Expression and activity of type 1 NAD(P)H dehydrogenase at different growth phases of the cyanobacterium, *Synechocystis* PCC 6803

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The expression and activity of type 1 NAD(P)H dehydrogenase (NDH-1) were investigated in *Synechocystis* PCC 6803 cells during different growth phases (i.e. lag, logarithmic, stationary and decline phases). The relative amount of NDH-1, estimated by Western blot analysis using antibodies against NdhH, NdhI and NdhK, increased more than two-fold during growth from the lag to the logarithmic phase and then decreased after the logarithmic phase to reach lowest levels after 15 days (decline phase). The activity of light-dependent NADPH oxidation and cyclic electron flow around photosystem I (PSI) changed nearly in parallel with the amount of NdhH, NdhI and NdhK in cells across the growth phases. In contrast, the activity of photosynthetic O₂ evolution and respiratory O₂ uptake was not significantly different across phases of growth; the fluctuation of the activity at different phases was within 40%. These results suggested that the activity of light-dependent NADPH oxidation and PSI-cyclic electron flow are restricted by the amount of NDH-1 and that other factor(s) are limiting the rates of photosynthesis and respiration.

Introduction

Cyanobacteria are globally distributed photosynthetic organisms that grow well in a wide range of natural environments. In laboratory batch cultures, cyanobacterial growth stages are divided into lag, logarithmic, stationary and decline phases, depending on growth rate and cell density. The decrease in growth rate after the logarithmic phase is considered to be a result of limited availability of light energy and/or nutrients. However, few studies have examined this in cells across different growth phases.

The enzyme type 1 NAD(P)H dehydrogenase (NDH-1) consists of two complexes in cyanobacteria. Each complex contains more than 15 subunits (Battchikova et al. 2005, Nixon et al. 1989, Prommeenate et al. 2004, Steinmüller 1992, Zhang et al. 2004), which are encoded by genes homologous to the chloroplast and mitochondrial *ndh* genes (Kaneko et al. 1996, Ohyama et al. 1986). Cyanobacterial NDH-1 complexes have a unique function in that they are involved in photosystem I (PSI)-cyclic electron flow and CO₂ uptake (Mi et al. 1992a, 1992b, 1994, 1995, Ogawa 1991). In addition, the activity and amounts of the subunits of NDH-1 complexes are inducible by changing environmental conditions, such as elevating light levels (Mi et al. 2001), reducing CO₂ (Deng et al. 2003a, 2003b, Zhang et al. 2004) and adding excess salt (Hibino

Abbreviations – FNR, ferredoxin-NADP⁺ oxidoreductase; NDH-1, type 1 NAD(P)H dehydrogenase; PSI, photosystem I; RFₚᵢ, initial rate of post-illumination increase in chlorophyll (Chl) fluorescence.
et al. 1996). This inducible property of NDH-1 suggests that it may have a protective role for the NDH-1 complexes under stressful conditions.

The current study examined the effects of growth phase on cellular levels and activity of NDH-1. The relative amounts of each of the three subunits of NDH-1 in *Synechocystis* PCC 6803 cells at different growth phases were measured, together with the activity of light-dependent NADPH oxidation, PSI-cyclic electron flow, photosynthetic O2 evolution and respiratory O2 uptake. The amount of NDH-1 was highly dependent on the growth phase and significantly correlated with the activity of NADPH oxidation and PSI-cyclic electron flow; however, there was not a significant correlation between NDH-1 levels and photosynthesis and respiratory activity.

### Materials and methods

#### Culture and determination of cell density

*Synechocystis* PCC 6803 cells were cultured at 30°C in BG-11 medium, (Allen 1968) buffered with Tris–HCl (5 mM, pH 8.0) and bubbled with 2% (v/v) CO2 in air under continuous illumination by fluorescent lamps (60 μE m−2 s−1). The cell density was determined every 24 h by measuring the absorbance at 730 nm using a spectrophotometer (UV3000, Shimadzu, Kyoto, Japan).

#### Isolation of whole-cell extracts

Cells harvested by centrifugation (5000 g for 5 min at 4°C) were suspended in medium A [10 mM HEPES-NaOH, 5 mM sodium phosphate (pH 7.5), 10 mM MgCl2 and 10 mM NaCl], supplemented with 25% glycerol and were disrupted by 5 × 20-s pulses with a Bead-beater (Biospec, Bartlesville, OK) followed by a 3-min incubation on ice. The homogenate was centrifuged at 5000 g for 5 min at 4°C to remove unbroken cells and debris. Membranes in the supernatant were solubilized with 1% (w/v) N-dodecyl-β-D-maltoside while shaking on ice for 1 h. The samples were then immediately subjected to SDS-PAGE.

#### Redox changes of plastoquinone and P700+ 

Cells were suspended in fresh BG-11 medium buffered with Tris–HCl (5 mM, pH 8.0) at a Chl concentration of 10 μg ml−1. Chl fluorescence and absorbance changes at 810 minus 830 nm were measured to monitor the redox changes of plastoquinone (PQ) and reduction of P700+ after far-red light illumination (FR, >705 nm, 5.2 μM m−2 s−1), respectively. A PAM Chl fluorometer (Walz, Effeltrich, Germany) and an emitter-detector-cuvette assembly (ED-101US) with a unit 101ED or a unit ED-P700DW-II were used for these measurements, as described by Klughammer and Schreiber (1998).

#### Western analysis and light-dependent NADPH oxidation

SDS-PAGE electrophoresis was carried out on 12% polyacrylamide gels according to the method of Laemmli (1970). Western blot analysis was performed with an ECL assay kit (Amersham Pharmacia, NJ), according to the manufacturer’s protocol. Antibodies against the NDH-1 subunits (NdhH, NdhI and NdhK) of *Synechocystis* PCC 6803 were raised in our laboratory. To amplify ndhH, ndhI and ndhK, the primer sequences as summarized in (Table 1) were designed. The polymerase chain reaction products were ligated into vector pET32a, and the construct was amplified in *Escherichia coli* DH-5a. The plasmid was used to transform *E. coli* strain BL21 (DE3) pLysS for expression. The gene expression products from *E. coli* were purified and used as antigens to immunize rabbits to produce polyclonal antibodies.

Light-dependent NADPH oxidation was performed as described by Deng et al. (2003a). Whole-cell extracts were diluted with medium A to a chlorophyll (Chl) concentration of 5 μg per 3 ml and supplemented with 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), 0.25 mM NaN₃, 0.1 mM KCN and 0.2% (v/v) Triton X-100. Reaction mixtures were pre-illuminated with actinic light (AL) for 2 min to activate NDH-1, and then 100 μM NADPH was added. The rates of NADPH oxidation were measured at 25°C as a decrease in 340-nm absorbance after application of AL (>660 nm, 200 μE m−2 s−1). An extinction coefficient of 6.22 mM−1 cm−1 was used to calculate the NADPH concentrations.

### Table 1. Primer sequences for amplifying ndhH, ndhI and ndhK.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers (5′–3′)</th>
<th>Reverse primers (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NdhH</td>
<td>TTAAGGATCCATGACCAAGATTTGAAACCAGA</td>
<td>GCAAAAGCTTCTAGCGGTCCACCGATCCCAT</td>
</tr>
<tr>
<td>NdhI</td>
<td>AGAGGATCCGATGTTTAAACACATTCTC</td>
<td>AACCTTGACGCTATTCTGGTTTACCACAA</td>
</tr>
<tr>
<td>NdhK</td>
<td>CCTAGGATCCATGAGCTCAACCCTGCTAAC</td>
<td>GTTCAGCTTTACGCCACTGGTTAATCTC</td>
</tr>
</tbody>
</table>

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Photosynthetic oxygen evolution and respiration

Oxygen evolution by photosynthesis and uptake by respiration were measured at 25°C (Hansatech, Hertfordshire, UK) with a Clark-type oxygen electrode according to the method described by Mi et al. (1995). Cells were suspended in BG-11 medium at a Chl concentration of 20 μg ml⁻¹. The intensity of AL used for the measurement of photosynthetic oxygen evolution was 800 μE m⁻² s⁻¹.

Results

Amount of NDH-1 in cells at different growth phases

*Synechocystis* PCC 6803 growth can be divided into four phases. The designation of growth phase depends on the growth rate and density of the cells, i.e. lag (0–2 days), logarithmic (2–8 days), stationary (8–15 days) and decline (after 15 days) phases (Fig. 1). Cells harvested 1, 4, 10 and 16 days (designated 1-, 4-, 10- and 16-day cells, respectively) after inoculation of stationary phase cells to a new culture medium were used to represent each phase. The amount of NDH-1 in cells is strongly dependent on their growth phase. Western blot analysis with antibodies against NdhH, NdhI and NdhK indicated that the amount of these subunits was highest in 4-day cells in the logarithmic phase and then decreased after this phase to very low levels in 16-day cells (Fig. 2 and curves a–c in Fig. 3).

PSI-cyclic electron transport

The activity of PSI-cyclic electron transport was monitored by measuring the activities of three different reactions: (1) light-induced NADPH oxidation, (2) post-illumination increase of Chl fluorescence and (3) initial rate of P700⁺ reduction. NADPH oxidation was measured in the light without adding artificial electron acceptor (Deng et al., 2003a). Under these conditions, there was no NADPH oxidation in the dark due to the diaphorase activity of ferredoxin-NADP⁺ oxidoreductase. The activity of light-dependent NADPH oxidation was 0.9, 2.3, 1.3 and 0.6 × 10⁻¹ mM min⁻¹ mg Chl⁻¹, respectively, in 1-, 4-, 10- and 16-day cells. The change of the amount of NDH-1 was corresponding with that of NADPH oxidation activity during different growth phases (curves a–c and f in Fig. 3A). The closed triangles showing the relative activity of NADPH oxidation at various levels of NDH-1 (the average values for NdhH, NdhI and NdhK are taken) were located near the line with the slope of 1 (Fig. 3B). Thus, there exists a significant correlation between the amount of NDH-1 complexes and the NADPH oxidation activity in cells of different phases. The results indicate that the PSI-driven NADPH oxidation reflects the NADPH-oxidizing activity of NDH-1. Western blot analysis of the thylakoid membranes after various dilutions indicated that there existed a linearity between the densities of the spots and the amount of proteins in the range of protein concentrations used in Fig. 2, except that the density dropped sharply off the linearity line when the protein concentration was below a certain level (data not shown). This would explain the extremely low levels of NdhH and NdhI in 16-day cells (Fig. 2).

Post-illumination increase in Chl fluorescence is considered to reflect the reduction of PQ by NAD(P)H or other reducing substances accumulated under light. In higher plants and cyanobacteria, this increase in Chl fluorescence represents the activity of PSI-cyclic electron transport (Asada et al. 1993, Mano et al. 1995, Mi et al. 1995). Typical Chl fluorescence kinetics of *Synechocystis* PCC 6803 is shown in Fig. 4A, and the area surrounded by the square shows a post-illumination increase in Chl

![Fig. 1. Growth curve of *Synechocystis* PCC 6803 cells and definition of various phases. The vertical bars indicate standard errors calculated from three independent experiments.](image)

![Fig. 2. Western blot analysis showing the relative amounts of NdhH, NdhI and NdhK in *Synechocystis* PCC 6803 cells harvested at different growth phases. Whole-cell extracts were subjected to SDS-PAGE gel electrophoresis and immunoblotted with antibodies against NdhH, NdhI and NdhK. Samples containing 5 μg chlorophyll were loaded in each lane. Cells were cultured for the periods indicated above the top lane.](image)
fluorescence (Deng et al. 2003a). Both the height and the relative rate of post-illumination increase in Chl fluorescence correspond to the amount of NDH-1 (Fig. 4B and curve e in Fig. 3A). Similar results were obtained on the initial rate of $P700^+$ reduction by far-red light ($\gamma$), the initial rise of post-illumination increase of chlorophyll (Chl) fluorescence ($\ell$), the light-dependent NADPH oxidation ($\ell$), photostatic $O_2$ evolution ($\ell$) and respiration ($\bullet$). The values in 4-day cells are taken as 100%. Each experiment was repeated four times and standard errors were calculated. (B) The relative activities of NADPH oxidation ($\ell$), post-illumination increase of Chl fluorescence ($\ell$), $P700^+$ reduction ($\bullet$) by far-red light, photostatic $O_2$ evolution ($\ell$) and respiration ($\bullet$) as a function of the relative amounts of NAD(P)H dehydrogenase (average values for NdhH, NdhI and NdhK). The linear line with the slope of 1 crosses 0 and 100%.

Photosynthesis and respiration

Photosynthetic oxygen evolution and dark respiration activity also depend on the growth phase of the cells, but the relationship was not significant (Fig. 3A and B). The fluctuation was within 40% for both photosynthesis and respiration (Table 2 and curves g and h in Fig. 3A), and the relative activities of oxygen evolution in photosynthesis or oxygen uptake in respiration plotted against the relative amount of NDH-1 were far above the line with the slope of 1 (Fig. 3B). Thus, the amount of NDH-1 is not limiting the processes of photosynthesis and respiration, at least under these conditions.

Discussion

In batch cultures, the growth stage of cells is divided into lag, logarithmic, stationary and decline phases depending on the growth rate and density of the cells. Growth rate in culture depends on light intensity and nutrient concentrations around the cells. During lag to logarithmic phases, cells are adapting to new
environments and may require more NDH-1 to increase the cyclic electron transport activity to optimize photosynthesis. This is reflected by the significant increases in the amount of NDH-1 and the activity of PSI-cyclic electron flow during this period. Zhang et al. (2004) reported that NDH-1L involved in respiration is a major fraction of NDH-1 in cells grown under high CO\textsubscript{2} condition, while NDH-1M involved in cyclic electron transport is less abundant in these cells. Although the present study did not distinguish NDH-1L and NDH-1M, we may assume that NDH-1L is a major fraction of NDH-1 in cells grown under high CO\textsubscript{2} condition used in this study and that the amount of NDH-1M is proportional to the total amount of NDH-1 complexes. The existence of the correlation between the amount of NDH-1 and the activity of light-induced NADPH oxidation and PSI-cyclic electron flow supports the notion that these reactions are mediated by NDH-1, most likely by NDH-1M (Deng et al. 2003a, Mi et al. 1995, Zhang et al. 2004).

A number of investigations have demonstrated a protective role for cyclic electron transport in response to stressful conditions, such as salt stress (Hibino et al. 1996, Tanaka et al. 1997), low CO\textsubscript{2} (Deng et al. 2003a) and strong light (Mi et al. 2001) in cyanobacteria and oxidative stress (Martin et al. 1996), water stress (Burrow et al. 1998) and photoinhibition (Endo et al. 1999) in higher plants. During the lag phase, cells may be exposed to oxidative and/or strong light stresses and possibly nutrient stress. Cyclic electron flow may be required to alleviate cells from these stresses. After the logarithmic phase, the growth rate slowed down to nearly zero after 16 days of culture, although there was no significant decrease in the activity of photosynthetic O\textsubscript{2} evolution when the initial rate was measured upon illumination with strong light. The availability of light and/or nutrients under the conditions of batch culture would limit the growth of the cells after the logarithmic phase. Our results indicate that the expression of NDH-1 was suppressed under such conditions. Although the NDH-1 complex is an important component of the respiratory electron flow, the correlation between the activity of respiration and the amount of NDH-1 was not significant. Because we grew the cells under high CO\textsubscript{2} conditions, the major fraction of NDH-1 is considered to be NDH-1L (Zhang et al. 2004). Thus, the amount and the activity of NDH-1L are high enough even in 16-day cells and do not appear to be limiting the respiratory activity. Furthermore, photosynthetic activity showed the same trend as the respiration activity and did not change significantly in cells of different growth phases. Therefore, the contribution of NDH-1-mediated cyclic electron flow to photosynthesis appears to be small under the conditions for the measurement of O\textsubscript{2} evolution. However, it is possible that the reduced activity of cyclic electron transport after the logarithmic phase is partially responsible for the deceleration of the growth rate. Taken together, these results suggested that the activity of light-dependent NADPH oxidation and PSI-cyclic electron flow are restricted by the amount of NDH-1, and other factor(s) are limiting the rates of photosynthesis and respiration.

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References


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