', PstS1, and SphX) and the high-affinity transporter Pst2 (PstS2). The IlvD protein participating in Glc metabolism (the Entner-Doudoroff pathway) was up-regulated. The expression of several proteins involved in photosynthesis was up-regulated in the ∆ndbC mutant, including the major subunits of the Cyt b6f complex (PetA,B,C,D) that were increased cooperatively, the PSI core subunits PsaB and PsaA, and the IsiA protein (Table III). In addition, the AbrB-like transcriptional regulator Slr0822, one of the subunits of the alternate respiratory terminal oxidase CtaCII, and the circadian clock-related response regulator RpaA were down-regulated in LC conditions in the ∆ndbC mutant as compared with the wild type. In LC, several proteins involved in the transport of amino acids, peptides, glucosylglycerol, and lipopolysaccharides were down-regulated in the ∆ndbC mutant, in contrast to up-regulated Fe, Mn, and K transporters. Furthermore, several proteins involved in DNA repair (Slr6097, RepA, and MutS) were down-regulated in the ∆ndbC mutant compared with the wild type only in LC.

**Targeted Proteomic Approach Strengthened and Expanded the Scope of Proteins Affected in the ∆ndbC Mutant**

We also applied to LC-grown wild-type and ∆ndbC cells a more precise and sensitive targeted quantification method called SRM (Anderson and Hunter, 2006; Ludwig et al., 2012; Vuorijoki et al., 2016). SRM was used in order to verify the results of DDA and,
Table III. Differential protein expression in the ΔndbC mutant versus the wild type

Proteins were quantified with DDA from cells grown in HC and LC and with SRM from LC-grown cells. The practical significance FC is in italic type when up-regulated (FC ≥ 1.3) and boldface type when down-regulated (FC ≤ −1.3; P ≤ 0.05) in the ΔndbC mutant relative to the wild type. NA, Not analyzed; ND, not detected.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Protein</th>
<th>Protein Symbol</th>
<th>HC DDA FC</th>
<th>LC DDA FC</th>
<th>LC SRM FC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell division</td>
<td>Slr1633</td>
<td>FtsZ</td>
<td>−1.47</td>
<td>−1.43</td>
<td>−1.54</td>
<td>6.36E-05</td>
</tr>
<tr>
<td></td>
<td>Slr0169</td>
<td>ZipN</td>
<td>−1.01</td>
<td>−1.40</td>
<td>−1.58</td>
<td>5.78E-05</td>
</tr>
<tr>
<td></td>
<td>Slr0288</td>
<td>MinC</td>
<td>−1.54</td>
<td>−1.33</td>
<td>−1.45</td>
<td>4.99E-02</td>
</tr>
<tr>
<td></td>
<td>Slr0289</td>
<td>MinD</td>
<td>−1.42</td>
<td>−1.61</td>
<td>−1.60</td>
<td>3.39E-05</td>
</tr>
<tr>
<td></td>
<td>Ssl0546</td>
<td>MinE</td>
<td>−1.56</td>
<td>−1.39</td>
<td>−1.57</td>
<td>8.99E-05</td>
</tr>
<tr>
<td>Circadian clock</td>
<td>Slr0757</td>
<td>KaiB1</td>
<td>−1.57</td>
<td>−1.69</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Slr0758</td>
<td>KaiC1</td>
<td>−1.58</td>
<td>−1.47</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Slr0115</td>
<td>RpaA</td>
<td>−1.08</td>
<td>−1.58</td>
<td>3.86E-03</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0108</td>
<td>Amt1</td>
<td>−1.75</td>
<td>−5.16</td>
<td>1.41E-03</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0224</td>
<td>1.25E-02</td>
<td>1.19E-02</td>
<td>1.67E-03</td>
<td>1.08</td>
<td>NA</td>
</tr>
<tr>
<td>DNA repair</td>
<td>Sll0659</td>
<td>RecA</td>
<td>−1.50</td>
<td>−1.66</td>
<td>2.35E-04</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0907</td>
<td>1.02E-02</td>
<td>1.19E-02</td>
<td>1.09</td>
<td>3.34E-01</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll1165</td>
<td>MutS</td>
<td>−1.19</td>
<td>−1.75</td>
<td>1.61E-02</td>
<td>NA</td>
</tr>
<tr>
<td>Transport and binding proteins</td>
<td>Sll0681</td>
<td>PstC1</td>
<td>1.22</td>
<td>18.67</td>
<td>6.27E-05</td>
<td>−12.21</td>
</tr>
<tr>
<td></td>
<td>Sll0683</td>
<td>PstB1</td>
<td>−1.00</td>
<td>−6.85</td>
<td>3.06E-04</td>
<td>−11.22</td>
</tr>
<tr>
<td></td>
<td>Sll0684</td>
<td>PstB1'</td>
<td>1.01</td>
<td>−8.10</td>
<td>4.64E-03</td>
<td>−8.26</td>
</tr>
<tr>
<td></td>
<td>Sll0680</td>
<td>PstS1</td>
<td>−1.12</td>
<td>−2.78</td>
<td>1.61E-05</td>
<td>−4.02</td>
</tr>
<tr>
<td></td>
<td>Sll0679</td>
<td>SphX</td>
<td>1.26</td>
<td>−1.60</td>
<td>3.41E-02</td>
<td>1.63</td>
</tr>
<tr>
<td></td>
<td>Sll1247</td>
<td>PstS2</td>
<td>1.42</td>
<td>−1.34</td>
<td>3.85E-03</td>
<td>−1.45</td>
</tr>
<tr>
<td></td>
<td>Sll0540</td>
<td>PstS</td>
<td>−1.35</td>
<td>−3.16</td>
<td>4.21E-04</td>
<td>3.09</td>
</tr>
<tr>
<td></td>
<td>Sll1451</td>
<td>NrtB</td>
<td>1.07</td>
<td>1.62</td>
<td>1.37E-03</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll1452</td>
<td>NrtC</td>
<td>−1.23</td>
<td>−2.24</td>
<td>1.96E-02</td>
<td>−1.58</td>
</tr>
<tr>
<td></td>
<td>Sll1453</td>
<td>NrtD</td>
<td>−1.05</td>
<td>−1.71</td>
<td>6.98E-04</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0108</td>
<td>UrtA</td>
<td>−1.67</td>
<td>−1.91</td>
<td>4.61E-04</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0764</td>
<td>UrtD</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0374</td>
<td>UrtE</td>
<td>−1.49</td>
<td>−1.34</td>
<td>1.45E-02</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll1527</td>
<td>YcbB</td>
<td>1.47</td>
<td>1.60</td>
<td>6.96E-01</td>
<td>−1.03</td>
</tr>
<tr>
<td>HCO₃⁻ uptake</td>
<td>Sll1927</td>
<td>SbtA</td>
<td>−1.45</td>
<td>1.00</td>
<td>9.60E-01</td>
<td>6.94E-01</td>
</tr>
<tr>
<td></td>
<td>Sll1513</td>
<td>SbtB</td>
<td>−2.19</td>
<td>−1.02</td>
<td>7.85E-01</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0982</td>
<td>RfbB</td>
<td>−1.95</td>
<td>−3.09</td>
<td>9.96E-06</td>
<td>NA</td>
</tr>
<tr>
<td>Others</td>
<td>Sll0064</td>
<td>1.61</td>
<td>1.88E-02</td>
<td>2.44</td>
<td>2.20</td>
<td>1.70E-04</td>
</tr>
<tr>
<td></td>
<td>Sll1216</td>
<td>MgtE</td>
<td>−1.99</td>
<td>−2.40</td>
<td>2.35E-04</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0559</td>
<td>NrtB</td>
<td>−1.21</td>
<td>−1.80</td>
<td>1.41E-03</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>Sll1740</td>
<td>AppA</td>
<td>−1.14</td>
<td>−1.70</td>
<td>4.64E-04</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0250</td>
<td>YcbBS</td>
<td>−1.08</td>
<td>−1.68</td>
<td>1.73E-02</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll1927</td>
<td>AppF</td>
<td>1.47</td>
<td>−1.64</td>
<td>2.89E-04</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0747</td>
<td>GctA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll1762</td>
<td>1.50</td>
<td>1.50E-04</td>
<td>−1.53</td>
<td>1.61E-02</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0415</td>
<td>1.13</td>
<td>4.00E-01</td>
<td>−1.50</td>
<td>3.41E-02</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll2131</td>
<td>AcrF</td>
<td>−1.49</td>
<td>−1.37</td>
<td>3.76E-02</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0369</td>
<td>EnvD</td>
<td>−1.15</td>
<td>−1.36</td>
<td>1.87E-03</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0224</td>
<td>1.30</td>
<td>1.20E-01</td>
<td>−1.31</td>
<td>9.42E-03</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0575</td>
<td>RfbB</td>
<td>−1.18</td>
<td>−1.31</td>
<td>4.34E-02</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll1270</td>
<td>BgtB</td>
<td>1.02</td>
<td>−1.39</td>
<td>6.99E-03</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll0672</td>
<td>PscL</td>
<td>1.18</td>
<td>1.53</td>
<td>4.05E-03</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll1295</td>
<td>SufA</td>
<td>1.05</td>
<td>1.58</td>
<td>6.72E-05</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll1598</td>
<td>MntC</td>
<td>ND</td>
<td>ND</td>
<td>1.65</td>
<td>5.79E-04</td>
</tr>
<tr>
<td></td>
<td>Sll1481</td>
<td>1.82</td>
<td>6.22E-03</td>
<td>1.80</td>
<td>2.05E-04</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll1729</td>
<td>KdpB</td>
<td>1.07</td>
<td>2.34</td>
<td>2.02E-03</td>
<td>NA</td>
</tr>
<tr>
<td>NDH-1</td>
<td>Sll1291</td>
<td>NdhD2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.65</td>
</tr>
<tr>
<td></td>
<td>Sll1733</td>
<td>NdhD3</td>
<td>1.37</td>
<td>2.19</td>
<td>1.29E-01</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td>Sll1735</td>
<td>CupS</td>
<td>−1.41</td>
<td>−1.54</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Sll0026</td>
<td>NdhF4</td>
<td>−1.36</td>
<td>−1.57</td>
<td>5.11E-03</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Sll1302</td>
<td>CupB</td>
<td>−1.09</td>
<td>−1.56</td>
<td>4.37E-02</td>
<td>−1.53</td>
</tr>
</tbody>
</table>

(Table continues on following page.)
importantly, to expand the scope of quantified membrane-embedded photosynthetic and respira-
tory proteins. For the SRM experiments, we selected 108 proteins, including those showing differential ex-
pression in DDA, plus several other targets that could provide complementary information on proteomeic 
changes of important metabolic routes in the ΔnbdC 
mutant. On the other hand, SRM revealed several 
newly divergently regulated proteins that were not 
detected in the global approach or were detected and 
quantified but with a high P value. Importantly, we 
discovered that various NDH-1 complexes respon-
ded differently to the NdbC mutation. SRM results 
showed that NDH-11, with the speci-
cNdhD1 sub-
unit, and NDH-13, with NdhF3, CupA, and 
Cup5, were not affected (Supplemental Table S6). In 
contrast, NdhD2, the specific subunit of the previ-
ously elusive NDH-1 complex, was up-regulated 
substantially (Table III), while NdhF4 and CupB, 
belonging to the NDH-14 complex, were distinctly 
down-regulated. Common NDH-1 subunits remained
unchanged (Supplemental Table S6). Furthermore, we discovered the down-regulation of SigE, the group 2 σ-factor, a decrease of NrtB and NrtD subunits of the NRT transporter, coordinately with NrtC, and an up-regulation of ChlH involved in Chl biosynthesis (Table III).

Western-Blot Analysis of Selected Proteins Provided Further Insights into the Consequences of ndbC Deletion

To verify the proteomic results described above, western-blot analysis of total proteins isolated from LC-grown cells was performed for several targets (Fig. 5A). The results obtained with protein-specific antibodies corroborated conclusions made based on mass spectrometry (MS) methods. In particular, it was confirmed that the Psab amount was slightly up-regulated in ΔndbC compared with the wild type (Fig. 5A). Moreover, the amount of the small form of ferredoxin-NADP⁺ oxidoreductase (FNR) was shown to be higher in ΔndbC compared with the wild type.

Since the proteomic results showed an interesting effect of the NdbC deletion on NDH-1 complexes, a possible interplay between NdbC and the NDH-1 variants was addressed. To this end, several NDH-1 mutants were grown in LC conditions, and total proteins were isolated and immunoblotted with α-NdbC (Fig. 5B). Intriguingly, NdbC was not expressed in detectable amounts if the functional NDH-1 complex (ΔndhB/M55 mutant) was missing (Fig. 5B). In line with this observation, the amount of NdbC was lower compared with the wild type when NdhD1 or NdhD2 was deleted, and the double deletion of these NDH-1 subunits decreased the NdbC amount even more. The deletion of NdhD3 or NdhD4 did not affect the NdbC amount compared with the wild type.

Furthermore, western blotting was used to reveal the localization of NdbC in the cell. Protein fractions representing the thylakoid membrane, soluble compartment, and the PM were analyzed with the NdbC-specific antibody. NdbC was clearly located only in the PM, while no signal was detected from the thylakoid membrane fraction or from the soluble proteins (Fig. 5C), in line with results obtained by proteomic studies (Pisareva et al., 2011; Liberton et al., 2016).

Response of ΔndbC to Light-Activated Heterotrophy and Photomixotrophic Conditions

Proteomics analysis of the ΔndbC mutant suggested a putative role for the NdbC protein in general carbon metabolism, which prompted us to test the behavior of the wild type and the ΔndbC mutant in the presence of an external carbon source. To this end, the wild-type and mutant cells were first subjected to LAHG conditions, where the growth medium was supported with 10 mM Glc and illumination was limited to 10 min (50 μmol photons m⁻² s⁻¹) every 24 h. Under these conditions, the ΔndbC mutant practically did not grow (Fig. 6A). Next, the length of the light period was increased to 4 h per day (4 h of 50 μmol photons m⁻² s⁻¹/20 h of dark) to change the growth conditions toward mixotrophy, which restored the growth of the ΔndbC mutant (Fig. 6B). During days 2 to 4, the growth of the ΔndbC mutant was faster compared with the wild type. Under mixotrophic conditions (50 μmol
photons m$^{-2}$ s$^{-1}$ continuous illumination + 10 mM Glc), there was no difference in the growth based on OD between the wild type and ΔndbC (Fig. 6C). Nevertheless, it is important to note that, also in the presence of Glc, the ΔndbC cells were considerably larger compared with the wild type (Supplemental Fig. S4), which increases OD and is in line with the phenotype observed under autotrophic growth conditions (Fig. 3A).

Redox State of the Electron Transport Chain in Darkness in the ΔndbC Mutant

To monitor more precisely the redox state of the ETC in ΔndbC during incubation in darkness, the cells were grown first in autotrophic conditions and then transferred to the dark for 24 h. QA$^-$ reoxidation kinetics and the fluorescence induction dynamics measured from the 24-h dark-adapted ΔndbC cells did not differ strikingly from those in the wild type (Fig. 7, A and B; Supplemental Fig S3, A–C). To address the possible effect of Glc on ETC in darkness, 24-h dark-incubated cells were supplemented with 10 mM Glc for 15 min before the Chl fluorescence measurements. A short incubation with Glc markedly slowed down QA$^-$ reoxidation kinetics in both the wild-type and ΔndbC cells, yet the kinetics became much slower in ΔndbC (Fig. 7C), implying a modification of the PSII acceptor side of the mutant, presumably due to the reduced PQ pool. When the flash-induced fluorescence yield was monitored in the presence of DCMU, reflecting more the donor side function of PSII, no clear difference was observed between the wild type and ΔndbC (Supplemental Fig. S3D). Next, the dynamic changes in the redox status of the PQ pool in dark-incubated cells supplemented with Glc were studied by Chl fluorescence measurements using a saturating pulse method (Fig. 7D). A sudden exposure of the dark-incubated wild-type cells to steady-state actinic light induced a transient increase in the F$s$ level with a concomitant decrease of $D_F$, reflecting a reduced PQ pool during the first 3 min of illumination. The ΔndbC cells demonstrated a higher $F_s$ level and low $F_m^D$ and $D_F$ values compared with the wild type (Fig. 7D). These results clearly showed that the addition of Glc in darkness triggers much stronger reduction of the PQ pool in ΔndbC than in the wild type and that the subsequent exposure to moderate light is capable of oxidizing the ETC.

DISCUSSION

In cyanobacteria, the physiological role of the NDH-2 enzymes remains largely unknown, particularly in comparison with the much better characterized NDH-1 complexes playing important roles in respiration, CET, and CO$_2$ uptake (for review, see Peltier et al., 2016). It was hypothesized previously that, in Synechocystis, the NDH-2s do not have a metabolically

![Figure 6](image-url)
relevant role in respiratory energy production and, instead, were postulated to be involved in regulatory functions, yet with no knowledge of specific targets so far (Howitt et al., 1999). Here, we addressed the physiological role of NdbC, one of the three NDH-2s encoded in the genome of *Synechocystis*. The deletion of NdbC (Fig. 1) revealed several significant rearrangements in the physiology, metabolism, and morphology under autotrophic growth conditions without affecting respiration (Table II) and, in addition, disclosed an indispensable function for NdbC under LAHG conditions. It is demonstrated that NdbC, localized in the PM (Fig. 5C), has a significant role in carbon allocation between the storage and various biosynthetic pathways (for LC conditions, this is presented schematically in Fig. 8).

Deletion of NdbC Distorts Cell Division

When the ΔnbdC mutant was grown under autotrophic conditions, in HC or LC, visible morphological changes were observed. The cell size became considerably bigger (Fig. 3A), with increase in the content of intracellular total protein, Chl, and dry weight compared with the wild type (Table I). Based on the cell number, ΔnbdC demonstrated substantially slower growth compared with the wild type in both LC and HC (Fig. 2, C and D). This was corroborated by the proteomic examination of ΔnbdC. The expression of specific cell division-related proteins (Mazouni et al., 2004) was clearly altered in ΔnbdC, including down-regulation of FtsZ, ZipN, MinC, MinD, and MinE proteins in HC and LC (Table III). It is possible, however, that the influence of the NdbC deletion on cell division may be a secondary effect, as some global regulators of gene expression, such as the group 2 σ-factor SigE (detected only in LC), the AbrB-like transcription factor Sll0822 (in LC), and components of the circadian clock (KaiB and KaiC), were likewise down-regulated in the ΔnbdC mutant. The influence of these proteins on cell division was demonstrated previously by Osanai et al. (2013) for SigE, by Yamauchi et al. (2011) for Sll0822, and in a review by Cohen and Golden (2015) for Kai proteins. In addition, several proteins encoded by pSYSX and pSYSA were markedly down-regulated in ΔnbdC compared with the wild type in both HC and LC conditions (Table III). Among them are CRISPR2 and CRISPR3 (Scholz et al., 2013), which belong to the CRISPR/Cas systems responsible for resistance to horizontal gene transfer, including phage transduction, transformation, and conjugation (Marraffini and Sontheimer, 2008; for review, see Sorek et al., 2013). However, the influence of NdbC deletion on these proteins remains unclear, since the mutant could have experienced problems with plasmid replication connected to the hindrance in cell division.

Carbon Catabolism Is Down-Regulated in the Absence of NdbC

Measurements of the intracellular glycogen showed that this important reserve of carbon was significantly higher in the ΔnbdC mutant compared with the wild type, both in LC and HC (Fig. 3D). The increase of intracellular glycogen per cell in the mutant was markedly more profound than the increase of the Chl amount or of the total protein content (Table I). In line with this, the electron microscopy of ΔnbdC cells in LC revealed the occurrence of multiple dark spots that, probably, are glycogen granules (Fig. 3B). The accumulation of glycogen could be due to a misbalance between synthesis and degradation. It is important to note, however, that amounts of enzymes performing...
glycogen synthesis (GlgA,B,C) or glycogen degradation (GlxP) were not coordinately altered in the ΔndbC mutant compared with the wild type either in LC or HC (Table III). Thus, the shift of the balance has to be caused by other reasons. Indeed, the proteomic analysis revealed alterations in protein contents of metabolic pathways involved in intracellular carbon allocation in LC and in HC; Gap1, Pgk, and Pyk1, the three enzymes catalyzing unidirectional reactions in glycolysis, were down-regulated in the ΔndbC mutant (Table III).

Considering the first steps of the glycolytic route, the OPP pathway and the Embden-Meyerhof-Parnas (EMP) pathway were down-regulated in both studied conditions. The amounts of two key enzymes, Glc-6-P dehydrogenase (Zwf) and 6-phosphogluconolactonase (Gnd), specific for OPP as well as PfkA involved in EMP were likewise reduced in the ΔndbC mutant (Table III). These results are in agreement with earlier studies showing that the mRNA amount of ndbC follows circadian rhythm peaking in the dark together with genes involved in sugar catabolism (Kucho et al., 2005; Layana and Diambra, 2011) as well as in the beginning of heterotrophy (Lee et al., 2007). Conversely, 6-PG hydratase (IlvD), which belongs to the recently discovered Entner-Doudoroff pathway in cyanobacteria (Chen et al., 2016), was up-regulated in ΔndbC in LC (Table III) and might partially compensate for the down-regulation of EMP and OPP.

Overall, the observed decrease in the expression levels of many glycolytic enzymes suggests that the NdbC deletion caused the slowdown of sugar catabolism, probably resulting in the reduction of pyruvate.

Figure 8. Schematic model representing the effects of NdbC deletion on protein expression in Synechocystis cells in LC conditions compared with the wild type. The up-regulated or accumulated protein complexes, metabolic routes, or compounds in the ΔndbC mutant are marked in red, and the down-regulated ones are marked in blue. Solid arrows represent metabolic routes or uptake, dashed arrows represent linear electron transfer, and dotted arrows represent CET. TM, Thylakoid membrane.
The Deletion of NdbC Alters the Expression of Multiple Transport and Binding Proteins Especially in LC

Even though similar changes in the proteome related to cell division and sugar catabolism were detected between the wild type and \( \Delta ndbC \) both in HC and LC, the deletion of NdbC had an evidently more profound effect on the expression of a large group of transporters in LC compared with HC. Most of these transporters were reported previously to localize in the PM (Pisareva et al., 2011), in accordance with the location of the NdbC protein (Fig. 5C). The transport of various nutrients, like potassium and metal ions, amino acids, and polysaccharides (Table III), demonstrated marked down-regulation in the \( \Delta ndbC \) mutant except for the \( \text{HCO}_3^- \) transporter BCT1 (Omata et al., 1999), which was up-regulated in the \( \Delta ndbC \) mutant compared with the wild type. The strongest down-regulation was observed for both ABC-type inorganic phosphate transporters (Table III), the constitutive low-affinity phosphate transporter Pst1 and the inducible, high-affinity phosphate transporter Pst2 (Pitt et al., 2010; Burut-Archanai et al., 2011). Furthermore, in the \( \Delta ndbC \) mutant, the levels of enzymes involved in nitrogen uptake, NRT transporters responsible for nitrate and nitrite uptake, as well as urea and ammonium transporters were down-regulated (Table III). Such coordinated down-regulation of different transporters in LC, when there is a shortage of terminal acceptors, unlike in HC, can be a protective response of the \( \Delta ndbC \) cells aimed to regulate a redox homeostasis of the cells by ion influx and efflux.

In addition to transporter proteins, the expression of several regulators of transporter functions is altered in \( \Delta ndbC \). While CmpR inducing BCT1 expression (Nishimura et al., 2008; Daley et al., 2012) was not affected (Supplemental Table S5), the SI0822 protein, which functions as a supplementary inducer of the Cmp operon (Orf et al., 2016), was down-regulated in \( \Delta ndbC \), as was already mentioned above (Table III). Thus, the up-regulation of the Cmp operon in \( \Delta ndbC \) was not caused by known regulating mechanisms. The regulators of the nitrogen assimilation genes PII and PipX (Llácer et al., 2010; Espinosa et al., 2014; for review, see Forchhammer, 2004) were reduced in the \( \Delta ndbC \) mutant (Table III), while the global nitrogen regulator NtcA remained unchanged (Supplemental Table S5). Decreased amounts of PII and PipX, which promote the deactivation of NtcA (Luque et al., 2004), as well as down-regulation of SI0822, also an inducer of nitrogen transport (Ishii and Hihara, 2008; Yamauchi et al., 2011), could cause the low accumulation of nitrogen transporters induced by the NdbC deletion, particularly in LC. Thus, in LC conditions, the deletion of NdbC disrupts the normal behavior of multiple transport systems, mainly of ABC-type transporters located in the PM.

Cross Talk between NdbC and the NDH-1 Complex: Low ATP Production from Sugar Catabolism in \( \Delta ndbC \) Is Compensated by CET

Cyanobacteria can incorporate inorganic carbon (\( \text{C}_i \)) in the form of \( \text{CO}_2 \) utilizing the low-\( \text{CO}_2 \)-inducible high-affinity \( \text{NDH-1}_3 \) complex and the constitutively expressed low-affinity \( \text{NDH-1}_4 \) complex (for review, see Battchikova et al., 2011). MIMS measurements revealed that the fast phase of \( \text{CO}_2 \) uptake was decreased in the \( \Delta ndbC \) mutant in LC (Table II). The proteomic results did not indicate changes in the expression of specific subunits of NDH-1\(_3\) in the \( \Delta ndbC \) mutant in LC (Table III). In contrast, NdF4 and CupB, specific subunits of NDH-1\(_4\), were coordinately down-regulated in \( \Delta ndbC \) compared with the wild type in LC (Table III), being in line with MIMS results. To get insights into the connection between the NdbC deletion and the expression of NDH-1\(_4\), we performed a western-blot analysis of several mutants lacking specific subunits of distinct NDH-1 variants using the NdbC-specific antibody (Fig. 5B). Remarkably, NdbC was severely down-regulated in the M55 strain, indicating some cross talk between this protein and NDH-1. However, it is important to note that the amount of NdbC in the \( \Delta ndhD4 \) mutant (and in \( \Delta ndhD3/D4 \)) was comparable with that in the wild type. Thus, NdbC is involved in the accumulation of NDH-1\(_4\), but not the other way around. Most probably, NdbC affects NDH-1\(_4\), indirectly, since they are located in different membrane compartments (Liberton et al., 2016).

In the \( \Delta ndbC \) mutant, CET around PSI was clearly elevated, as deduced from the high amplitude of \( F_0 \) rise (Fig. 4D; Mi et al., 1992a; Holland et al., 2015) and from slower oxidation and faster rereduction kinetics of \( F_700 \)
(Fig. 4C; Mi et al., 1992b; Fan et al., 2007). Proteomic analysis revealed that two variants of the NDH-1 complex, NDH-1, and NDH-12, which are involved in CET (Mi et al., 1992a), demonstrate different responses to NdbC deletion. NDH-1 was not affected, according to the levels of NdhD1 specific for this complex (Supplemental Table S6). In contrast, marked up-regulation was observed for NdhD2 (Table III), the specific subunit of NDH-12. The latter complex is present in Synechocystis cells in much smaller amounts than NDH-1 (Herranen et al., 2004), but, despite a low abundance, NDH-1, which accumulated in the ΔndbC mutant contributed to the significant increase in CET (Fig. 4, C and D). Western-blot analysis demonstrated that in the ΔndhD2 mutant (and in ΔndhD1/D2), the NdbC amount was clearly lower compared with the wild type (Fig. 5B). Thus, NdbC and NDH-12 affect each other, in contrast to NDH-1.

Furthermore, according to western-blot analysis, FNR, was up-regulated in ΔndbC (Fig. 5A). This form of FNR oxidizes NADPH and accumulates in heterotrophic conditions, possibly enhancing CET by transferring electrons via ferredoxin to NDH-1 (Thomas et al., 2010). The elevated CET in the mutant was accompanied by an increase of the Cyt $bc_6$ complex (PetA,B,C,D; Table III). Enhanced CET and the corresponding increase in ATP production can compensate for lower ATP yield from glycolytic reactions in the mutant. In addition, the up-regulation of oxygen photo-reduction (Table II), operated by the increased Flv1 and Flv3 proteins (Table III), possibly contributes to ATP production via a functional water-water cycle and increases the photo-protection of photosynthetic protein complexes, especially PSI (Allahverdiyeva et al., 2015).

Even though the carbon catabolic reactions were distorted due to the deletion of NdbC, photosynthesis in general was not compromised dramatically either in LC or in HC; no significant difference was detected in $F_v/F_m$, in $F_{m}'$ or in Y(II) between the wild type and ΔndbC (Fig. 4B; Supplemental Table S1). However, both the gross and net oxygen evolution (Table II), as well as Y(II) (Fig. 4A), of ΔndbC were somewhat lower compared with the wild type, demonstrating excess electron pressure downstream of PSI, which is in agreement with elevated CET. Cooley and Vermaas (2011) showed that the PQ pool of Synechocystis deficient of all three NDH-2s (ΔndbABC) was more oxidized during illumination, which is contrary to the redox status of the PQ pool in ΔndbC. Thus, we cannot exclude the possibility that the redox state of the NADH pool (by NDH-2) and the NADPH pool (via NDH-1) can influence each other, at least in the case of ΔndbC.

**NdbC Is Indispensable in LAHG Conditions**

The consequences of the blockage of the glycolytic route, evident even in autotrophic conditions, should become more profound when photosynthesis is limited and all the energy for cellular needs must be derived from the degradation of sugars. This was experimentally proven by the inability of the ΔndbC mutant to grow under LAHG conditions (Fig. 6A). In heterotrophic growth conditions, 90% of supplemented Glc is directed to OPP and only about 5% enters glycolysis (Yang et al., 2002). In ΔndbC, OPP is strongly down-regulated even in autotrophic conditions, and this is expected to have serious consequences on nucleotide biosynthesis upon LAHG conditions, due to the fact that OPP is the only source for required intermediates in darkness (Kruger and von Schaewen, 2003). Similarly, ΔsigE, which is deficient in sugar catabolism, shows retarded growth in LAHG conditions (Osanai et al., 2005). Fluorescence measurements further confirmed the problems of ΔndbC. The ETC became severely and rapidly over-reduced in the dark and during the dark-light transition upon supplementation of the culture with Glc (Fig. 7). The reason for Glc toxicity could be a reprogramming of carbon metabolism and a high production of reactive oxygen species triggering oxidative stress and preventing cell growth, eventually causing cell death (Latifi et al., 2009; Narainsamy et al., 2013). In mixotrophic conditions, when the active Calvin-Benson cycle functions together with the OPP pathway (Yang et al., 2002), the ΔndbC mutant was able to grow (Fig. 6C). The up-regulated Entner-Doudoroff pathway in ΔndbC (in LC), which is physiologically significant under mixotrophy (Chen et al., 2016), also could contribute to carbon flux distribution in the cells. Nevertheless, similar problems were present in cell division, as demonstrated in autotrophic conditions (Supplemental Fig. S4). Thus, the maintenance of ΔndbC growth in the medium supplemented with Glc is dependent on the regular interruption of darkness with light periods (Fig. 6B) to efficiently oxidize the ETC (Fig. 7D).

**CONCLUSION**

NADH and NADPH are fundamental reducing equivalents for the maintenance of cellular metabolism. In phototrophic organisms, the NADH/NAD$^+$ status is known to regulate the coordination of nitrogen assimilation and carbon metabolism by various mechanisms (Dutilleul et al., 2005; Kämäräinen et al., 2017; Pétiauq et al., 2017). By applying a number of physiological measurements and the proteomics approach, we showed that the deletion of NdbC has a distinct effect on the metabolism of Synechocystis. We postulate that NdbC located in the PM is an important component in the regulation of the cytosolic NADH/NAD$^+$ balance. In photoautotrophic growth conditions with active photosynthetic carbon assimilation, Synechocystis cells do survive in the absence of NdbC. Yet, importantly, glycolytic enzymes are down-regulated in the ΔndbC mutant under these growth conditions (Fig. 8), apparently due to the elevated NADH/NAD$^+$ ratio.
Consequently, cells need to compensate for the lack of the enzyme by the modulation of several metabolic routes. This provides evidence that NdbC is important in fine-tuning the balance between various metabolic pathways when the cytosolic redox balance is influenced mainly by light reactions in the thylakoid membrane. However, the function of NdbC becomes crucial for cell viability in heterotrophic growth conditions. This indicates an essential role of NdbC in the regulation of the cytosolic redox environment when glycolysis is the sole pathway to assimilate carbon for metabolic needs.

MATERIALS AND METHODS

Strains and Culture Conditions

A Glc-tolerant *Synechocystis* sp. PCC 6803 (wild type) was used as the reference strain. The ΔndeB mutant was obtained by disruption of the ndeB gene by Zeocin resistance cassette (for details, see “Mutagenesis” below). Preexperimential cultures were always grown in BG11 medium buffered with 20 mM HEPES-NaOH (pH 7.5) under continuous white light of 50 μmol photons m⁻² s⁻¹ at 30°C, under air enriched with 3% CO₂ (HC) with agitation of 150 rpm. All experimental cultures were grown in BG11 medium buffered with 20 mM HEPES-NaOH (pH 7.5) under continuous illumination of 50 μmol photons m⁻² s⁻¹, unless mentioned otherwise, and in growth chambers with cool-white light-emitting diodes (AlgaeTron AG130 by PSI Instruments). In some experiments, cells were grown in different dark-light cycles where illumination was 50 μmol photons m⁻² s⁻¹ during the light period. During experimental cultivations, cells were grown at 30°C with agitation of 150 rpm without antibiotics, and OD₅₇₀ was measured using the Lambda 25 UV/VIS spectrometer (PerkinElmer). Glc (10 mM) was provided when mentioned. For physiological measurements and protein extraction, cells were harvested from preexperimental cultures at the midlogarithmic phase, inoculated in fresh BG11 medium at OD₅₇₀ = 0.1, and grown at HC or inoculated to OD₅₇₀ = 0.5 and shifted to ambient air (LC) for 3 d. For activity measurements, cells were harvested and resuspended in fresh BG11 medium at the desired Chl concentration and acclimated under the respective growth conditions before the measurements.

Mutagenesis

To ensure the correct wild-type background, the interrupted ndeB gene (sll1484) with Zeocin resistance cassette and its surroundings were amplified by PCR with forward primer 5’-GCTGGTACCGTGAAAAAGTCT-3’ (in sll1483) and reverse primer 5’-CTACCGATTCTATACCCTGGCTA-3’ (in the end of sll1484) from the ΔndeB mutant constructed by Howitt et al. (1999). The resulting PCR product was transformed into the wild-type cells using a standard established protocol (Eaton-Rye, 2011). Transformants were segregated by fluorescence microscopy and protein extraction, cells were harvested from preexperimental cultures at the midlogarithmic phase, inoculated in fresh BG11 medium at OD₅₇₀ = 0.1, and grown at HC or inoculated to OD₅₇₀ = 0.5 and shifted to ambient air (LC) for 3 d.

Light Microscopy and Cell Counting

Cell suspensions in BG11 were examined using the Leitz Orthoplan Large Field Research Microscope and photographed with a digital microscope camera (Leica DFC420C). Digital slide photograph brightness and contrast were optimized with Leica Application Suite version 4.1. and CorelDRAW X7. A Bürker counting chamber (Marienfeld-Superior) was applied to quantify the number of cells in a culture (normalized to OD₅₇₀ = 1).

Dry Weight and Chl Determination

Twenty milliliters of cell culture was passed through a prewashed and weighed glass-fiber filter (Millipore). Filters were dried at 60°C for 24 h, kept in a desiccator for 24 h, weighed, and normalized to premeasured OD₅₇₀. Chl was extracted from cells with 100% methanol, and Chl concentrations were determined by measuring OD₆₆₅ and multiplying it with extinction coefficient factor 12.7 (Meeks and Castenholz, 1971).

Transmission Electron Microscopy

Preparation of samples was done with 5% glutaraldehyde in 0.16M s-collidin buffer, pH 7.4. The rest of the sample preparation and microscopy work was done by the Laboratory of Electron Microscopy, University of Turku.

Glycogen Determination

Ten milliliters of cultures at OD₅₇₀ = 1 was collected, and cells were lysed by sonication (Bioruptor; CosmoBio). Glycogen determination was performed with the Total Starch Assay Kit (Megazyme) according to the manufacturer’s instructions.

MIMS

MIMS measurements were performed as described by Ermakova et al. (2016). Gas-exchange kinetics and rates were determined according to Beckmann et al. (2009).

Fluorescence Measurements

The Chl fluorescence from intact cells was recorded with a pulse amplitude-modulated fluorometer (Dual-PAM-100; Walz). Before measurements, cell suspensions at a Chl concentration of 15 μg mL⁻¹ were dark adapted for 10 min. Saturating pulses of 5,000 μmol photons m⁻² s⁻¹ (300 ms) and strong FR light (720 nm, 75 W m⁻²) were applied to samples when required. Y(II) was calculated as \(\frac{F_{m} - F_{0}}{F_{m}}\). The \(F_{m}\) was recorded after the first saturating pulse without actinic light, the \(F_{0}\) was recorded subsequent to 8 s of strong FR illumination combined with saturating pulse, and the maximum fluorescence during illumination (\(F_{m}'\)) was recorded upon saturating pulse during illumination. The maximum quantum yield of PSII was calculated as \(\frac{F_{m}' - F_{0}}{F_{m}'}\) and measured in the presence of 20 μM DCMU from dark-adapted cells upon the application of red actinic light of 200 μmol photons m⁻² s⁻¹ for 1 min.

The \(F_{r}\) rise was measured in darkness after the termination of actinic light. Cell suspensions at a Chl concentration of 15 μg mL⁻¹ were dark adapted for 10 min before the measurements and exposed to 1 min of red actinic light intensity of 50 μmol photons m⁻² s⁻¹.

The kinetics of the Chl fluorescence decay after a single-turnover saturating flash was monitored using a fluorometer (FL 3500; PSI Instruments) according to Vass et al. (1999). Cells were adjusted to a Chl concentration of 7.5 μg mL⁻¹ and dark adapted for 5 min before measurements. When indicated, measurements were performed in the presence of 20 μM DCMU.

The Maximum and Effective Yield of PSI and Oxidoreduction of P700

The P700 signal was recorded with a pulse amplitude-modulated fluorometer (Dual-PAM-100; Walz). Before measurements, cell suspensions at a Chl concentration of 15 μg mL⁻¹ were dark adapted for 10 min. The maximal change of P700 upon transformation of P700 from the fully reduced to the fully oxidized state, \(P_{m}\), was achieved by the application of a saturation pulse after preillumination with FR light. The Y(I) was calculated as \(Y(I) = \frac{(P_{m} - P_{0})}{P_{m}}\).

Oxidation and rereduction of P700 were monitored from the cell suspensions at a Chl concentration of 20 μg mL⁻¹. The cells were dark adapted for 2 min before measurements. For P700 oxidation, cells were illuminated with strong FR light (720 nm, 75 W m⁻²) for 5 s, and the subsequent rereduction was recorded in darkness.

Proteomics

Western Blotting: Protein Isolation, Electrophoresis, and Immunodetection

Total protein extracts of *Synechocystis* cells were isolated as described by Zhang et al. (2009). Proteins were separated by 12% (w/v) SDS-PAGE.
containing 6 M urea, transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and analyzed with protein-specific antibodies. The membrane fractions to confirm the localization of NdbC were prepared as described by Zhang et al., (2004).

**Sample Preparation for MS**

Total proteins were isolated from cell cultures as described by Zhang et al. (2009). An equivalent of 30 μg of total protein was mixed with 2× Laemml buffer (with 8 M urea) in a 1:1 ratio and loaded on a 12% (50% acrylamide and 1.3% bis-acrylamide) stacking gel (0.5 M Tris-HCl, pH 6.8) containing 6 M urea and no SDS. Gel bands containing all proteins were reduced, alkylated, and digested according to the protocol described by Shevchenko et al. (1996, 2006). Extracted peptides were vacuum dried and further solubilized in 2% acetonitrile (ACN) and 0.1% formic acid (FA) just before MS analysis. Samples were prepared in four biological replicates from the wild type and the ΔndbC mutant.

**MS Analyses**

**DDA**

The peptide mixtures from the wild type and the ΔndbC mutant were analyzed on a liquid chromatography-MS/MS system with the DDA method in three biological replicates. An equivalent of 200 ng of peptides was separated on a nano-liquid chromatography system (EasyNanoLC 1000; Thermo Fisher Scientific) coupled to QExactive (Thermo Fisher Scientific). The samples were first loaded on a trapping column and separated in line on a 15-cm C18 column (75 μm × 15 cm, Magic 5-μm 200 Å C18, Michrom BioResources). The mobile phase consisted of water:ACN (98:2, v/v) with 0.2% FA (solvent A) or ACN:water (95:5, v/v) with 0.2% FA (solvent B). To separate the peptide mixture, a 110-min gradient was used that started with 2% B, increased to 20% B in 70 min and further to 40% B in 30 min, and then reached 100% in 5 min. MS data were acquired automatically in positive ionization mode with 2.3-kV (75 μm × 15 cm, Magic 5-μm 200 Å C18, Michrom BioResources). The mobile phase consisted of water:ACN (98:2, v/v) with 0.2% FA (solvent A) or ACN:water (95:5, v/v) with 0.2% FA (solvent B). To separate the peptide mixture, a 110-min gradient was used that started with 2% B, increased to 20% B in 70 min and further to 40% B in 30 min, and then reached 100% in 5 min. MS data were acquired automatically in positive ionization mode with 2.3-kV ionization potential using Thermo Xcalibur software (Thermo Fisher Scientific). The MS method combined an MS survey scan of mass range 300 to 2,000 mass-to-charge ratio and MS/MS scans for up to 10 most intense peaks from the full scan, with charge +2 or +3. The spectra were registered with a resolution of mass-to-charge ratio and MS/MS scans for up to 10 most intense peaks from the full scan, with charge +2 or +3. The spectra were registered with a resolution of 15,000 and 17,500 (at mass-to-charge ratio 200) for full scan and for fragment ions, respectively. Fragmentation of ions occurred in high collision dissociation mode.

**Protein Identification**

The raw files were searched against the Synechocystis protein database retrieved from Cyanobase (Kaneko et al., 1996) using an in-house Mascot (version 2.4) search engine (Perkins et al., 1999) and further analyzed using Proteome Discoverer (version 1.4) software (Thermo Scientific). The Mascot search parameters were set to trypsin as an enzyme with two miscleavages allowed, Met oxidation as variable modification, and carbamidomethylation as the fixed one. Precursor mass tolerance was restricted to monoisotopic mass of ±4 ppm and fragment ion to ±0.02 D. For validation of the spectrum identifications, the Percolator algorithm was used with a relaxed false discovery rate of 0.05.

**DDA Quantification**

Global label-free quantification for DDA data was performed using Progenesis QI for proteomics, LC-MS 4.0 (Nonlinear Dynamics). Peptide ions were identified with identification results from the DDA experiment. Proteins were identified and quantified with at least two peptides. Protein abundances were estimated based on volumes of the peaks representing its peptides detected in three biological replicates. Relative protein quantification was performed using all identified with ±10 ppm mass error for precursor mass, with nonconflicting peptides per protein. The statistical significance threshold in ANOVA was set to \( P < 0.05 \), and the practical significance threshold for FC was set to ±1.3.

**Targeted Quantification**

For targeted quantification with SRM, a spectral library was created in Skyline (MacLean et al., 2010) based on the DDA results. SRM analysis was performed on wild-type and ΔndbC mutant samples injected with four biological replicates according to the protocol described by Vuorioki et al. (2016), with several modifications. The samples were injected with two transition lists, comprising assays for 109 proteins (106 targets and three housekeeping proteins), to ensure dwell time of at least 20 ms with cycle time of 2.5 s. The mobile phases used in chromatographic separation were 0.1% FA (A) and 80% ACN:0.1% FA (B). The 55-min gradient started with 8% B, which increased to 28% in 35 min and then to 45% in 15 min. The SRM assays for *Synechocystis* proteins were loaded from the Panorama Public (Sharma et al., 2014) data repository, and the assays for the missing targets were developed according to the published protocol. Data analysis was performed in RStudio with MSstats algorithm version 3.5.1 (Choi et al., 2014). Data were normalized with global standard normalization with the following housekeeping peptides: slr1818, FSELEPLDR and SYTDQFQICGR; slr6638, GVIATVER; and slr0145, ASSLDSLGLITPNNDGK.

**Supplemental Data**

The following supplemental materials are available.

- **Supplemental Figure S1.** PCR analysis to verify full segregation of the ΔndbC mutant.
- **Supplemental Figure S2.** Reverse transcription-quantitative PCR analysis of slr1485 and slr1486 expression in the wild type and the ΔndbC mutant.
- **Supplemental Figure S3.** Relaxation of the flash-induced fluorescence yield in darkness from the wild type and the ΔndbC mutant in different conditions.
- **Supplemental Figure S4.** Light microscope images from wild-type and ΔndbC cells grown under 4 h of 50 μmol photons m⁻² s⁻¹/20 h of dark + 10 μJ Glc and under mixotrophic conditions.
- **Supplemental Table S1.** Photosynthetic parameters of the wild type and the ΔndbC mutant grown in LC and HC conditions.
- **Supplemental Table S2.** Identification of proteins in the wild type and the ΔndbC mutant grown under HC conditions with DDA.
- **Supplemental Table S3.** Identification of proteins in the wild type and the ΔndbC mutant grown under LC conditions with DDA.
- **Supplemental Table S4.** Results of global protein quantification for the ΔndbC mutant versus the control (wild type) grown under HC conditions \( (P < 0.05) \).
- **Supplemental Table S5.** Results of global protein quantification for the ΔndbC mutant versus the control (wild type) grown under LC conditions \( (P < 0.05) \).
- **Supplemental Table S6.** Results of SRM protein quantification for the ΔndbC mutant versus the control (wild type) grown under LC conditions.

**ACKNOWLEDGMENTS**

We thank Prof. Teruo Ogawa and Prof. Wim Vermaas for sharing *Synechocystis* mutants as well as Anita Santana Sanchez for helping with MIMS measurements.

Received March 23, 2017; accepted May 19, 2017; published May 22, 2017.

**LITERATURE CITED**


Nishimura T, Takahashi Y, Yamaguchi O, Suzuki H, Maeda S, Omata T (2014) Co-existence of photosynthetic and respiratory ac-

Perkins DN, Pappin DJ, Creasy DM, Cottrell JS

gnosis for mass spectrometric characterization of proteins and pro-
teomcs. Nat Protoc 1: 2856–2860


