Electron Flow from NAD(P)H Dehydrogenase to Photosystem I is Required for Adaptation to Salt Shock in the Cyanobacterium Synechocystis sp. PCC 6803

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The role of the NAD(P)H-dehydrogenase complex in adaptation to salt stress was examined in an ndhb-inactivated mutant of the cyanobacterium Synechocystis sp. PCC 6803. Wild-type cells and ndhb-inactivated mutant cells grew at similar rates under conditions of low salinity (<0.6 M NaCl) and high CO 2 (3%). However, when the concentration of NaCl in the culture medium was higher than 0.6 M, the mutant cells grew much more slowly than the wild-type cells. Upon addition of high concentrations of NaCl, the oxygen-evolving activity was rapidly inhibited but then it recovered, with the rate of recovery depending on the concentration of NaCl. The recovery of the mutant cells was significantly delayed when the concentration of NaCl was above 0.3 M. At 0.9 M NaCl, wild-type cells recovered with a half time of about 40 min, while mutant cells did not recover. The kinetics of changes in Chi fluorescence confirmed these results. In wild-type cells, input of electrons from the cytosol to PSI via the NAD(P)H-dehydrogenase complex increased upon salt shock. It appears, therefore, that the electron flow from the cytosol to PSI via NAD(P)H-dehydrogenase is essential for the adaptation of cyanobacteria to salt shock.

Key words: Cyclic electron transport — NAD(P)H-dehydrogenase — ndhb gene — Photosystem I — Salt stress — Synechocystis sp. PCC 6803.

Salinity is an important limiting factor in crop productivity. Organisms thrive in hypersaline environments have specific mechanisms that allow them to adjust their internal osmotic status. One such mechanism involves the ability to accumulate organic “compatible” solutes of low molecular weight, such as sugars, certain amino acids and quaternary ammonium compounds, which are believed to be essential for the adaptation of plant cells to high salinity (Gorham et al. 1985, Storey and Wyn Jones 1977). Another mechanism for adaptation to high salinity involves the exclusion of Na + ions from cells via a H + /Na + antiport function (Erber et al. 1986, Fry et al. 1986, Molitor et al. 1986). The process of adaptation to salt stress can be separated to two phases: short-term responses that occur from 1 ms to 1 h after transfer to high salinity and long-term acclimation that occurs over the course of several hours to several days. The uptake and exclusion of ions are believed to play important roles in short-term responses and the synthesis of osmoprotectant(s) in addition to metabolic adaptation, is the major response in long-term acclimation (Reed et al. 1985, Ishitani et al. 1993). Both the synthesis of compatible solutes and the exclusion of Na + ions require energy, which can be provided by photosynthesis and respiration. Indeed, activation of respiratory electron-transport systems by salt stress has been reported (Fry et al. 1986, Molitor et al. 1986, Moser et al. 1991, Jeanjean et al. 1993). However, mechanisms of adaptation of photosynthetic thylakoid membrane systems to salt stress are not well understood. In cyanobacteria and eukaryotic algae, increased cyclic transport of electrons around PSI appears to provide the energy for adaptation to high-salt conditions (Gilmour et al. 1985, Canaani 1990, Fork and Herbert 1993, Jeanjean et al. 1993, Endo et al. 1995, Hibino et al. 1996). The pathway of the PSI-driven cyclic flow of electrons remains a subject of debate (Fork and Herbert 1993, Ben-dall and Manasse 1993). From the Cyt b6/f complex to ferredoxin, the cyclic flow of electrons is believed to follow the same path as the linear flow of electrons. The return part of the cycle from ferredoxin to the Cyt b6/f complex remains largely unknown. One possible pathway involves the transfer of electrons from ferredoxin to plastoquinone via a putative ferredoxin-plastoquinone reductase (Bendall and Manasse 1995, Miyake et al. 1995). Biochemical evidence for the existence of a ferredoxin-plastoquinone reductase is lacking but this pathway would be independent of NAD(P)H. Other possibility is cycling through NAD(P)H-plastoquinone reductase. In this pathway, the involvement of NAD(P)H-dehydrogenase (NDH-1), which has many subunits homologous to those of mitochondrial complex I and bacterial NDH-1 (Fearnley and Walker 1992, Sugiuara 1992, Berger et al. 1993, Yagi 1993, Kaneko et al. 1996), has been demonstrated (Ogawa 1991, Mi et al. 1992, 1994, 1995). The presence of a second NAD(P)H-dehydrogenase (NDH-2), which consists of single polypeptide and does

Abbreviations: M55, ndhb-inactivated mutant; NDH, NAD(P)H dehydrogenase; WT, wild-type.
not pump protons in bacteria (Fearnley and Walker 1992, Yagi 1993), has not yet been demonstrated. However, the existence of three forms of NDH-2 in Synechocystis sp. PCC 6803 has been deduced from the complete nucleotide sequence of the genome (Kaneko et al. 1996). The physiological functions of these two types of NDH are unknown and it is unclear whether or not NDH (NDH-1 and/or NDH-2) is involved in the adaptation to salt stress.

In this study, we examined the effects of cyclic electron flow through the NDH-1 complex on the adaptation to salt stress. Using an ndhB-inactivated mutant of Synechocystis sp. PCC 6803 (Ogawa 1991), we showed that the flow of electrons from NDH-1 to PSI is required for adaptation to salt shock in the cyanobacterium Synechocystis sp. PCC 6803. We also discovered that light is essential for the short-term adaptation of Synechocystis to salt shock.

**Materials and Methods**

**Cell culture**—Wild-type (WT) cells and an NDH-defective mutant (M55) of Synechocystis sp. PCC 6803 were cultured photoautotrophically at 30°C under light from fluorescent lamps at 60 μE m⁻² s⁻¹ in BG 11 medium that contained 10 mM HEPES-KOH (pH 8.0) and was bubbled with 3% CO₂ in air (Ogawa 1991). In the M55 mutant, a kanamycin-resistance cartridge had been inserted at the BamHI site of the ndhB gene. WT and M55 cells at the late logarithmic phase of growth were subjected to salt stress by addition of an appropriate volume of growth medium supplemented with 4 M NaCl.

**Measurements of the linear transport of electrons and respiratory activity**—Linear electron-transport activity was monitored in terms of the evolution of oxygen by cells (0.15-0.25 μg Chl ml⁻¹) with a Clark-type oxygen electrode in BG-11 medium that contained 5 mM KHCO₃, the electron acceptor, under white light at 800 μE m⁻² s⁻¹ from a halogen lamp.

**Measurements of Chi fluorescence and the redox state of P700**—Chi fluorescence was measured with a PAM fluorometer (Walz, Effeltrich, Germany). Pulse-modulated excitation was obtained from a light-emitting diode with peak emission at 650 nm. Modulated fluorescence was measured at λ > 710 nm (RG9 long-pass filter, Schott, Wiesbaden). The accessory modules PAM-102 and PAM-103 (Walz) were used to control the actinic light and saturation pulses, respectively. The redox state of P700 was monitored in terms of the change in absorbance at 830 nm in a PAM Chl fluorometer with the emitter-detector unit ED 800T (Schreiber et al. 1988). Saturating multiple-turnover light (25-ms pulse length, 15,000 μE m⁻² s⁻¹) was applied to samples via a multibranched fiber optic system with a xenon discharge lamp (XMT103; Walz). Chl a was extracted in 90% methanol and quantitated from its absorbance at 665 nm, as described by Tandeau de Marsac and Houmard (1988).

**Results**

**Growth of wild-type and M55 mutant cells under salt-stress conditions**—The growth rates of wild-type cells and ndhB-inactivated mutant cells of Synechocystis sp. PCC 6803 were measured at various concentrations of NaCl by following increases in the absorbance of the culture at 730 nm. The growth rate at the logarithmic phase was taken as the reciprocal of the doubling time during the logarithmic phase of growth.

**Changes in the photosynthetic evolution of O₂ upon salt shock**—Addition of high concentrations of NaCl to the BG11 medium transiently inhibited the growth of cells and then cells returned to logarithmic growth after a few days. Figure 2 shows the photosynthetic evolution of O₂ from
wild-type and ndhB mutant cells, as determined 5 min and 45 min after the addition of NaCl at various concentrations to the growth medium. The photosynthetic evolution of O2 by wild-type and ndhB mutant cells 5 min after salt shock, which might reflect the extent of inhibition, decreased with increasing concentrations of NaCl (Fig. 2). At 0.3 M NaCl, the mutant cells had much lower activity than the wild-type cells. The difference between mutant and wild-type cells was smaller at 0.6 M NaCl, and it was almost non-existent at 0.9 M NaCl. After salt shock, the O2-evolving activity of both wild-type and ndhB mutant cells recovered at rates that depended on the concentration of NaCl. The O2-evolving activity of wild-type and ndhB mutant cells returned to close to the original level within 45 min when the concentration of NaCl was below 0.3 M (Fig. 2). The extent of recovery decreased when the concentration of NaCl was above 0.6 M. In particular, in the mutant cells, the activity 45 min after salt shock at above 0.6 M was much lower than that of the wild-type cells. These results indicate that NDH-1 is important for the recovery process after salt shock, at least under high-salinity conditions (>0.6 M NaCl). The data obtained with NaCl at 0.3 and 0.6 M also suggest the existence of a mechanism for the protection of NDH-1 against inactivation by salt.

Typical time courses of the photosynthetic evolution of O2 in wild-type and ndhB mutant cells after transfer to high salinity (0.9 M NaCl) are shown in Figure 3. The photosynthetic evolution of O2 was completely inhibited upon salt shock in both wild-type and ndhB mutant cells. In wild-type cells, the activity recovered gradually and returned to the original level within 2 h after salt shock. By contrast, the mutant cells did not recover over the course of 3 h. Thus, the ndhB mutant cells were unable to adapt to salt shock.

Changes of Chl fluorescence upon salt shock—Changes in photosynthetic activity upon salt shock were monitored in terms of Chl fluorescence. When the actinic light was switched on, both wild-type and ndhB mutant cells showed an increase in the stationary fluorescence yield, F, an indication of the accumulation of electrons in the intersystem chain due to the activity of PSII. The gradual increase in the maximum fluorescence upon a saturating pulse in the wild-type cells after the onset of actinic light (Fm'), shown in Fig. 4A, upper panel, indicated the actinic light-induced transition to state 1, in which light energy is transferred preferentially to the PSII reaction center rather than to PSII (Allen 1992, Schreiber et al. 1993). The minimal increase in the maximum fluorescence upon a saturating pulse (Fm') in ndhB mutant cells (Fig. 4A, lower panel) suggests the absence of the state transition in the mutant, and this result is consistent with the observations by Schreiber et al. (1993). Upon salt shock, (Fm'—F)/Fm', a measure of the quantum yield in PSII under illumination (Genty et al. 1989), decreased in both wild-type and ndhB mutant cells (Fig. 4B), indicating the inhibition of the linear electron-transport activity. Close to the original value of (Fm'—F)/Fm' was obtained within 90 min in wild-type cells (upper panel of Fig. 4C). By contrast, in the mutant cells, no recovery of (Fm'—F)/Fm' was observed, even 90 min after salt shock (Fig. 4C, lower panel), indicating that the activity in the mutant cells had been irreversibly lost. These results of fluorescence quenching analysis are consistent with those obtained in the analysis of the photosynthetic O2-evolving activity.

When the actinic light was turned off, the stationary fluorescence yield of wild-type and ndhB mutant cells decreased rapidly (Fig. 4). In the case of wild-type cells (upper panel), the fluorescence yield increased transiently and then decreased but such was not the case in ndhB mutant cells. The largest increase was observed 90 min after salt shock, as shown in panel C for wild-type cells. The increase in fluorescence yield after the actinic light had been turned off was due to the increased input of electrons from the cytosol to the intersystem chain (Asada et al. 1993), suggesting the activation of the cyclic flow of electrons around PSI under high salinity conditions in wild-type but not in ndhB mutant cells.

Effects of light and HgCl2 on the recovery of O2-evolving activity after salt shock—We examined the role of the NDH-1 complex in the recovery of O2-evolving activity after salt shock using HgCl2. Wild-type cells were treated for 2 min with indicated concentrations of HgCl2. Then 0.9 M NaCl was added. No NaCl was added in the case of the control cells. After a 30-min incubation in the light, the photosynthetic evolution of O2 was measured. HgCl2 from 1.0 to 2.0 μM, which inhibits the activity of the NDH-1 complex but not of NDH-2 (Mi et al. 1992), did not affect the O2-evolving activity in the control cells. However, the
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Wild type

A) -NuCI H \(>0.8\) M NaCl

B) 0.8 M NaCl

5 min

C) 0.8 M NaCl

90 min

ndhB' mutant

- NaCl

0.8 M NaCl

5 min

0.8 M NaCl

90 min

Fig. 4 Comparison of light-induced changes in Chl fluorescence in wild-type and ndhB mutant cells of *Synechocystis* sp. PCC 6803 upon exposure to salt stress. *Synechocystis* sp. PCC 6803 wild-type and ndhB mutant cells were transferred to medium that contained 0.8 M NaCl. Chl fluorescence was measured with a PAM fluorometer. A, Before salt shock (control); B, 5 min after the addition of NaCl; C, 90 min after the addition of NaCl. A light pulse of 400 ms (300 W m\(^{-2}\)) was applied at 40-s intervals. Actinic light (AL; 650 nm, 2 W m\(^{-2}\)) was turned on and off as indicated.

same concentrations of HgCl\(_2\) strongly inhibited the recovery of O\(_2\)-evolving activity after salt shock (Fig. 5). These data indicate that the transfer of electrons to the intersystem electron carriers through the NDH-1 complex in thylakoid membranes participates in the adaptation to salt shock. High concentrations of HgCl\(_2\) (>2 \(\mu\)M) inhibited the activities of both the O\(_2\)-evolving system and the NDH-1 complex.

We examined the effects of light on the recovery of O\(_2\)-evolving activity. Addition of 0.9 M NaCl inhibited the photosynthetic evolution of O\(_2\) in wild-type cells by about 83%, as shown in Table 1. After 45-min cultivation in darkness, the rate of photosynthetic evolution of O\(_2\) remained low, at 25% of the original rate, whereas the activity recovered to 72% of the original level if cultivation for 45 min was carried out under illumination. When salt shocked cells that had been cultured for 45 min in darkness were transferred to the light and cultured for an additional 45 min, O\(_2\)-evolving activity recovered to 67% of the original value. These results indicated that light was required for the recovery processes after salt shock.

Effects of osmotic stress on the O\(_2\)-evolving activity of wild-type and ndhB mutant cells—The inhibition of photosynthetic activity upon salt shock might have been due to osmotic as well as ionic effects of NaCl. To examine osmotic effects specifically, we added glycerol to the growth medium. The presence of 1.8 M glycerol, which has the same osmotic pressure as 0.9 M NaCl, decreased the O\(_2\)-evolving activity of wild-type and ndhB mutant cells to about 70% and 60% of that of control cells, respectively.
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2.0 HgCl₂

Fig. 5 Effects of HgCl₂ on the photosynthetic evolution of O₂ by Synechocystis sp. PCC 6803 cells, 30 min after salt stress. Wild-type cells were transferred to medium that contained 0.9 M NaCl. HgCl₂ at the indicated concentrations was added to the medium 2 min before salt shock. The photosynthetic evolution of O₂ from whole cells was measured 30 min after the transfer to salt-stress conditions. The activity was expressed as a percentage of the activity of cells treated without the inhibitor and it was compared with the effects of the inhibitor on the same activity of control cells that were not exposed to salt stress.

(Table 1). The extent of inhibition was much lower than that observed with NaCl. The activities of both wild-type and ndhB mutant cells remained the same even after illumination for 45 min. These results indicated that glycerol inhibited the O₂-evolving activity to some extent but that inhibition by glycerol was not specific to the ndhB mutant. Thus, the inhibition by NaCl was due primarily to ionic effects and the NDH-1 complex seemed to be essential for recovery from the ionic inhibition by NaCl.

Cyclic electron flow after salt stress—Changes in the redox kinetics of P700 in wild-type cells upon salt shock were examined. As shown in Figure 6, P700 was oxidized to P700⁺ upon flash illumination and then P700⁺ was rapidly reduced when the salinity in the growth medium was low. When the linear flow of electrons from PSII was inhibited by the addition of DCMU, the rate of reduction of P700⁺ in wild-type cells increased upon salt shock. By contrast, when the flow of electrons through the NDH-1 complex was inhibited by the addition of HgCl₂, the rate of reduction of P700⁺ in wild-type cells decreased upon salt shock. These results indicate that input of electrons from the NDH-1 complex into the intersystem electron transfer chain was activated upon salt shock whereas the flow of electrons from PSII to PSI decreased under the same conditions. Electrons provided by the NDH-1 complex were apparently transferred to P700⁺ through the Cyt b₅/f complex since the addition of 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB) inhibited the reduction of P700⁺ (data not shown).

Discussion

The present study clearly showed that input of electrons into the intersystem chains via the NDH-1 complex was essential for the recovery of the photosynthetic evolution of O₂ after high-salinity stress. Light was required for these processes and dark respiration was not involved. Stimulation of the donation of electrons from the cytosol to the intersystem chain via the NDH-1 complex and an increased rate of reduction of P700⁺ were also observed after salt shock. Donation of electrons from the cytosol to the quinone pool resulted in the cyclic flow of electrons around PSI since the electrons of reduced quinone could apparently be used for the reduction of P700⁺ in illuminated cells. The consequent activity of PSI resulted in generation of

Table 1 Effects of light and glycerol on the recovery of photosynthetic oxygen-evolving activity in wild-type and mutant Synechocystis sp. PCC 6803 after salt shock

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity [nmol O₂ (mg Chl)⁻¹ min⁻¹]</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>Control</td>
<td>1.78</td>
</tr>
<tr>
<td>0.9 M NaCl</td>
<td>0 min</td>
</tr>
<tr>
<td></td>
<td>45 min (light)</td>
</tr>
<tr>
<td></td>
<td>45 min (dark)</td>
</tr>
<tr>
<td></td>
<td>45 min (dark)+45 min (light)</td>
</tr>
<tr>
<td>1.8 M glycerol</td>
<td>0 min</td>
</tr>
<tr>
<td></td>
<td>45 min (light)</td>
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</tbody>
</table>

Oxygen-evolving activity was measured as described in Materials and Methods. The wild-type and mutant cells (0.20 µg Chl ml⁻¹) were illuminated with white light (800 µE m⁻² s⁻¹). The growth medium (BG-11) contained 5 mM KHCO₃ as an electron acceptor. nd, Not determined.
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NAD(P)H, which was again able to reduce the quinone pool. The possibility of such cyclic flow of electrons around PSI has been proposed in cyanobacteria (Ogawa 1991, Mi et al. 1992, 1994, 1995), green algae (Bennoun 1982, Peltier et al. 1987, Garab et al. 1989) and plants (Herbert et al. 1991, Asada et al. 1993). However, the involvement of the NDH-1 complex in the cyclic flow of electrons around PSI was only clearly demonstrated by biochemical analysis in cyanobacteria (Mi et al. 1992, 1994, 1995, Hibino et al. 1996). Therefore, it appears that the cyclic flow of electrons through the PSI complex, NADPH, the NDH-1 complex, and the Cyt b$_6$f complex is stimulated upon salt shock, at least in cyanobacteria. Although NDH-2 also catalyzes the transfer of electrons from NAD(P)H to plastoquinone, it is unclear from our present data whether NDH-2 might be involved in the adaptation to salt shock.

It was reported that, upon a shift to high-salinity conditions (550 mM NaCl), the activities of Cyt c oxidase, PSI and cyclic photophosphorylation were increased in *Synechocystis* sp. PCC 6803, whereas the activities of NADH/Cyt c reductase, ubiquinol-10/Cyt c reductase, and PSII were unchanged (Jeanjean et al. 1993). These observations suggest that the electrons for the stimulation of Cyt c oxidase and cyclic photophosphorylation must be provided by some components other than NADH/Cyt c reductase and ubiquinol-10/Cyt c reductase. Since our present data suggest the stimulation of electron flow through NADPH dehydrogenase (NDH-1), the NADH/Cyt c reductase activity observed by Jeanjean et al. (1993) might have been due to an enzyme other than NDH-1. In the experiment for which results are shown in Figure 4B (lower panel), a high stationary fluorescence (F) in response to background actinic light was observed with the ndhB mutant cells, suggesting that the plastoquinone pool is highly reduced. We might have expected a different result when the NDH-1 complex was inactivated by mutation and the linear flow of electrons was inhibited by salt shock. One

**Fig. 6** Kinetics of the reduction of P700$^+$ in *Synechocystis* sp. PCC 6803 cells before and after salt shock. Wild-type cells were incubated for 5 min with 5 $\mu$M DCMU or 100 $\mu$M HgCl$_2$ as indicated. The extent of re-reduction of P700$^+$ was measured before and 5 min after the transfer to 0.8 M NaCl. Changes in the redox state of P700 after the application of multiple-turnover light flashes (50 ms) were monitored in terms of the change of absorbance at 830 nm with a PAM fluorometer, as described in the text.
possible explanation for this discrepancy is the activation of other forms of NDH, such as NDH-2, that can reduce plastoquinone. In such a case, recovery would not be achieved because of the absence of proton-pumping ability by NDH-2. Further studies are necessary to clarify these issues.

It has been reported that, after a rapid influx of Na+ ions upon salt shock, Na+ ions are excluded within 20 min (Reed et al. 1986). We found that the photosynthetic evolution of O2 was inhibited to a considerable extent for the first 15 minutes or so (Fig. 2). By contrast, donation of electrons from the cytosol to P700+ via the NDH-1 complex was stimulated (Fig. 6). These results suggest that the cyclic flow of electrons through the NDH-1 complex might play an important role in the exclusion of Na+ ions from salt-stressed cells. The cyclic flow of electrons around PSI might produce more ATP (Fork and Herbert 1993, Bendall and Manasse 1995) and our conclusion is reasonable if ATP is required for the exclusion of Na+ ions.

The observed enhancement of the cyclic flow of electrons upon salt shock is consistent with the fact that the transition from state 1 to state 2 occurs under salt-stressed conditions in Chlamydomonas reinhardtii (Endo et al. 1995). This transition causes a decrease in the ratio of PSII/PSI and the transfer of energy from phycobilisomes to PSII, with a consequent increase in the “spillover” of electrons from the cytosol to P700+ via the NDH-1 complex. Further studies are necessary to clarify these issues.

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