Two Types of Functionally Distinct NAD(P)H Dehydrogenases in
Synechocystis sp. Strain PCC6803*

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The ndhD gene encodes a membrane protein component of NAD(P)H dehydrogenase. The genome of Synechocystis sp. PCC6803 contains 6 ndhD genes. Three mutants were constructed by disrupting highly homologous ndhD genes in pairs. Only the ∆ndhD1/∆ndhD2 (∆ndhD1/D2) mutant was able to grow under photoheterotrophic conditions and exhibited low respiration rate, although the mutant grew normally under phototrophic conditions in air. The ∆ndhD3/∆ndhD4 (∆ndhD3/D4) mutant grew very slowly in air and did not take up CO₂. The results demonstrated the presence of two types of functionally distinct NAD(P)H dehydrogenases in Synechocystis PCC6803 cells. The ∆ndhD5/∆ndhD6 (∆ndhD5/D6) mutant grew like the wild-type strain. Under far-red light (>710 nm), the level of P700 was high in ∆ndhD1/D2 and M55 (ndhB-less mutant) at low intensities. The capacity of Qₐ (tightly bound plastoquinone) reduction by plastoquinone pool, as measured by the fluorescence increase in darkness upon addition of KCN, was much less in ∆ndhD1/D2 and M55 than in ∆ndhD3/D4 and ∆ndhD5/D6. We conclude that electrons from NADPH are transferred to the plastoquinone pool mainly by the NdhD1-NdhD2 type of NAD(P)H dehydrogenases.

The Type I NAD(P)H dehydrogenase complex (NDH-1) in cyanobacteria is involved in both the respiratory and photosynthetic electron transport chains (1). The whole genome sequence data base for Synechocystis sp. PCC6803 has shown the presence of genes for 12 subunits of NDH-1 with the large, hydrophobic NdhB, NdhD, and NdhF subunits being core membrane components (2). The data base also reveals that ndhD and ndhF are present as gene families with six and three members, respectively (note that NdhF4 has homology to NdhD5 and has been designated as NdhD6 (3)), although most ndh genes are present as single copies. This suggests that several types of NDH-1 exist in cyanobacteria, each with different NdhD and/or NdhF subunits, and with each potential complex having differing functions (4–6). In fact, of the five ndhD-less mutants, ∆ndhD3 is the only mutant that displays the phenotype of slow growth at limiting CO₂ (i.e. 50 ppm CO₂) and reduced affinity for CO₂ uptake, whereas the other ndhD-less mutants (∆ndhD1, ∆ndhD2, ∆ndhD4, and ∆ndhD5) do not show such phenotype (6).

It has been demonstrated that NDH-1 is essential for inorganic carbon (CO₂ and HCO₃⁻; designated C₅) transport in cyanobacteria (3–11), and it was assumed that ATP produced by NDH-1-dependent cyclic electron flow is essential to energize the C₅ transporter (7, 12). However, a recent observation indicated that mutations in ndh genes lead to inhibition of CO₂ uptake rather than HCO₃⁻ uptake (6). This suggested that CO₂ uptake is energized differently from HCO₃⁻ uptake. The presence of an ATP-dependent HCO₃⁻ transporter in Synechococcus PCC7942 has been recently demonstrated (13). In an attempt to see if there are functionally distinct NDH-1 complexes, we constructed double mutants of Synechocystis sp. PCC6803 by disrupting highly homologous ndhD genes in pairs and analyzed their growth under various conditions, CO₂ uptake and redox levels of P700 and plastoquinone (PQ) pool. The results suggested the presence of two types of functionally distinct NDH-1 complexes, one essential for phototrophic growth of the cells and the other essential for CO₂ uptake.

EXPERIMENTAL PROCEDURES

Growth Conditions—Wild-type and mutant cells of Synechocystis sp. PCC6803 were grown at 30 °C in BG11 medium (14) buffered with 20 mM Mes-KOH (pH 8.0) and bubbled with 3% CO₂ in air (v/v) or air (about 400 μl of CO₂ liter⁻¹). Solid medium was BG11-supplemented with 1.5% agar, 5 mM sodium thiosulfate, and 20 mM of the same buffer. Continuous illumination was provided by fluorescent lamps, generating photosynthetically active radiation of 60 μmol of photons m⁻² s⁻¹.

Construction of Mutants—Construction of single ndhD mutants, e.g. slr0331 (ndhD1), slr1291 (ndhD2), slr1733 (ndhD3), slr0027 (ndhD4), and slr2007 (ndhD5)-less mutants, has been described in a previous study (6). Descriptions of these mutants and the slr2009 (ndhD6)-less mutant have been deposited on the Web (CyanobMutants), where the drug resistance cassette used for each inactivation and the sites of insertion into the target genes are shown. The constructs used to generate the single mutants were used to transform various appropriate single mutants of Synechocystis sp. PCC6803 to generate the double mutants, i.e. ∆ndhD1/∆ndhD2 (∆ndhD1/D2), ∆ndhD3/∆ndhD4 (∆ndhD3/D4), and ∆ndhD5/∆ndhD6 (∆ndhD5/D6). M55 is the mutant constructed by inserting a Km² cartridge at the BamHI site in the Slr5033 site in nbdB, as described previously (7). The mutated ndhD genes in the transformants were segregated to homoogeneity (by successive streak purification) as determined by polymerase chain reaction amplification.

Determination of Growth Characteristics—Wild-type and mutant strains grown under 3% CO₂ were collected and resuspended in fresh BG11 medium to an OD₇₃₀nm of 1.0, 0.1, or 0.01. 2 μl of the cell suspensions was spotted onto BG11 agar plates buffered at various pHs in the absence or presence of 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 5 mM glucose. The plates were incubated under 3% CO₂ in air (v/v) or air for 5 days with continuous illumination by fluorescent lamps under a photosynthetically active radiation intensity of 60 μmol of photons m⁻² s⁻¹. The OD₇₃₀nm was measured using a recording spectrophotometer, model UV2200 (Shimadzu Co., Kyoto, Japan).
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TABLE I

Amino acid sequence similarity of the NdhD subunits in Synechocystis sp. PCC6803

The similarity was calculated using the Clustal W-based program from DNASTAR software.

|     | NdhD1 | NdhD2 | NdhD3 | NdhD4 | NdhD5 | NdhD6 |
|-----|-------|-------|-------|-------|-------|-------|
|     | %     |       |       |       |       |       |
| NdhD1 | 60.0  | 31.9  | 33.1  | 15.0  | 15.8  |       |
| NdhD2 | 33.1  | 35.7  | 17.7  | 17.6  |       |       |
| NdhD3 | 52.8  |       | 17.0  | 17.4  |       |       |
| NdhD4 |       | 18.3  | 18.8  |       |       |       |
| NdhD5 |       |       | 52.8  |       |       |       |
| NdhD6 |       |       |       |       |       |       |

RESULTS

Six ndhD Genes—The Synechocystis sp. PCC6803 genome contains six ndhD genes that were denoted as ndhD1 (sll0027), ndhD2 (sll1291), ndhD3 (sll1733), ndhD4 (sll10267), ndhD5 (sll0267), and ndhD6 (sll0269) (9). Table I summarizes the amino acid sequence similarity of the products of these genes. NdhD1, NdhD3, and NdhD5 showed the highest homology to NdhD2, NdhD4, and NdhD6, respectively. Double mutants were constructed by disrupting highly homologous ndhD genes in pairs, i.e., ndhD1/ndhD2, ndhD3/ndhD4, and ndhD5/ndhD6. Growth Characteristics—To explore how the inactivation of ndhD genes affects the growth characteristics of the cells, growth of the mutant strains was examined under photoautotrophic conditions on solid BG11 medium buffered at various pHs in the presence of 3% CO2 or air as well as under photoheterotrophic conditions on the same medium (pH 8.0) containing 5 mM glucose and 10 μM DCMU with air. There was a striking difference between the growth characteristics of ΔndhD1/ΔD and ΔndhD3/ΔD. The ΔndhD1/ΔD mutant grew as fast as the wild-type at pH levels of 8.0 and 7.0 in air but was unable to grow under photoheterotrophic conditions (Fig. 1, middle column panels). In contrast, ΔndhD3/ΔD grew very slowly in air, although the mutant grew as fast as the wild-type under photoheterotrophic conditions. These results demonstrated that there are at least two types of functionally distinct NdhD1 complexes in Synechocystis. One type of NdhD1 is essential for the photoheterotrophic growth of cells, and the other type is essential for growth under low CO2 concentrations. No significant difference was observed between the wild-type and ΔndhD5/ΔD strains in their growth characteristics under the conditions examined. Both ΔndhD1/ΔD and ΔndhD3/ΔD grew more slowly than the wild-type at pH 6.5 even under 3% CO2 (Fig. 1, panels C). The growth of ΔndhD5/ΔD was also slower than the wild-type at pH 6.5 in air. Thus, all the ndhD genes appear to be needed for cells to grow under acidic conditions.

The panels on the right column in Fig. 1 show the growth of four single ndhD-less mutants and M55 in air under photoautotrophic and photoheterotrophic conditions. The ΔndhD1 mutant exhibited partially the phenotype of ΔndhD1/ΔD and grew poorly under photoheterotrophic conditions. On the other hand, ΔndhD3 exhibited slow growth in air. However, none of these single mutants showed the severe phenotypes of the double mutants ΔndhD1/ΔD and ΔndhD3/ΔD. Double mutants constructed by inactivating ndhD genes in different combinations did not show the phenotype of ΔndhD1/ΔD or ΔndhD3/ΔD (data not shown). As expected, M55 exhibited the phenotypes of both ΔndhD1/ΔD and ΔndhD3/ΔD and did not grow under air as well as under photoheterotrophic conditions (Fig. 1, right column panels).

Respiration—The rates of respiration of the wild-type and mutant cells in the presence of glucose are summarized in Table II. Glucose enhanced the respiration rates by 50–400%.
Constant rates of oxygen uptake were attained 0.5–2 min after addition of glucose and continued until oxygen in the cell suspension was exhausted. Out of the three double mutants, only ΔndhD1/D2 showed significantly reduced respiration rate, which was about one-fourth the wild-type activity but was 40% higher than that of M55. The respiration rates of ΔndhD3/D4 and ΔndhD5/D6 were not significantly different from the rate of the wild-type.

CO2 Uptake—Fig. 2 shows changes in the rates of CO2 exchange of the wild-type and mutant cells upon switching the light on and off, as measured using the gas analysis system (15, 16). When the suspension of the wild-type cells was illuminated, the rate of CO2 uptake increased and reached the maximum level in 20 s. An efflux of CO2 from the cells was observed immediately after turning the light off. Similar CO2 exchange profiles have been observed with the wild-type cells of Synechococcus PCC7942 (15, 16) and Anabaena variabilis (21) and have been shown to reflect the CO2 uptake in the light by a CO2-concentrating mechanism and the release of intracellular C2 as CO2 after darkening. The ΔndhD3/D4 mutant did not take up CO2 at all in the light, indicating that the CO2 uptake system is impaired in this mutant. A similar result has been observed with M55 (7) and was confirmed in this study (Fig. 2). The ΔndhD1/D2 and ΔndhD5/D6 mutants showed similar CO2 exchange profiles to the wild-type, and their CO2 uptake activities were not significantly different from the activity in the wild-type. The ΔndhD3 mutant showed about 30% of the CO2 uptake activity in the wild-type under our experimental conditions (340 μl of CO2 liter–1).

Redox Level of P700 under FR Light—In cyanobacteria, NADPH produced by photosystem I (PSI) reaction donates electrons to the PQ pool via NDH-1, thus constituting NDH-1-dependent PSI cyclic electron flow (12). The donation of electrons from NADPH to the PQ pool can be observed by measuring the redox changes of P700. Fig. 3 shows the redox levels of P700 in the wild-type and mutant cells as a function of the intensity of FR light. The values in this figure are normalized based on the assumption that P700 was completely oxidized when the cell suspension was illuminated with white light (50-μs duration) superimposed on strong FR light (98 μeq m–2 s–1). When the cell suspension of M55 or ΔndhD1/D2 was illuminated with strong FR light, P700 was oxidized completely and no further oxidation of P700 was observed when the white flash light was superimposed on the FR light. In contrast, oxidation of P700 in the wild-type and other mutants was not complete with the strong FR light alone and further oxidation of P700 was observed with the flash light. P700 in M55 and ΔndhD1/D2 was highly oxidized even under weak FR light, whereas the oxidation levels of P700 in the wild-type and other mutants were low under the same conditions. At 3.3 μeq m–2 s–1 of FR light, the levels of P700+ in wild-type, ΔndhD1/D2, ΔndhD3/D4, ΔndhD5/D6, and M55 strains were 2, 56, 7, 10, and 72%, respectively. Evidently, in mutants M55 and ΔndhD1/D2 the reduction of P700 is strongly inhibited and the transport of electrons from NADPH to the PQ pool, and thereby to P700, is mediated mainly by the NdhD1-NdhD2 type of NDH-1 complexes. The higher oxidation levels of P700 in ΔndhD3/D4 and ΔndhD5/D6 than in the wild-type under weak FR light indicate that the NdhD3-NdhD4 and NdhD5-NdhD6 NDH-1 complexes also have a role in mediating transfer of electrons from NADPH to the PQ pool. The capacity of electron donation from NADPH to the PQ pool by these NDH-1 complexes, however, appears to be much less than that mediated by the NdhD1-NdhD2 NDH-1 complexes.

The Redox State of the PQ Pool—The redox state of the PQ pool in thylakoids was monitored indirectly by determining the chlorophyll fluorescence yield in darkness upon addition of KCN, reflecting an increase in the redox state of Q4 that is in redox equilibrium with the PQ pool. Therefore, the rate of reduction of the PQ pool can be monitored by measuring the chlorophyll fluorescence yield after oxidation of the PQ pool is blocked by KCN in the dark (22).

The wild-type cells exhibited a rapid increase in the fluorescence yield upon addition of KCN, reflecting an increase in the Q4 level due to PQ pool reduction. When the wild-type and mutant cells were illuminated in the presence of DCMU, the

### Table II

| Mutant          | Oxygen uptake (μmol of O2 mg chlorophyll–1 h–1) |
|-----------------|-----------------------------------------------|
| Wild-type       | 24.0 ± 3.6                                    |
| ΔndhD1/D2       | 6.6 ± 2.7                                     |
| ΔndhD3/D4       | 26.1 ± 1.5                                    |
| ΔndhD5/D6       | 18.9 ± 2.9                                    |
| M55             | 4.6 ± 0.57                                    |

**Fig. 2.** CO2 exchange of wild-type and mutant cells upon switching the light on and off, measured by an open gas analysis system. This system records the rate of CO2 exchange as a function of time (15, 16).

**Fig. 3.** Effects of the FR light intensity on the oxidation level of P700 in the wild-type and mutant cells. The P700+ levels were normalized assuming that P700 was completely oxidized when a 50-μs pulse of saturating white light (1500 watts m–2) was given to the cell suspension superimposed to strong FR light (98 μeq m–2 s–1). Wild-type (○), ΔndhD1/D2 (●), ΔndhD3/D4 (□), ΔndhD5/D6 (△), M55 (●).
were kept in darkness, and KCN (1 mM) was added at weak illumination that did not have any actinic effect. Cells were kept in darkness, and KCN (1 mM) was added at weak illumination that did not have any actinic effect. Cells

**DISCUSSION**

The ability of cells to grow under phototrophic conditions strongly depends on their respiratory activity. The \( \Delta ndhD1/D2 \) and M55 mutants, which exhibited low respiratory rates, were unable to grow under phototrophic conditions (Fig. 1 and Table II). Reduction of the PQ pool and P700 was also strongly inhibited in these mutants (Figs. 3 and 4). This indicated that the \( \text{NdhD}1/D2 \) type of NDH-1 complex mediates the transport of electrons from NADPH to the PQ pool and thereby to P700, thus constituting a PSI-dependent cyclic electron transport pathway. The \( \text{NdhD}3/D4 \) and \( \text{NdhD}5/\text{NdhD}6 \) complexes of NDH-1 complexes also contribute to this reaction, as seen from the higher oxidation levels of P700 in \( \Delta ndhD3/D4 \) and \( \Delta ndhD5/D6 \) than in the wild-type under FR light (Fig. 3). The contribution of these complexes of NDH-1 to the PSI-dependent cyclic electron transport appears to be much smaller than that of the \( \text{NdhD}1/D2 \) NDH-1 complex (Figs. 3 and 4, Table II).

The absence of the rapid increase in the fluorescence yield in \( \Delta ndhD1/D2 \) and M55 upon addition of KCN suggested that this fluorescence increase reflects the electron donation to the PQ pool mediated by the \( \text{NdhD}1/D2 \) NDH-1 complexes (Fig. 4). This contradicts the report by Cooley et al. (22) that the rapid fluorescence increase reflects the reduction of the PQ pool mediated by SDH. It appears possible that inactivation of SDH led to decreased formation of NADPH, thereby decreasing the NDH-1-dependent reduction of the PQ pool. Adversely, inactivation of NDH-1 might lead to the decrease of succinate formation. Inactivation of \( ndhB \) or \( ndhD1 \) plus \( ndhD2 \) had much stronger effect on the fluorescence increase than the inactivation of \( sl1625 \) plus \( sl10823 \) (Fig. 4; also see Fig. 6 in Ref. 22), and hence, we prefer the former possibility.

Among the single and double \( ndh \) mutants constructed in this and previous studies (5, 6), only \( \Delta ndhD3/D4 \) showed the accentuated phenotype of M55 (i.e. complete loss of CO2 uptake activity). It is evident that inability of these mutants to grow under low CO2 conditions is a result of inactivation of their CO2 uptake activity (Figs. 1 and 2) and that only the \( \text{NdhD}3/\text{NdhD}4 \) NDH-1 complexes are essential for CO2 uptake. We have previously proposed a model in which ATP produced by NDH-1-dependent PSI cyclic electron transport drives the “transport” of CO2 (23). However, inactivation of \( \text{ndhD}1 \) and \( \text{ndhD}2 \), which are essential to the PSI cyclic electron transport, did not have any significant effect on CO2 uptake (Fig. 2). Thus, this previous model appears to be unlikely.

The mechanism of CO2 uptake and the role of \( \text{NdhD}3/\text{NdhD}4 \) NDH-1 complex types in CO2 uptake are not known. The CO2 uptake reaction is postulated to be an energy-dependent unidirectional conversion of CO2 to HCO3 (11, 24). Kaplan and Reinhold proposed a model in which an “alkaline pocket” produced in the light functions as a converter of CO2 to HCO3 (11). The information presented in this paper does allow for limited speculation on how a particular type of NDH-1 complex might be specifically involved in CO2 uptake. A vectorial carbonic anhydrase-like reaction could be closely associated with the \( \text{NdhD3/} \text{NdhD4} \) NDH-1 complex type such that OH– ions might be produced in a “localized pocket” and used to drive the conversion of CO2 to HCO3. Such a pocket could be produced at the site(s) of protonation of NADP and/or PQ, and a relatively high electron transfer rate would be required to account for the high CO2 uptake rate. The low rate of electron transfer from NADPH to PQ pool in the \( \Delta ndhD3/\text{D4} \) mutant would suggest that the \( \text{NdhD3/} \text{NdhD4} \) NDH-1 complexes donate electrons mainly to an alternate electron acceptor. Further studies are in progress to identify the electron acceptor.

In a previous paper, the following phenotypes were reported to characterize the \( ndhB \)-less mutant (M55) of Synechocystis sp. strain PCC6803 (7). 1) It grows very slowly under air and is unable to take up CO2, 2) it is unable to grow under phototrophic conditions, and 3) it has a low respiration rate. The first phenotype was observed in the \( \Delta ndhD3/D4 \) mutant described in this study and the second and third phenotypes were found for the \( \text{ndhD}1/\text{D2} \) mutant (Figs. 1 and 2, Table II). Thus, it appears evident that there are two types of functionally distinct NDH-1 complexes in Synechocystis sp. PCC6803, and M55 exhibits the phenotype of both types of the mutants. The presence of the homologues of the six \( ndhD \) genes in the genome of Anabaena sp. PCC7120 (the Web, CyanoBase) and Synechococcus sp. PCC7002 (4) strongly suggests that the presence of the two types of functionally distinct NDH-1 complexes is common among various strains of cyanobacteria.

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