

Expression and Functional Roles of the Two Distinct NDH-1 Complexes and the Carbon Acquisition Complex NdhD3/NdhF3/CupA/SII1735 in *Synechocystis* sp PCC 6803

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To investigate the (co)expression, interaction, and membrane location of multifunctional NAD(P)H dehydrogenase type 1 (NDH-1) complexes and their involvement in carbon acquisition, cyclic photosystem I, and respiration, we grew the wild type and specific *ndh* gene knockout mutants of *Synechocystis* sp PCC 6803 under different CO₂ and pH conditions, followed by a proteome analysis of their membrane protein complexes. Typical NDH-1 complexes were represented by NDH-1L (large) and NDH-1M (medium size), located in the thylakoid membrane. The NDH-1L complex, missing from the Δ NdhD1/D2 mutant, was a prerequisite for photoheterotrophic growth and thus apparently involved in cellular respiration. The amount of NDH-1M and the rate of P700⁺ rereduction in darkness in the Δ NdhD1/D2 mutant grown at low CO₂ were similar to those in the wild type, whereas in the M55 mutant (Δ NdhB), lacking both NDH-1L and NDH-1M, the rate of P700⁺ rereduction was very slow. The NDH-1S (small) complex, localized to the thylakoid membrane and composed of only NdhD3, NdhF3, CupA, and SII1735, was strongly induced at low CO₂ in the wild type as well as in Δ NdhD1/D2 and M55. In contrast with the wild type and Δ NdhD1/D2, which show normal CO₂ uptake, M55 is unable to take up CO₂ even when the NDH-1S complex is present. Conversely, the Δ NdhD3/D4 mutant, also unable to take up CO₂, lacked NDH-1S but exhibited wild-type levels of NDH-1M at low CO₂. These results demonstrate that both NDH-1S and NDH-1M are essential for CO₂ uptake and that NDH-1M is a functional complex. We also show that the Na⁺/HCO₃⁻ transporter (SbtA complex) is located in the plasma membrane and is strongly induced in the wild type and mutants at low CO₂.

INTRODUCTION

NAD(P)H dehydrogenase type 1 (NDH-1) complexes have been reported to have multiple functions both in cyanobacteria and plant chloroplasts. Common for both organisms seems to be the function in respiration (chlororespiration in chloroplasts) and in cyclic photosystem I (PSI) (Ogawa, 1991; Mi et al., 1992; Burrows et al., 1998; Munekage et al., 2004). The genome analysis has revealed 11 genes encoding NDH-1 subunits (NdhA to NdhK) in *Synechocystis* 6803 (Kaneko et al., 1996) as well as in chloroplasts of several plant species (Friedrich et al., 1995). Most of these genes are present as single copies in cyanobacterial genomes, except for the *ndhD* and *ndhF* genes, which in *Synechocystis* 6803 comprise small gene families of six and three members, respectively (Kaneko et al., 1996; Shibata et al., 2001). Reverse genetics has been essential in revealing the roles of specific Ndh subunits (Price et al., 1998; Ogawa and Kaplan,

2003). NDH-1 complexes containing NdhD1 and NdhD2 proteins, together with NdhF1, have been postulated to function in PSI-associated cyclic electron flow as well as in cellular respiration (Mi et al., 1992, 1995; Ohkawa et al., 2000a). NDH-1 complexes with other NdhD and NdhF gene products have been suggested to have additional functions in carbon concentrating mechanisms in cyanobacteria (Ohkawa et al., 1998, 2000a; Price et al., 1998; Klughammer et al., 1999; Shibata et al., 2001; Maeda et al., 2002). These mechanisms are important in aquatic organisms to overcome the low affinity of their ribulose-1,5-bisphosphate carboxylase/oxygenase to CO₂ (Volokita et al., 1984; Kaplan and Reinhold, 1999; Badger and Spalding, 2000; Price et al., 2002).

Four distinct inorganic carbon (Ci) acquisition systems have been identified in cyanobacteria by reverse genetics approaches (Ogawa and Kaplan, 2003). Two of them are specialized in CO₂ uptake (Ohkawa et al., 2000a; Shibata et al., 2001; Maeda et al., 2002). One is a constitutively expressed low-affinity CO₂ uptake system, and the other one is a high-affinity CO₂ uptake system induced at limiting CO₂ conditions. Reverse genetics with cyanobacteria has demonstrated that the inducible CO₂ uptake system involves the NdhD3 and NdhF3 proteins, whereas the constitutively expressed CO₂ uptake system involves NdhD4 and NdhF4 proteins (Ohkawa et al., 2000a; Shibata et al., 2001; Maeda et al., 2002). Moreover, the two homologous proteins CupA and CupB are essential for inducible and constitutive CO₂

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uptake, respectively (Shibata et al., 2001; Maeda et al., 2002). Function of both these CO₂ uptake systems has been suggested to occur via specialized NDH-1 complexes (Ohkawa et al., 2000a). The other two Ci acquisition systems in cyanobacteria are involved in bicarbonate transport. The more important one in *Synechocystis* 6803 is a Na⁺-dependent HCO₃⁻ transporter, which is strongly inducible under Ci limitation. This transporter is encoded by the *sbtA* gene and appears to operate as a Na⁺/HCO₃⁻ symporter (Shibata et al., 2002). The ATP binding cassette transporter, BCT1 (Omata and Ogawa, 1986; Omata et al., 1999), on the other hand, has been shown to have only little, if any, effect on HCO₃⁻ transport activity in *Synechocystis* 6803 (Shibata et al., 2002).

Although considerable progress has been made during the past few years in elucidating the functions of the NDH-1 complexes in cyanobacteria and chloroplasts, the structural bases and cooperation of various complexes still requires elaborate research. One major obstacle in this elucidation has been the lack of profound knowledge on protein level of the multiplicity and the composition of the complexes participating in different functions. To understand the structural and functional basis of cyanobacterial membrane complexes, we have started a functional proteomics project with *Synechocystis* 6803. Recently, we reported the presence of four distinct complexes containing *ndh* gene products in *Synechocystis* 6803 membrane: NDH-1L (large), NDH-1M (medium size), NDH-1S₁ (small₁), and NDH-1S₂ (small₂) (Herranen et al., 2004). Here, we have investigated the function, cooperation and subunit composition of these complexes by performing membrane proteomics of specific *ndh* gene knockout mutants of *Synechocystis* 6803 grown under high and low CO₂ and pH conditions. We provide evidence that the thylakoid membrane-associated low CO₂ inducible complex NDH-1S, composed of NdhD3, NdhF3, CupA, and Sll1735, is functionally active in CO₂ uptake when coexpressed with the NDH-1M complex. NDH-1M complex contains all known single copy *ndh* gene products but lacks the NdhD and NdhF subunits. This complex is efficient in P700⁺ rereduction, implying the function in PSI cyclic electron flow. The NDH-1L complex closely resembles in subunit composition the NDH-1 complex of *Escherichia coli* and the chloroplast thylakoid NDH-1 complex (Friedrich and Scheide, 2000). This complex is constitutively expressed and particularly important when cellular energy production relies on respiration.

RESULTS

Growth of the Wild Type and *ndh* Mutant Strains at Different pH and CO₂ Conditions

To address the vitality of cells by induction of Ci acquisition systems (and/or various Ndh-containing complexes) at low CO₂, we first cultured *Synechocystis* wild type and Ci acquisition mutant strains M55 (Δ NdhB), Δ NdhD3, Δ NdhD4, Δ NdhD3/D4, and Δ NdhD3/D4/SbtA at 3% CO₂, pH 7.5, and then shifted the cells to low (air level) CO₂, pH 7.5 or 8.3 (in former pH the Ci species are depleted in HCO₃⁻, whereas at alkaline pH Ci is mostly present as HCO₃⁻). Although the Ndh mutants used in this

study have previously been characterized in their capacity for CO₂ uptake, respiration and cyclic PSI electron flow under some of the growth conditions described above (Ohkawa et al., 2000a), we found it crucial to compare their growth capacities at CO₂ downshift both at pH 7.5 and 8.3, the conditions that induced differential expression of the Ci acquisition and Ndh complexes in different *Synechocystis* strains (see later). It is also important to note that the cells were always cultured in liquid medium because it is known from previous studies that the Ci acquisition systems might be displayed differently when cells are grown on plates directly in contact with air (Ohkawa et al., 2000b).

As shown in Figure 1A, the wild type, M55, and Δ ndhD3 grew equally well at high CO₂ (as also the Δ NdhD4, Δ NdhD3/D4, and Δ NdhD3/D4/SbtA strains; data not shown). At low CO₂, only Δ NdhD3, Δ NdhD4, and the wild type were capable of growing at pH 7.5, however, with a substantially slower rate than at high CO₂ (Figure 1B). The mutants M55 (deficient in NdhB protein; Ogawa, 1991) and Δ NdhD3/D4 did not grow under these conditions. Moreover, M55 became bleached and died after a few days of incubation (as also the triple mutant Δ NhdD3/D4/SbtA; data not shown), whereas Δ NdhD3/D4 remained green, yet the growth of this mutant was suppressed. When the cells were shifted from high CO₂ to low CO₂ and pH 8.3 (Figure 1C), both M55 and the Δ NhdD3/D4 double mutant were capable of growing, though the latter one exhibiting a retarded growth rate compared with the other strains. Only the triple mutant Δ NhdD3/D4/SbtA could not sustain viability and became bleached upon culturing at low CO₂ and pH 8.3.

Identification and Diversity of the Ndh-Containing Complexes and the SbtA Complex in M55 Strain as Compared with Wild-Type Cells

Crude thylakoid membrane fractions were isolated from wild-type and M55 mutant cells grown in liquid cultures either at high CO₂, pH 7.5, or at low CO₂, pH 8.3. The membranes were then subjected to blue-native (BN)-PAGE separation of the intrinsic membrane protein complexes (Figure 2). The cytochrome b₆f complex as monomers and dimers, PSII as monomers and dimers, ATP synthase, and PSI monomers and trimers were well distinguished in all membranes (Figure 2A) (for matrix-assisted laser-desorption ionization time of flight [MALDI-TOF] identification, see Herranen et al., 2004). The NdhJ- and NdhK-specific antibodies were used in immunoblotting to identify the NDH-1 complexes. These antibodies reacted with two membrane protein complexes, NDH-1L and NDH-1M (Figures 2B and 2C, respectively). These complexes were present in wild-type cells under both growth conditions tested here but absent from M55 membranes independently of the growth conditions. It is noteworthy that in wild-type membranes under high CO₂ conditions the NDH-1L complex was more abundant than the NDH-1M complex, whereas at low CO₂ and pH 8.3 the NDH-1M complex was clearly the more dominant one. After immunoblotting with the NdhJ and NdhK antibodies, the membranes were probed with the NdhD3-, NdhF3-, and SbtA-specific antibodies. Two considerably smaller protein complexes were detected with the NdhD3 antibody (Figure 2B) and also with the NdhF3 antibody (data not shown). These complexes, NDH-1S₁ and NDH-1S₂,

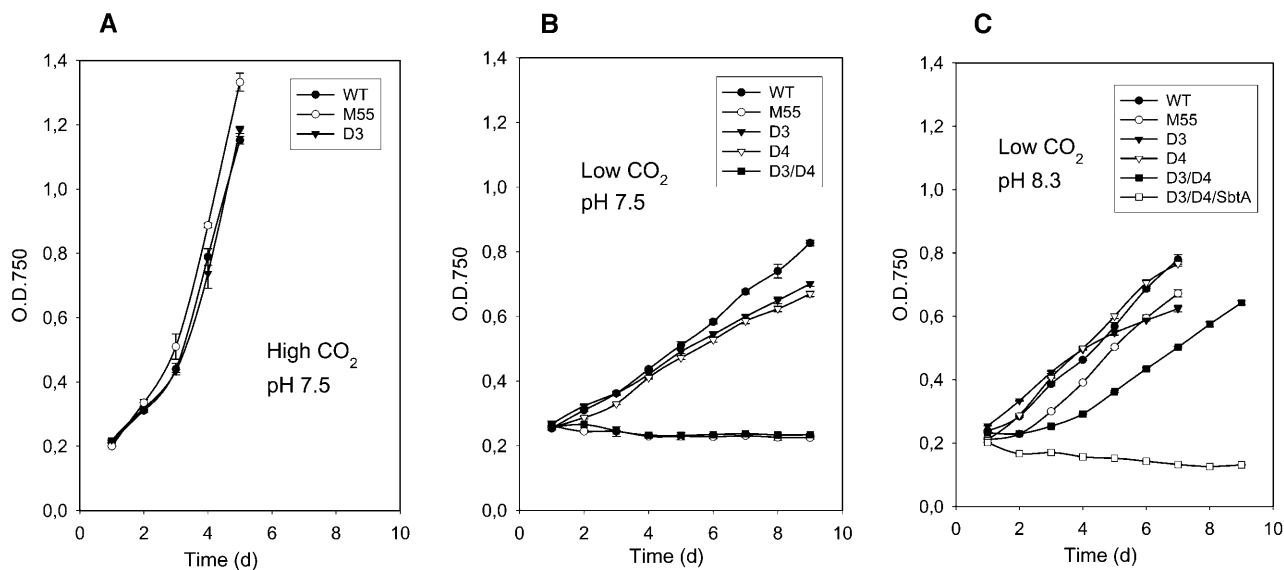


Figure 1. Growth Curves of Wild-Type *Synechocystis* 6803 Cells and M55, Δ NdhD3, Δ NdhD4, Δ NdhD3/D4 Double Mutant, and Δ NdhD3/D4/SbtA Triple Mutant.

(A) Growth in BG-11 medium, pH 7.5, 3% CO_2 .

(B) Growth in BG-11 medium, pH 7.5, air level of CO_2 .

(C) Growth in BG-11 medium, pH 8.3, air level of CO_2 .

appeared in both wild-type and M55 cells grown in low CO_2 and were absent in high CO_2 . The SbtA antibody reacted with a protein complex between the two NDH-1S complexes (Figure 2C). The SbtA complex appeared only when the cells were grown at low CO_2 , showing a similar expression profile as the NDH-1S complexes. The apparent molecular mass of the SbtA complex was estimated to be ~ 160 kD.

To study the subunit diversity of Ndh-containing complexes in more detail, the BN gel lanes of the wild type and M55 (low CO_2 , pH 8.3) (Figure 2) were subjected to SDS-PAGE in the second dimension (Figure 3A). General features of *Synechocystis* 6803 membrane proteome were previously described in Herranen et al. (2004). The two-dimensional (2-D) gels were analyzed by sequential immunoblotting with the NdhJ, NdhK, NdhD3, NdhF3, and SbtA antibodies. Immunoblots clearly indicated the absence of NdhD3 and NdhF3 proteins from the NDH-1L and NDH-1M complexes, and conversely, the complete absence of NdhJ and NdhK proteins from the NDH-1S complexes (Figure 3B). It is also noteworthy that neither NdhJ nor NdhK were found as free proteins in the thylakoid membrane. Distinct of wild-type cells grown at low CO_2 and high pH (8.3) was an abundant NDH-1M complex (Figures 3A and 3B). Both the NDH-1L and NDH-1M complexes, composed of at least 10 different subunits (Figure 3), were absent from M55 cells deficient in the NdhB protein. As already noted from one-dimensional BN gels, both wild-type and M55 cells, grown at low CO_2 , exhibited considerable amounts of both the NDH-1S and SbtA complexes. The identities of NdhK, CupA, and SbtA proteins, present in NDH-1L/M, NDH-1S₁, and SbtA complexes, respectively, were further verified by MALDI-TOF mass spectrometry (Figure 4).

In the SbtA complex, several protein bands with slightly different mobility in SDS-PAGE were detected in silver stained gels (Figure 3A). Analysis by two independent methods, MALDI and immunoblotting, identified them as SbtA, suggesting an involvement of posttranslational modifications of this protein.

Coexpression of the Ndh-Containing and SbtA Complexes in Wild-Type and *Ci* Acquisition Mutants as Influenced by Growth Conditions

To get further insights into the induction, coexpression, and function of the Ndh-containing and SbtA complexes, we subjected the wild type and different *ndh* mutant strains to proteome analysis before and after the CO_2 downshift at pH 7.5 and 8.3 for 24 h, if not otherwise indicated (conditions corresponding to those in Figure 1). In addition to the Δ NdhB mutant M55, the membrane protein complexes of several other *Ci* acquisition mutants, Δ NdhD3, Δ NdhD4, Δ NdhD3/D4, and Δ NdhD3/D4/SbtA, were subjected to 2-D BN/SDS-PAGE analysis. Sections of silver-stained gels in Figure 5, enclosing the major components of the Ndh-containing complexes and the SbtA complex, make it possible to evaluate the coexpression of these protein complexes with each other.

Under high CO_2 conditions, the presence of a dominant NDH-1L complex was distinct to the membranes of all strains except M55 (Figure 5, left panel), and all other complexes harboring *ndh* gene products, as well as the SbtA complex, were either absent or present only in very low amounts (NDH-1M).

At low CO_2 and pH 7.5, the *Ci* acquisition and NDH-1 complexes showed somewhat varying patterns between the

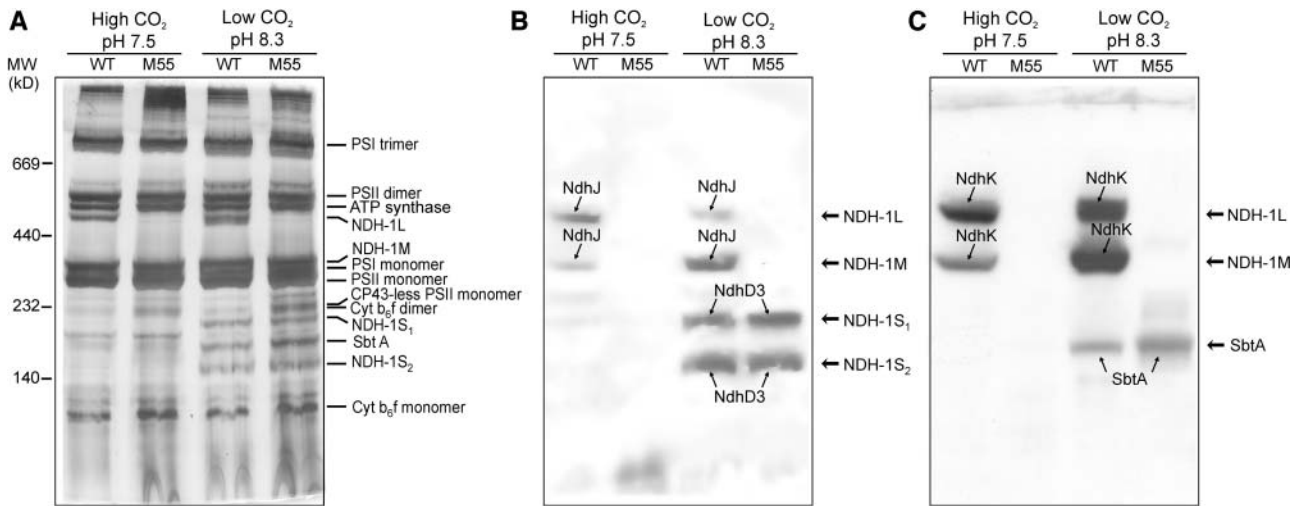


Figure 2. Membrane Protein Complexes of *Synechocystis* 6803 Wild Type and the M55 Strain.

Wild-type and M55 cells were grown at 3% CO₂ in BG-11 medium at pH 7.5 or at air level of CO₂ in BG-11 medium at pH 8.3. Cells were then harvested and crude thylakoid membranes isolated as described in Methods. After solubilization of membranes with 1.5% *n*-dodecyl- β -D-maltoside, the protein complexes were separated by BN-PAGE.

(A) The BN-gel was stained with silver. Molecular markers are indicated to the left, and the assignment of the major protein complexes is given to the right.

(B) Protein complexes were electroblotted to a polyvinylidene difluoride (PVDF) membrane, and the membrane was first probed with anti-NdhJ to identify the NDH-1 complexes (NDH-1L and NDH-1M) and subsequently with anti-NdhD3, which recognized the NDH-1S₁ and NDH-1S₂ complexes.

(C) PVDF membrane was first probed with anti-NdhK and subsequently with anti-SbtA to recognize the Na⁺/HCO₃⁻ transporter. Protein bands indicated by arrows interacted with respective antibodies.

different mutant strains (Figure 5, middle panel). Typical for wild-type cells was a strong expression of NDH-1M, NDH-1S₁, and NDH-1S₂ as well as of the bicarbonate transporter SbtA. Both NDH-1L and NDH-1M were absent from M55. The membrane proteome of M55 showed initially, within 24 h after CO₂ downshift, some transient upregulation of the NDH-1S complexes (Figure 5). However, already after 48 h of incubation at low CO₂, the newly synthesized NDH-1S complexes were degraded (data not shown). Another distinct feature of the M55 cells at low CO₂ pH 7.5 was the lack of accumulation of the SbtA transporter, in sharp contrast with that observed in the wild-type cells. It is also noteworthy that free CP47 protein (identified by MALDI-TOF; data not shown) of photosystem II (PSII) accumulated in M55 membranes under these conditions, referring to oxidative stress and destruction of the PSII complexes (Aro et al., 2005). The Δ NdhD3 and Δ NdhD3/D4 mutants showed yet a different membrane proteome with regard to Ci transporters and NDH-1 complexes when incubated at low CO₂, pH 7.5. The NDH-1S complex was absent but particularly the NDH-1M complex accumulated heavily. Intriguingly, the NdhD3/D4 double mutant showed a prominent expression of the SbtA transporter as well (Figure 5).

Low CO₂ at pH 8.3 induced different patterns of Ci acquisition and NDH-1 complexes compared with that at pH 7.5, especially in the M55 strain (Figure 5, right panel). At elevated pH (8.3), M55 showed an evident upregulation of both the NDH-1S and SbtA complexes, which at pH 7.5 remained at a low level or were completely unexpressed, respectively (Figure 5). Patterns of the

carbon acquisition complexes of the wild type, Δ NdhD3, Δ NdhD4, and Δ NdhD3/D4, on the other hand, did not qualitatively differ from those occurring at low CO₂ at pH 7.5.

The membrane proteome of the Δ NdhD4 mutant did not significantly differ from that of the wild-type cells (Figure 5). It seems that our proteome approach did not recognize the constitutively expressed CO₂ uptake system composed of NdhD4/NdhF4/CupB (Ohkawa et al., 2000a), possibly because of a low abundance of this complex.

The growth capacity and the expression of various NDH-1 and Ci acquisition complexes of the wild type and the Ci acquisition mutants at high CO₂ and after CO₂ downshift (pH 7.5 and 8.3) are summarized in Table 1.

Expression of NDH-1 Complexes in PSI-Less and Δ NdhD1/D2 Mutants of *Synechocystis* 6803

To further clarify the functional role(s) of the NDH-1L and NDH-1M complexes, we investigated the membrane proteomes of yet two other strains, the PSI-less mutant, which cannot perform cyclic PSI electron transfer, and the Δ NdhD1/D2 mutant, which is not capable of photoheterotrophic growth (Ohkawa et al., 2000a; confirmed here, data not shown).

The PSI-less mutant grew only heterotrophically in the presence of glucose. Thus, the energy for cell growth and metabolism was likely to be derived from respiration. NDH-1L was by far the most dominating NDH-1 complex in this strain, and only traces of NDH-1M were detected (Figure 6A). This was in sharp contrast

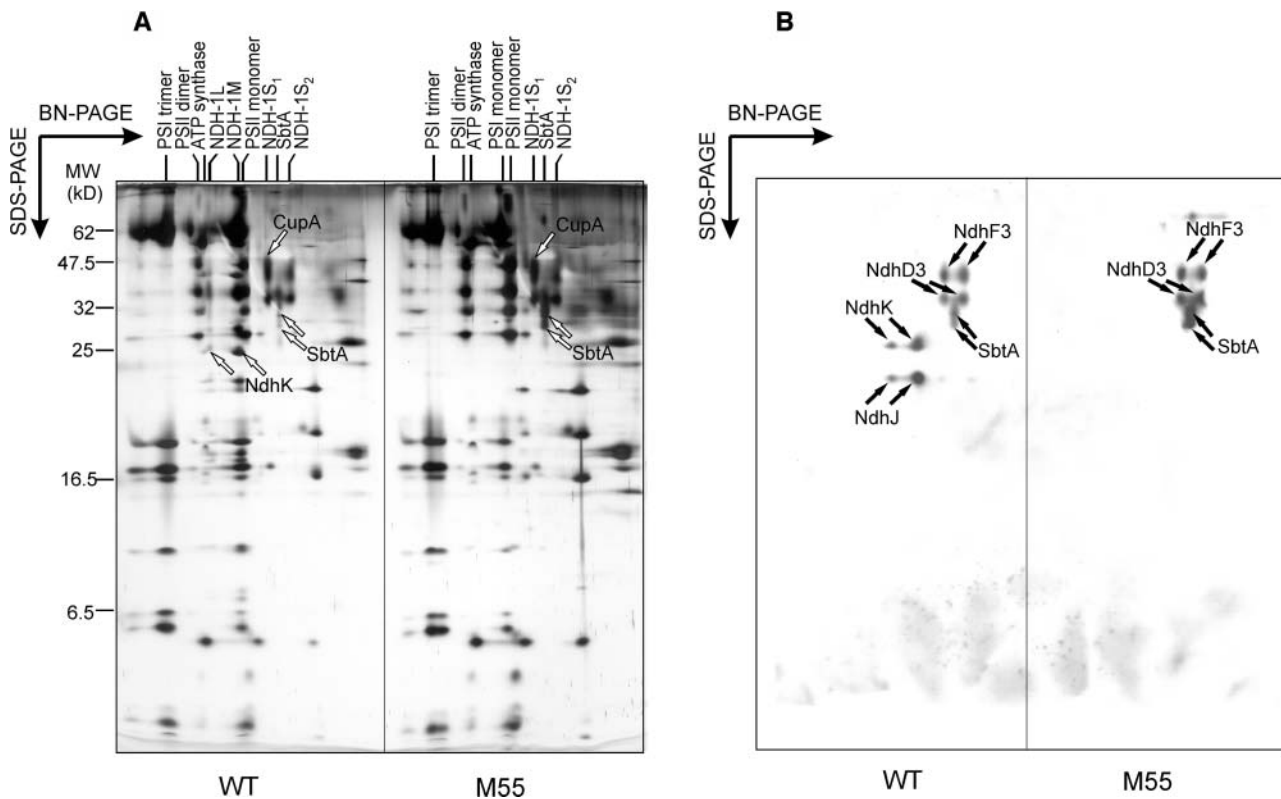


Figure 3. Two-Dimensional Analysis of *Synechocystis* 6803 Wild Type and M55 Membrane Protein Complexes from Cells Grown at Low CO_2 , pH 8.3.

After separation of the protein complexes in the BN gel, the lane was cut out, solubilized with Laemmli buffer, and subjected to SDS-PAGE.

(A) Silver stained gels of wild-type and M55 membranes (crude thylakoid preparations).

(B) Gels were electroblotted to a PVDF membrane and probed sequentially with NdhK, NdhJ, NdhD3, NdhF3, and SbtA antibodies revealing the presence or absence, as well as the positions, of the NDH-1L, NDH-1M, NDH-1S complexes (S_1 and S_2), and the SbtA complex.

with wild-type cells grown under similar conditions in the presence of glucose, having only small amounts of NDH-1L as compared with the dominating NDH-1M complex (Figure 6A). Moreover, the NDH-1S and SbtA complexes present in the wild type were completely missing in the PSI-less mutant.

The $\Delta\text{NdhD1/D2}$ mutant, on the other hand, was capable of growing in normal BG-11 medium at low CO_2 and pH 7.5, the conditions sustaining the expression of all Ndh-containing complexes in wild-type cells (Figure 6B). The $\Delta\text{NdhD1/D2}$ mutant, however, lacked the NDH-1L complex (Figure 6B) but interestingly enough, had a relatively high level of the NDH-1M complex, similar to that in the wild type. Shift of the $\Delta\text{NdhD1/D2}$ mutant to high CO_2 drastically decreased the amount of NDH-1M (data not shown). Addition of glucose and DCMU to the growth medium induced bleaching of the $\Delta\text{NdhD1/D2}$ mutant as well as M55, whereas wild-type cells continued growing and upregulated the expression of the NDH-1L complex (data not shown). The NDH-1L complex thus appeared to be a prerequisite for photoheterotrophic growth and could not be replaced by the NDH-1M complex.

As evidenced by the $\Delta\text{NdhD1/D2}$ mutant, the NdhD1 (or D2) subunit is clearly missing from the NDH-1M complex. The other difference between the NDH-1L and NDH-1M complexes was

the absence of the NdhF1 subunit from the NDH-1M complex (Figure 6B). Besides recognition of NdhF1 by protein-specific antibody, the identity of the NdhF1 subunit missing from NDH-1M was further proven by N-terminal sequencing, which gave similar results to those by Prommeenate et al. (2004). It is noteworthy that the NdhF1 antibody did not recognize any smaller complexes or free NdhF1 proteins in the thylakoid membrane.

To obtain insights into the function of the NDH-1M complex, we next measured the cyclic PSI electron transfer by monitoring the reduction rate of P700^+ in darkness after illumination of cells with far red light. As shown in Table 2, the half-time of P700^+ rereduction in the $\Delta\text{NdhD1/D2}$ mutant was similar to that in wild-type cells, in accordance with similar amounts of the NDH-1M complex in these two strains under the given growth conditions. For comparison, the cyclic electron transfer rates for wild-type and M55 cells, grown at high CO_2 , were also measured (Table 2). The wild type showed two times and M55 10 times slower rereduction of P700^+ in darkness as compared with wild-type cells grown at low CO_2 conditions. This slow rate of P700^+ rereduction was accompanied with a small amount of NDH-1M in wild-type cells at high CO_2 and a complete lack of NDH-1M (and NDH-1L) in M55 cells (Figures 2 and 5).

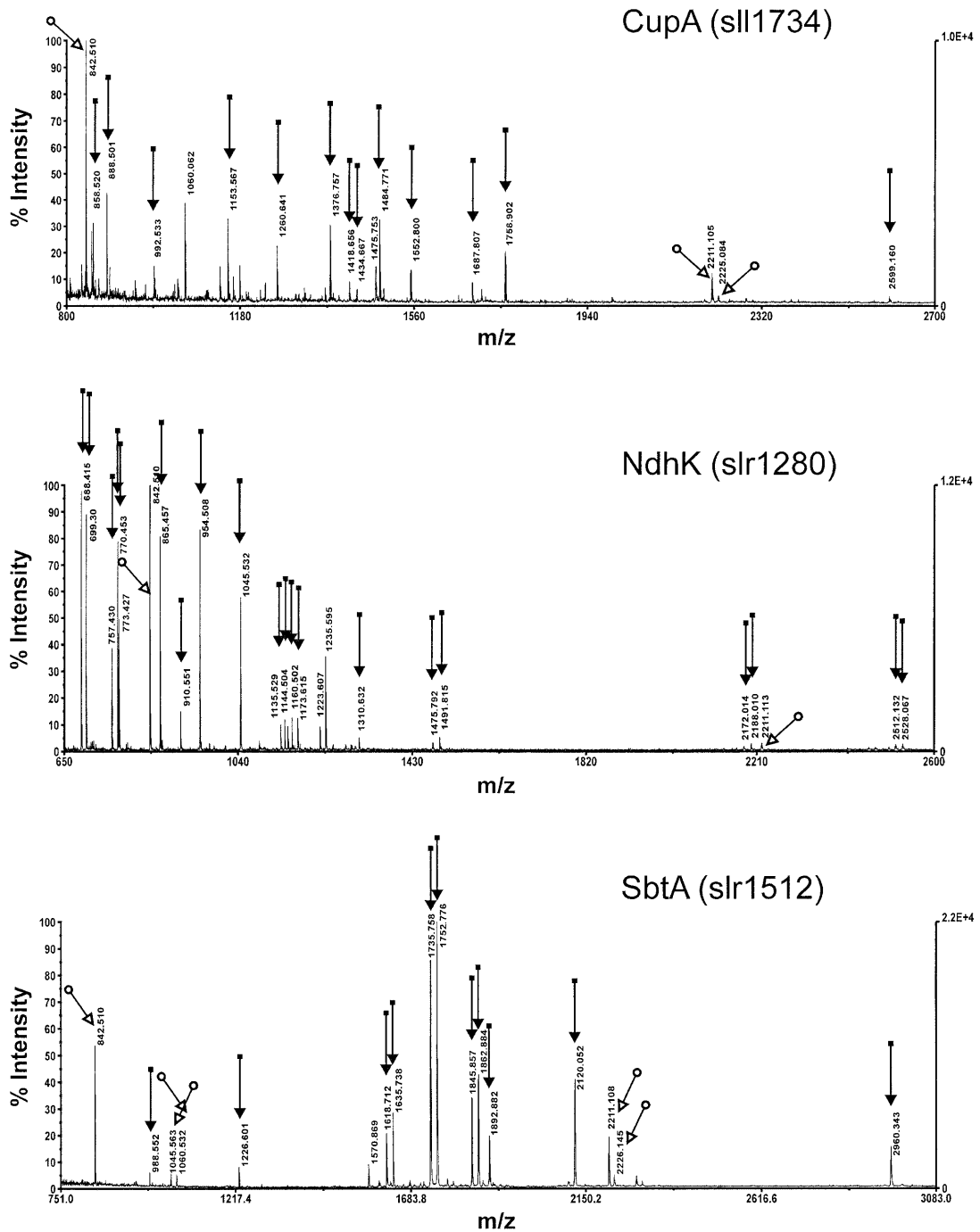


Figure 4. Identification by MALDI-TOF Mass Spectrometry of the NdhK Protein Present in the NDH-1L and NDH-1M Complexes, the CupA Protein Present in the NDH-1S₁ Complex, and the SbtA Protein in the HCO₃⁻ Transporter Complex.

Protein spots for identification were taken from the gel in Figure 3. Essentially similar SbtA spectra were obtained from three different partially overlapping spots. m/z, mass-to-charge ratio.

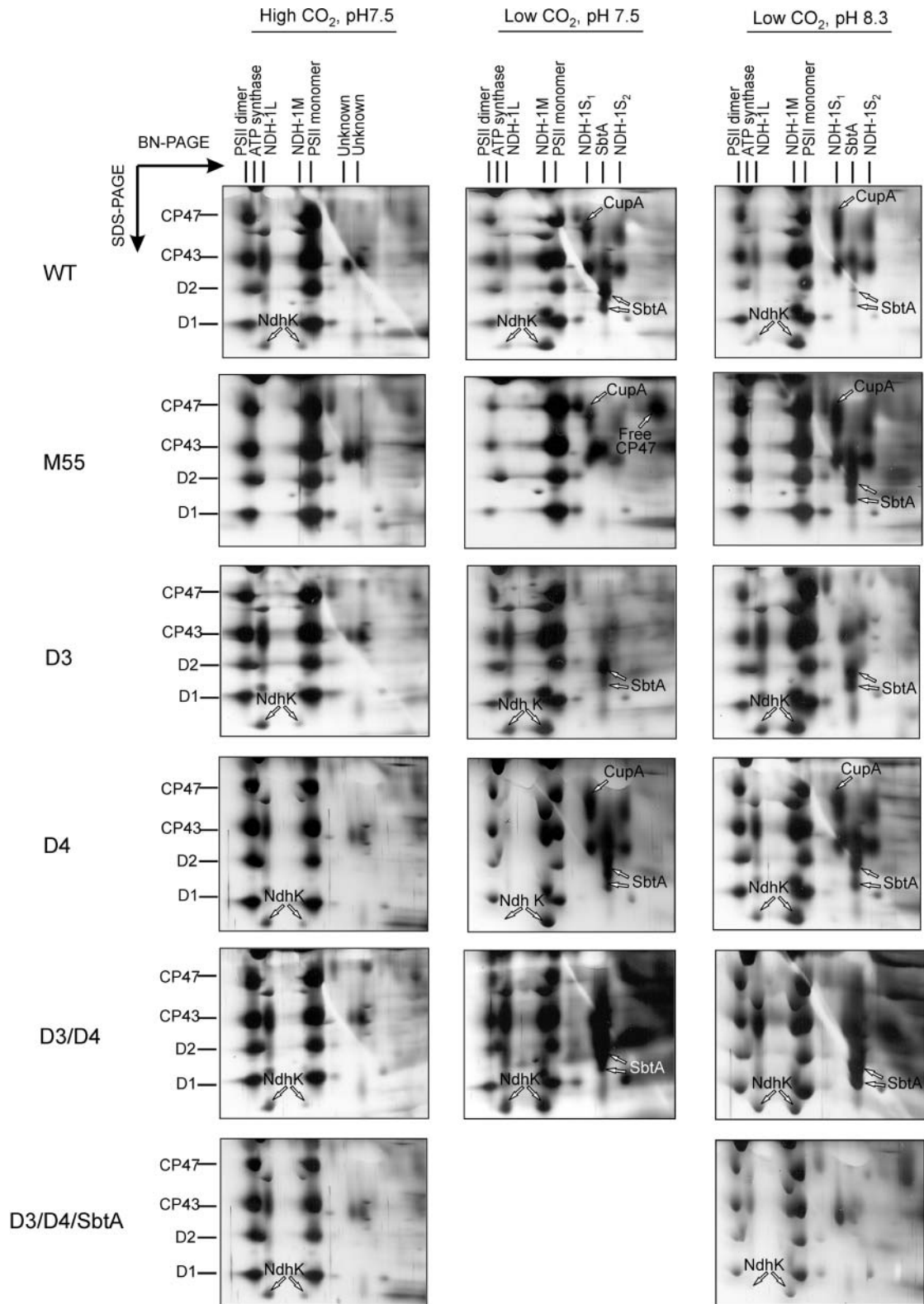


Figure 5. Sections of Silver-Stained 2-D BN/SDS-PAGE Gels from *Synechocystis* Wild Type and M55, Δ NdhD3, Δ NdhD4, Δ NdhD3/D4 Double Mutant, and Δ NdhD3/D4/SbtA Triple Mutant, Enclosing the Major Components of the Carbon Acquisition Complexes and the PSII Monomer and Dimer Complexes.

Table 1. Summary of the Growth and Expression of the Ndh and SbtA Containing Protein Complexes in the Wild Type and Different Carbon Acquisition Mutants of *Synechocystis* 6803

| Strain | Growth/ Complex | Growth Conditions | | |
|------------|--------------------|----------------------------------|---------------------------------|---------------------------------|
| | | High CO ₂ , pH 7.5 | Low CO ₂ , pH 7.5 | Low CO ₂ , pH 8.3 |
| Wild type | Growth | ++ | + | + |
| | NDH-1L | ++ | +/- | + |
| | NDH-1M | +/- | ++ | ++ |
| | NDH-1S | - | ++ | ++ |
| | SbtA | - | + | + |
| M55 | Growth | ++ | - | + |
| | NDH-1L | - | - | - |
| | NDH-1M | - | - | - |
| | NDH-1S | - | +/- | ++ |
| | SbtA | - | - | +++* |
| D3 | Growth | ++ | + | + |
| | NDH-1L | ++ | + | + |
| | NDH-1M | +/- | ++ | ++ |
| | NDH-1S | - | - | - |
| | SbtA | - | + | + |
| D4 | Growth | ++ | + | + |
| | NDH-1L | ++ | +/- | + |
| | NDH-1M | +/- | ++ | ++ |
| | NDH-1S | - | ++ | ++ |
| | SbtA | - | + | + |
| D3/D4 | Growth | ++ | +/- | + |
| | NDH-1L | ++ | ++ | ++ |
| | NDH-1M | +/- | ++ | ++ |
| | NDH-1S | - | - | - |
| | SbtA | - | ++ | ++ |
| D3/D4/SbtA | Growth | ++ | - | - |
| | NDH-1L | ++ | n.a. | +/- |
| | NDH-1M | +/- | n.a. | + |
| | NDH-1S | - | n.a. | - |
| | SbtA | - | n.a. | - |

+++ , Exceptionally strong; ++ , strong; + , moderate; +/- , poor; - , none (cell bleached); n.a. , not analyzed; * , see Figure 9.

Ndh-Containing Complexes in *Thermosynechococcus elongatus*

The diversity and integrity of the thylakoid Ndh protein complexes was also tested with *T. elongatus*, a cyanobacterial strain known for the stability of the membrane protein complexes and, therefore, generally used for structural analysis and crystallization of membrane complexes. The proteome of the thylakoid membrane protein complexes, isolated from cells grown at high and low CO₂, pH 8.3, is shown in Figure 7. Compared with *Synechocystis* 6803 (Figures 3 to 6), a distinct feature was the integrity of the NDH-1S complex—only one NDH-1S complex,

corresponding to NDH-1S₁, was detected in *T. elongatus*. Also importantly, both the NDH-1M and NDH-1L complexes were present (Figure 7). Moreover, the regulation of the abundances of these complexes by CO₂ was similar to that in *Synechocystis* 6803, the NDH-1M complex being the dominating one under low CO₂ conditions.

Localization of SbtA and the Ndh Proteins to the Plasma and the Thylakoid Membrane

To localize different NDH-1 complexes and the SbtA proteins to the thylakoid and the plasma membrane of *Synechocystis* 6803, we took advantage of the two-phase partitioning system in purification of the two membrane compartments. As a criterion for the purity of the plasma and thylakoid membranes, we probed the membrane fractions with the plasma membrane-specific NrtA and the thylakoid membrane-specific CP43 antibodies (Norling et al., 1998). As shown in Figure 8, the obtained membrane fractions were pure, and indeed the marker proteins were exclusively present in the plasma membrane (NrtA) or the thylakoid membrane (CP43). All tested Ndh proteins, NdhD3, NdhF3, NdhJ, and NdhK, were detected only in the thylakoid membrane fraction, as previously reported also for the NdhH protein (Ohkawa et al., 2001). On the other hand, SbtA was recorded solely from the plasma membrane fraction. Thus, SbtA observed in the crude thylakoid preparation both here and in our previous article (Herranen et al., 2004) is because of cytoplasmic membrane contamination. Indeed, there seems to be a strict compartmentalization of all Ndh proteins to the thylakoid membrane and the Na⁺/HCO₃⁻ transporter SbtA to the plasma membrane.

Abundance of Distinct Ndh Proteins and SbtA in *Synechocystis* Membranes of Different Strains in Response to Growth Conditions

Because of different membrane locations of the Ndh and SbtA proteins, the comparison of the abundances of these proteins in various *Synechocystis* 6803 strains acclimated to high and low CO₂, pH either 7.5 or 8.3, was performed using total (thylakoid plus plasma) membrane fractions isolated from the wild type, M55, ΔNdhD3, ΔNdhD4, ΔNdhD3/D4, and ΔNdhD3/D4/SbtA. Immunoblots (Figure 9) were made based on the assumption that NdhD3/F3 proteins represent the NDH-1S complex, NdhF1 the NDH-1L complex, NdhK stands for both the NDH-1L and NDH-1M complexes, and SbtA for the HCO₃⁻ transporter in cellular membranes.

When the strains were grown at high CO₂, pH 7.5 (Figure 9, lane 1), NdhK was the only protein detected in all strains except for M55. Upon CO₂ downshift (Figure 9, lanes 2 and 3), the strains reacted in different ways. In wild-type cells, the expression of

Figure 5. (continued).

Major PSII proteins CP47, CP43, D2, and D1 present in PSII monomers and dimers, identified by MALDI (data not shown), are indicated to the left. Membranes were isolated from cells grown under high CO₂, pH 7.5, and after a CO₂ downshift at pH 7.5 and 8.3 for 24 h. The NdhK protein in NDH-1L and NDH-1M complexes, CupA in the NDH-1S₁ complex, and SbtA are indicated by arrows. CP47 released from the PSII monomer in M55 cells shifted to low CO₂, pH 7.5, was identified with mass spectrometry (data not shown) and is likewise indicated by an arrow.

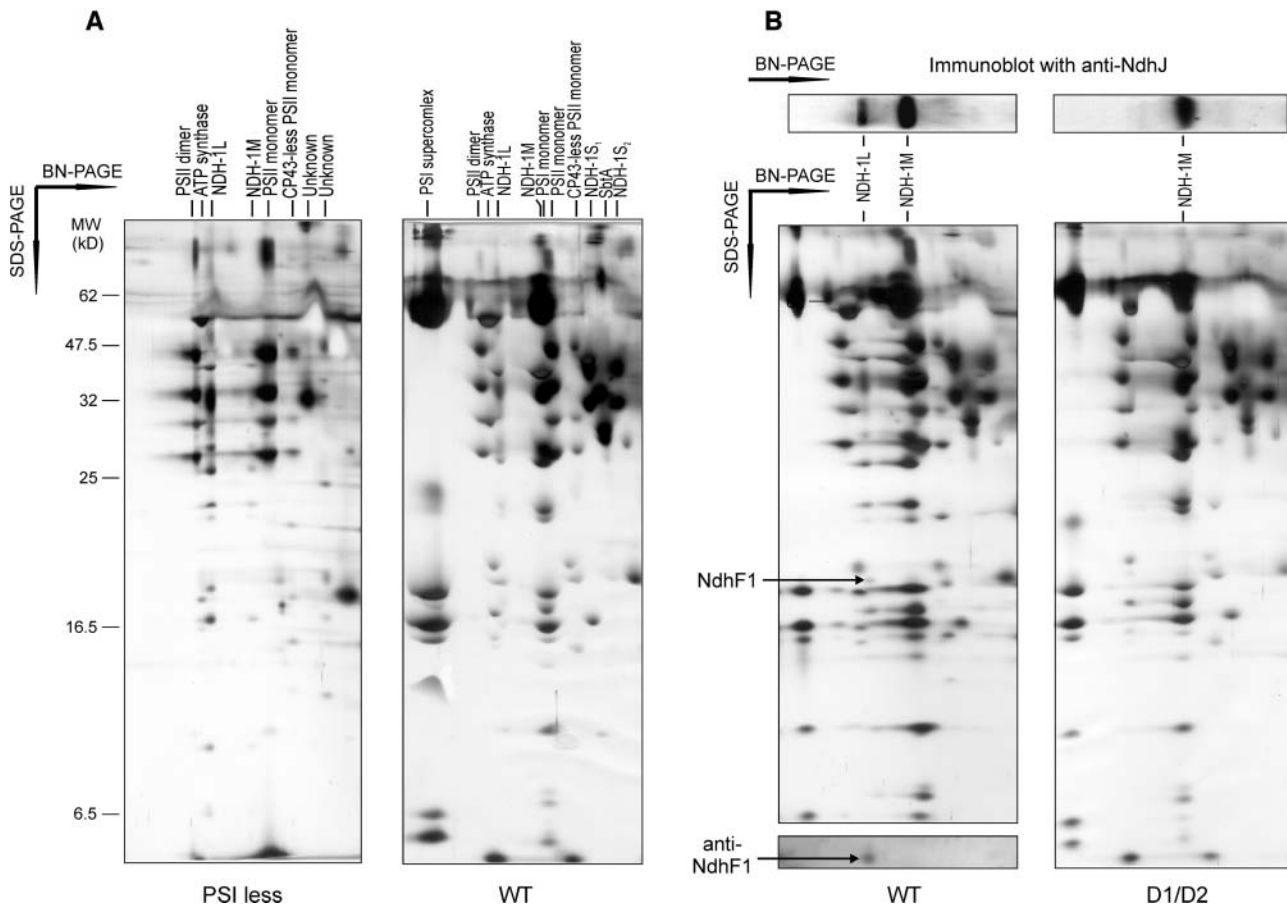


Figure 6. Proteomes of the Membrane Protein Complexes of the PSI-Less Mutant and the Δ NdhD1/D2 Mutant as Compared with the Wild-Type Strain Grown under Similar Conditions.

(A) The wild type and the PSI-less mutant were grown in BG-11 medium supplemented with 5 mM glucose at low CO_2 , pH 7.5, $5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The crude thylakoid membrane fraction was isolated and subjected to 2-D BN/SDS-PAGE, and the gel was stained with silver.

(B) The wild type and the Δ NdhD1/D2 mutant were grown at low CO_2 , pH 7.5, and $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. On the top of the silver-stained 2-D gels is shown an immunoblot of one-dimensional BN gel probed with anti-NdhJ to demonstrate the locations and abundances of the NDH-1L and NDH-1M complexes in silver-stained gels below, prepared after 2-D BN/SDS-PAGE. Below wild-type membranes is shown an NdhF1 immunoblot after 2-D BN/SDS-PAGE. Arrows indicate the spot reacting with anti-NdhF1.

NdhD3, NdhF3, NdhK, and SbtA was enhanced irrespectively of the pH of the growth medium (Figure 9, wild type, lanes 2 and 3), whereas a drastic decrease in the NdhF1 subunit took place upon CO_2 downshift.

In M55, the response to low CO_2 was strongly dependent on pH. The induction of NdhD3, NdhF3, and SbtA was weak but detectable at pH 7.5 (Figure 9, M55, lane 2), in contrast with pH 8.3, where NdhD3 and NdhF3 were upregulated to wild-type levels while the accumulation of SbtA was exceptionally strong (Figure 9, M55, lane 3). The Δ NdhD3 and Δ NdhD4 mutants behaved upon CO_2 downshift in a pH-independent manner showing an upregulation of NdhK and SbtA and a downregulation of NdhF1 similar to the wild type (Figure 9, D3 and D4, lanes 2 and 3). NdhD3 and NdhF3 proteins, which belong to the same NDH-1S complex, were both absent from the Δ NdhD3 mutant (Figure 9, D3, lanes 2 and 3) but present in the Δ NdhD4 mutant on wild-type levels (Figure 9, D4, lanes 2 and 3). The Δ NdhD3/D4 double

mutant, lacking the NdhD3 and NdhF3 proteins, showed a lower level of NdhK accumulation at pH 7.5 compared with pH 8.3 and a more abundant accumulation of SbtA at CO_2 downshift as compared with wild-type membranes (Figure 9, D3 and D4, lanes 2 and 3). The triple mutant Δ NdhD3/D4/SbtA showed a response only with the NdhK antibody without any upregulation at CO_2 downshift (Figure 9, D3/D4/SbtA), and this mutant eventually bleached and died in the course of incubation at low CO_2 concentration.

DISCUSSION

Composition and Location of the Ndh- and SbtA-Containing Protein Complexes in *Synechocystis* 6803

Based on extensive reverse genetics studies, it has been postulated that different forms of NDH-1 complexes reside in

Table 2. Half-Time of P700⁺ Reduction in Darkness after Far Red Light Illumination of Wild-Type, Δ NdhD1/D2, and M55 (Δ NdhB) Mutants Grown at High and Low CO₂

| Strain | Growth Conditions | t _{1/2} of P700 ⁺ Reduction |
|-------------------|----------------------|---|
| Wild type | Low CO ₂ | 252 ms ± 29 ms |
| Δ NdhD1/D2 | Low CO ₂ | 220 ms ± 32 ms |
| Wild type | High CO ₂ | 529 ms ± 31 ms |
| M55 | High CO ₂ | 2162 ms ± 154 ms |

Measurements were made at 26.5 W m⁻² far red light with maximum at 715 nm. Results are a mean ± SE of at least three independent cultures.

Synechocystis 6803 membranes (Ohkawa et al., 1998; Price et al., 1998; Klughammer et al., 1999). These NDH-1 complexes were hypothesized to contain the same Ndh single copy gene products but different members of the NdhD/F family. NdhD3 and NdhF3 as well as NdhD4 and NdhF4 were hypothesized to form, together with the single copy gene products, two specific NDH-1 complexes functioning in inducible and constitutive CO₂ transport, respectively. NdhD1/D2 and NdhF1, on the other hand, were postulated to be components of the NDH-1 complexes involved in respiration and cyclic electron flow around PSI (Ohkawa et al., 2000a). However, the structural basis of the Ndh-containing complexes has turned out to be much more diverse (Herranen et al., 2004), which prompted us to investigate distinct *ndh* gene deletion mutants to specify the diversity of the NDH-1 complexes and their coexpression with Ci acquisition complexes in *Synechocystis* 6803 membranes under different growth conditions.

Four different Ndh-containing complexes, all localized to the thylakoid membrane, were present in *Synechocystis* 6803 cells grown at low CO₂: the NDH-1L (large, ~490 kD), NDH-1M (medium size, ~350 kD), and the NDH-1S₁ and NDH-1S₂ (small, ~200 and 140 kD, respectively) complexes (Figure 2, the mass estimations were based on the known mass of the PSII, PSI, cytochrome b₆f, and ATPase). Analysis of the protein complexes in the second dimension by SDS-PAGE (Figures 3A and 3B) revealed more than 10 protein spots in NDH-1L and NDH-1M, and their patterns imply structural similarity between these two complexes. The presence of the NdhH, NdhI, NdhJ, and NdhK proteins in these complexes was verified (Figures 2 and 3; Herranen et al., 2004). Therefore, both NDH-1L and NDH-1M contain the subunits homologous to NuoB, -C, -D, and -I comprising the interconnecting module of the *E. coli* NDH-1 complex (Leif et al., 1995; Holt et al., 2003), which is presumed to connect the membrane module of the complex with catalytically active subunits still unknown in cyanobacteria. Furthermore, the absence of both NDH-1L and NDH-1M from M55 strain lacking the functional *ndhB* gene (Figures 2A to 2C, 3A, and 3B) implies that the membrane protein NdhB (NuoN) is also an intrinsic subunit of both NDH-1L and NDH-1M. Two other hydrophobic membrane subunits, NdhD1(D2) (NuoM) and NdhF1 (NuoL), on the other hand, were present only in the NDH-1L complex (Figure 6B). Our NDH-1L complex most probably corresponds to the NDH-1 complex recently isolated from *Synechocystis* 6803 cells with 10 identified Ndh subunits (including NdhD1 and the C terminus of NdhF1) and two previously unidentified subunits

(Prommeenate et al., 2004). The NDH-1M complex, on the other hand, lacks the NdhD1(D2) and the NdhF1 subunits but otherwise seems to be identical in the subunit composition with the NDH-1L complex (N. Battchikova, P. Zhang, S. Rudd, T. Ogawa, and E.-M. Aro, unpublished data). In overstained gels, we detected 10 subunits in NDH-1M, and they all were present also in NDH-1L, probably corresponding to NdhA, B, C, G, H, I, J, K, and two novel subunits, as reported by Prommeenate et al. (2004).

Contrary to NDH-1M and NDH-1L, NDH-1S₁ is a small complex with a simple protein composition comprising NdhD3 (Figure 2B), NdhF3, CupA (Figure 4), and SII1735 proteins, whereas NDH-1S₂ is composed of only NdhD3 and NdhF3 (Herranen et al., 2004). The presence of only the NDH-1S₁ complex in *T. elongatus* strongly suggests that the NDH-1S₂ complex easily disassembles from NDH-1S₁ in *Synechocystis* 6803. Hereafter we refer to these two complexes in *Synechocystis* 6803 collectively as the NDH-1S complex. This complex functions in CO₂ uptake and is strongly induced by low CO₂ conditions (Figures 2 and 5).

Another low CO₂-induced membrane complex of ~160 kD (Figures 2A and 2C) was localized to the plasma membrane and was composed of SbtA proteins of slightly varying molecular masses, which might be an indication of protein posttranslational modifications. Also, the ABC type bicarbonate transporter (BCT1) has been localized to the plasma membrane (Omata

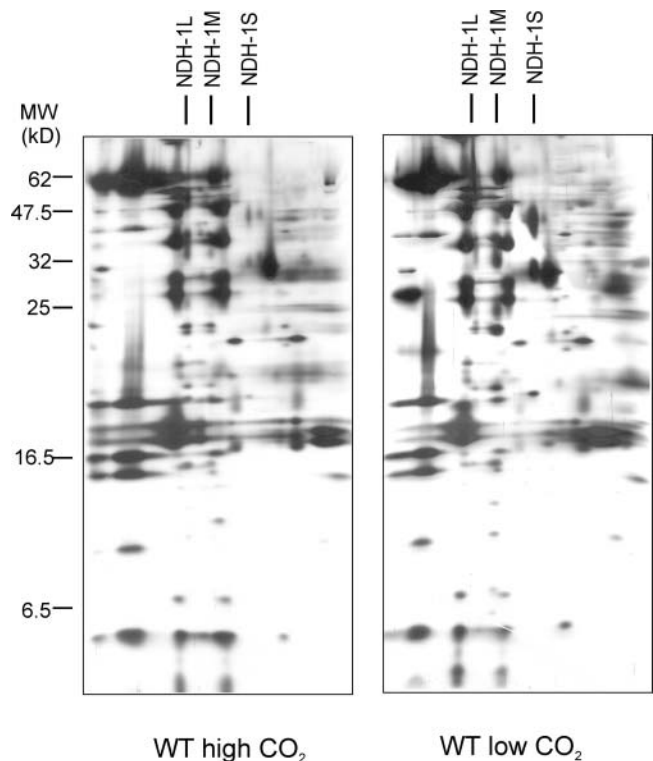


Figure 7. Two-Dimensional Analysis of Membrane Protein Complexes from *T. elongatus* Cells Grown at High and Low CO₂, pH 8.3.

NDH-1L, NDH-1M, and NDH-1S complexes are marked on the top of the gel.

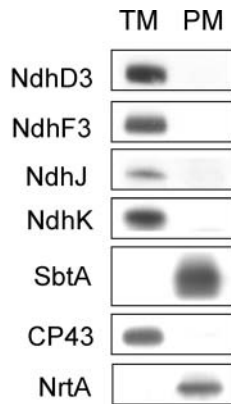


Figure 8. Location of the Various Carbon Acquisition Systems and NDH-1 Complexes in the Thylakoid Membrane and the Plasma Membrane.

The purified membrane fractions were obtained by sucrose density fractionation and subsequent purification of the thylakoid and plasma membranes in the two-phase partitioning system composed of dextran and polyethylene glycol. CP43 and NrtA were used as markers for the purity of the plasma and the thylakoid membrane fractions, respectively. Anti-NdhD3 and anti-NdhF3 were used to localize the marker proteins of the NDH-1S complexes and anti-NdhJ and anti-NdhK the marker proteins of the NDH-1L and NDH-1M complexes to the thylakoid membrane, whereas Anti-SbtA localizes the $\text{Na}^+/\text{HCO}_3^-$ transporter to the plasma membrane.

and Ogawa, 1986). It is therefore evident that from the inducible Ci acquisition complexes, the bicarbonate transporters function in the plasma membrane, whereas the inducible CO_2 uptake system (NDH-1S) and the typical multisubunit NDH-1 complexes (NDH-1L and NDH-1M) are specific for the thylakoid membrane.

Both NDH-1S and NDH-1M Complexes Are Involved in Inducible CO_2 Uptake

In wild-type cells, the expression of the NDH-1S complex starts at CO_2 downshift and closely coincides with the upregulation of the NDH-1M complex. Such coexpression might suggest also functional cooperation between the NDH-1S and NDH-1M complexes. Under particular conditions, the NDH-1S complex was, however, expressed also independently of both NDH-1L and NDH-1M. This was unambiguously demonstrated in M55 cells, which despite the absence of both the NDH-1L and NDH-1M complex, accumulated NDH-1S upon CO_2 downshift (Figure 5). Conversely, a strong expression of the NDH-1M complex was characteristic to the $\Delta\text{NdhD3/D4}$ mutant completely devoid of the NDH-1S complex (Figure 5). Common to both the M55 and $\Delta\text{NdhD3/D4}$ mutant was a suppression of growth at low CO_2 , pH 7.5 (Figure 1) as a result of inefficient CO_2 uptake (Shibata et al., 2001). Thus, both the NDH-1S and NDH-1M complexes are essential for inducible CO_2 uptake and cell survival at low CO_2 and low pH. At high pH, on the other hand, Ci mainly occurs in a form of bicarbonate ions that are efficiently taken up by the SbtA transporter, which was strongly induced both in M55 and $\Delta\text{NdhD3/D4}$ mutants making the growth possible.

It was recently demonstrated by a genome-wide DNA microarray analysis that the *ndhF3/ndhD3/cupA* operon and the *sbtA* gene are both upregulated as a result of inactivation of the *ndhR* gene, a LysR family regulator of Ci uptake (Wang et al., 2004). It is conceivable that Ci availability and associated pH homeostasis under given growth conditions regulate the *ndhR* gene expression, which in turn coordinately controls the expression of both the CO_2 and HCO_3^- uptake systems. In this regard, it is interesting that the single copy *ndh* genes, whose products constitute the NDH-1M complex, are not under a direct control of NdhR (Wang et al., 2004), thus possibly allowing, when

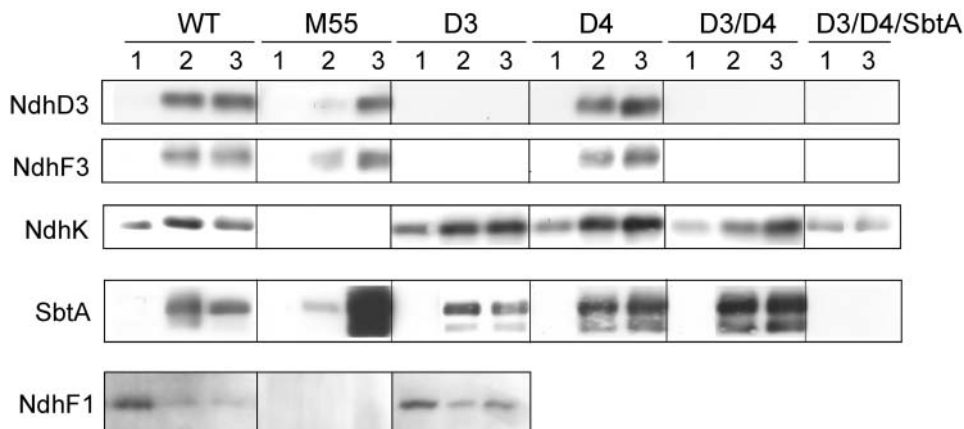


Figure 9. Immunoblots Demonstrating the Accumulation of the NdhD3, NdhF3, NdhF1, NdhK, and SbtA Proteins in the Total Membrane Fractions of Synechocystis Wild Type and Several Ci Acquisition Mutant Strains.

The cells were first grown at high CO_2 (lane 1) and then shifted to low CO_2 at pH 7.5 (lane 2) or 8.3 (lane 3) for 24 h before isolation of the total membrane fractions of the cells. To work on the linear region of the immunoresponse with different antibodies, 5 μg of membrane proteins were loaded in the well for detection with SbtA antibody, 20 μg protein for detection with the NdhK, NdhD3, and NdhF3 antibodies, and 40 μg protein for detection with the NdhF1 antibody.

appropriate, an independent expression of the CO₂ uptake (NDH-1S) complex from NDH-1M. Generally at CO₂ downshift, the upregulation is particularly prominent for genes directly involved in Ci acquisition (the *ndhF3/ndhD3/cupA* operon and the *sbtA* gene) but also occurs for the single copy *ndh* genes (Wang et al., 2004), whose products constitute the NDH-1M complex, thus being in accordance with the abundance of both the NDH-1S and NDH-1M complexes at low CO₂ conditions (Figure 5). Interestingly, the *ndhD1(D2)* and *ndhF1* genes rather respond negatively to CO₂ downshift (Wang et al., 2004), which in turn is reflected in a low abundance of the NdhF1 protein (Figure 9) and the NDH-1L complex at low CO₂ conditions (Figure 5).

NDH-1M and NDH-1L Are Different Protein Complexes with Distinct Functions

As discussed above, the transfer of *Synechocystis* cells from high to low CO₂ induced an upregulation of the NDH-1M complex, particularly in the wild type and the Δ NdhD3, Δ NdhD4, and Δ NdhD3/D4 mutants (Figures 2 and 5, Table 1), whereas the contents of NDH-1L were downregulated. It has been reported previously that the shift of cyanobacterial cells to low CO₂ also increases cyclic electron flow around PSI (Deng et al., 2003; Table 2 for wild-type cells). NDH-1-mediated cyclic PSI electron transfer was first reported in cyanobacteria (Ogawa, 1991; Mi et al., 1995), and recently this pathway was shown to be essential for efficient photosynthesis also in plant chloroplasts (Munekage et al., 2004). It is conceivable that in *Synechocystis* 6803 the NDH-1M complex, which showed distinct upregulation at low CO₂, is specifically involved in cyclic PSI. Therefore, we analyzed the capacity for PSI cyclic electron flow (rereduction of P700⁺ in darkness) in the Δ NdhD1/D2 mutant lacking the NDH-1L complex but having a prominent NDH-1M complex at low CO₂ (Figure 6B). The Δ NdhD1/D2 mutant showed wild-type rates of P700⁺ rereduction at low pH and low CO₂ (Table 2), and the same mutant was previously shown also to have wild-type levels of CO₂ uptake under similar growth conditions (Ohkawa et al., 2000a). Thus, the upregulation of PSI cyclic electron transfer is likely to be energetically important for inducible CO₂ uptake systems in cells grown at low CO₂ (Tchernov et al., 2001).

In the absence of the NDH-1M and NDH-1L complexes (M55 strain), the cells died upon the CO₂ downshift at pH 7.5. This was probably because of oxidative stress resulting from the failure of the cells in CO₂ uptake in the absence of NDH-1M (Figure 5), thereby limiting the intracellular contents of the terminal photosynthetic electron acceptor CO₂ and hence inducing the production of active oxygen species. It is conceivable that the function of NDH-1M is important for upregulation and function of NDH-1S complexes at low pH (7.5), whereas at elevated pH (8.3) the upregulation of both the SbtA and NDH-1S complexes occurs independently of NDH-1M, as was shown here with the M55 cells. The function of NDH-1M elevates cytosolic pH, which seems to be essential for upregulation of Ci transporters (Shibata et al., 2002) at neutral pH of the growth medium, whereas the growth of cells at higher pH probably modulates the intracellular pH independently of the function of the NDH-1M and NDH-1L complexes, resulting in sustained growth of also the M55 cells. Thus, the NDH-1M-supported cyclic electron flow around PSI is

probably essential for CO₂ uptake both in modifying the cytosolic pH suitable for upregulation of NDH-1S at low pH of the growth medium and for providing energy for the function of the Ci acquisition systems.

Proteome studies of the wild type and various *ndh* gene knockout mutants of *Synechocystis* 6803 demonstrated that the NDH-1L complex is generally expressed under all growth conditions (Figure 5). The Δ NdhD1/D2 mutant, lacking the NDH-1L complex (Figure 6B), exhibits wild-type levels of cyclic electron flow (Table 2) and reduced rates of respiration and is not capable of photoheterotrophic growth in the presence of glucose and DCMU (Ohkawa et al., 2000a; confirmed by us, data not shown). These phenotypes of the NdhD1/D2 mutant strongly suggest the role of the NDH-1L complex in cellular respiration. Because the respiratory pathways in *Synechocystis* 6803 are probably very complex (e.g., Cooley and Vermaas, 2001), the exact function, either direct or indirect, of the NDH-1L complex in cellular respiration is difficult to assess. Studies with the PSI-less mutant further supported the involvement of NDH-1L in cellular respiration. This mutant, capable of only heterotrophic growth in the presence of glucose (Shen et al., 1993), thus strongly relying on respiration, showed a distinguished expression of the NDH-1L complex in the thylakoid membrane (Figure 6A). It is presently not clear whether the NdhD1(D2) and NdhF1 subunits as such confer the specificity of NDH-1L and NDH-1M to respiratory and cyclic electron flow, respectively, or whether posttranslational modifications of the Ndh subunits are possibly involved as well.

We conclude that the specific low CO₂-inducible CO₂ uptake complex in *Synechocystis* 6803, composed of the NdhD3, NdhF3, CupA, and Sll1735 proteins, is located exclusively in the thylakoid membrane and is functionally dependent on the NDH-1M complex. The NDH-1M complex contains single gene copy Ndh proteins, components of both the hydrophilic and the membrane domains of the NDH-1 complexes, whereas NDH-1L additionally comprises the NdhD1/D2 and the NdhF1 subunits. Both NDH-1M and NDH-1L are located in the thylakoid membrane. NDH-1M is capable of fast rereduction of P700⁺ in darkness and is strongly coexpressed with the NDH-1S complex, suggesting that NDH-1M fuels the thylakoid-associated CO₂ uptake systems. NDH-1M might function as a ferredoxin-plastoquinone oxidoreductase with less complicated structural requirements as compared with respiratory NDH-1 complexes using NAD(P)H as an electron source (Sapra et al., 2003). Only the NDH-1L complex, composed of the most complete set of *ndh* gene products, also including the NdhD1/D2 protein and the NdhF1 subunit, is capable of supporting photoheterotrophic growth of *Synechocystis* 6803, yet the electron donation domain still remains unknown. It will be interesting to find out whether any differentiation of the NDH-1 complexes occurs in plant chloroplasts, possibly being differentially involved in cyclic PSI electron flow and chlororespiration.

METHODS

Cell Culture Conditions

Synechocystis 6803 glucose tolerant strain (wild type) and the *ndh* gene inactivation mutants Δ ndhB (M55), Δ ndhD3, Δ ndhD4, Δ ndhD3/ndhD4,

Δ ndhD3/D4/sbtA, and Δ ndhD1/ndhD2 (Ogawa, 1991; Ohkawa et al., 2000a; Shibata et al., 2002) were grown in BG-11 medium (Williams, 1988) at 32°C under 50 μ mol photons $m^{-2} s^{-1}$ in 200-mL batch cultures under gentle agitation. The Δ PSI mutant (Shen et al., 1993) was grown in BG-11 medium supplemented with 5 mM glucose at 32°C under 5 μ mol photons $m^{-2} s^{-1}$. The mutant strains were grown in the presence of appropriate antibiotics. The experimental conditions used for culturing *Synechocystis* wild-type and mutant strains were as follows: high CO₂ (3% CO₂ in air) at pH 7.5 (buffered with 20 mM Hepes-NaOH) and low CO₂ (air level) both at pH 7.5 and 8.3 (buffered with 20 mM Tes-KOH).

Thermosynechococcus elongatus BP1 was grown in BG-11 medium at 50°C under 50 μ mol photons $m^{-2} s^{-1}$.

Isolation of Cyanobacterial Membranes

Isolation of the Total Membrane Fraction

The cells (1 liter cultures) were harvested when the cultures had reached the optical density of 1.2 at 730 nm and were washed and resuspended in 3 mL of disruption buffer (20 mM potassium phosphate, pH 7.8). Glass beads (150 to 212 μ m) were added to the cell suspension, and the cells were broken by vortexing three times at the highest speed for 2 min with 1 min cooling on ice between the runs. To remove the glass beads, the sample was centrifuged at 2000g for 10 min, and the membranes were subsequently collected by ultracentrifugation at 150,000g for 40 min.

Isolation of Crude Thylakoid Membranes

The cell cultures (200 mL) were harvested at the logarithmic phase and washed twice by suspending in 20 mL of washing buffer (50 mM Hepes-NaOH, pH 7.5, and 30 mM CaCl₂), and the thylakoids were isolated according to Gombos et al. (1994) as follows. The cells suspended in 2 mL of isolation buffer (50 mM Hepes-NaOH, pH 7.5, 30 mM CaCl₂, 800 mM sorbitol, and 1 mM ϵ -amino-*n*-caproic acid) were supplemented by glass beads and disrupted by vortexing eight times at the highest speed for 1 min at 4°C with 1 min cooling on ice between the runs. The crude extract was centrifuged at 3000g for 5 min to remove the glass beads and unbroken cells. Membranes were pelleted by centrifugation at 17,000g for 20 min and resuspended in storage buffer (50 mM Tricine-NaOH, pH 7.5, 600 mM sucrose, 30 mM CaCl₂, and 1 M glycinebetaine).

Aqueous Polymer Two-Phase Partitioning of the Plasma and Thylakoid Membranes

Plasma and thylakoid membranes were isolated from *Synechocystis* 6803 cells by aqueous polymer two-phase partitioning. In this process, the total *Synechocystis* membrane pellet (isolated as described above) was first fractionated by sucrose density gradient centrifugation and thereafter, according to the surface properties of the membrane fractions, by two-phase partitioning using the polymers Dextran T-500 and PEG 3350 (Norling et al., 1998; Jansén et al., 2002). CP43 and NrtA proteins were used as markers of the purity of the thylakoid and the plasma membranes (Norling et al., 1998).

Electrophoresis and Immunoblotting

The BN-PAGE of *Synechocystis* 6803 membranes was performed basically as described earlier (Kügler et al., 1997) with modifications from Cline and Mori (2001) and Herranen et al. (2004).

Isolated membranes were prepared for BN-PAGE as follows. Membranes were washed with 330 mM sorbitol, 50 mM Bis-Tris, pH 7.0, and 250 μ g/mL of pefabloc and subsequently suspended in 20% glycerol (w/v), 25 mM Bis-Tris, pH 7.0, 10 mM MgCl₂, and 0.01 unit/ μ L RNase-Free

DNase RQ1 (Promega, Madison, WI) at the final concentration of 20 μ g protein/ μ L. The samples were incubated on ice for 10 min, and the equal volume of 3% *n*-dodecyl- β -D-maltoside was added. Solubilization was performed for 10 min on ice followed by incubation at room temperature for 20 min. Insoluble material was removed by centrifugation at 18,000g for 15 min. The collected supernatant was mixed with one-tenth volume of 0.1 M EDTA and one-tenth volume of sample buffer (5% Serva blue G, 200 mM Bis-Tris, pH 7.0, 75% sucrose, and 1 M ϵ -amino-*n*-caproic acid) and applied to 0.75-mm-thick 5 to 12.5% acrylamide gradient gel (Hoefer Mighty Small mini-vertical unit; San Francisco, CA). Samples were loaded on an equal protein basis of 150 μ g per well. Electrophoresis was performed at 4°C by increasing voltage gradually from 50 V up to 200 V during the 5.5-h run.

For electrophoresis in the second dimension, a lane of the BN gel was cut out and incubated in Laemmli SDS sample buffer containing 5% β -mercaptoethanol and 6 M urea for 1 h at 23°C. The lane was then laid onto a 1-mm-thick 14% SDS-PAGE gel with 6 M urea (Laemmli, 1970). After electrophoresis, the proteins were visualized by silver staining (Blum et al., 1987).

For immunoblotting, the proteins were electrotransferred to a PVDF membrane (Immobilon P; Millipore, Bedford, MA) and detected by protein-specific antibodies using the CDP-Star chemiluminescent detection kit (New England Biolabs, Beverly, MA). The NdhD3 antibody was prepared against amino acids 185 to 196 and 346 to 359, the NdhF3 antibody against amino acids 28 to 41 and 439 to 453, and the NdhF1 antibody against amino acids 495 to 509 and 610 to 624 of the respective proteins of *Synechocystis* 6803 (Eurogentec, Seraing, Belgium). SbtA antibody was prepared against amino acids 184 to 203. The antibody for NrtA (a subunit of an ABC-type nitrate transporter located in the plasma membrane) was provided by B. Norling (Stockholm University, Sweden). The antibody for CP43 (a chlorophyll *a* binding protein located in the thylakoid membrane) was obtained from R. Barbato (University of Piemonte Orientale, Alessandria, Italy) and the NdhJ and NdhK antibodies from J. Appel (Institute of Botany, Kiel, Germany) and P. Nixon (Imperial College London, UK), respectively.

Identification of Proteins by MALDI-TOF

Silver-stained protein spots were excised from gels and digested with modified porcine trypsin (Promega) according to Shevchenko et al. (1996). Trypsin digests were concentrated and purified from salts using self-made reverse-phase POROS R3 (Perseptive Biosystems, Framingham, MA) microcolumns (Gobom et al., 1999). Peptides were eluted from a column directly onto the MALDI plate using a solution of α -cyano-4-hydroxycinnamic acid (10 mg/mL) in 60% acetonitrile and 0.3% trifluoroacetic acid. MALDI-TOF analysis was performed in reflector mode on the Voyager-DE PRO mass spectrometer (Applied Biosystems, Foster City, CA). Calibration of spectra was based on masses of trypsin autodigestion products (842.510, 1045.564, and 2211.105 D). Proteins were identified by searching in the National Center for Biotechnology Information database using Mascot (www.matrixscience.com). The search parameters allowed for carbamidomethylation of cysteine, one miscleavage of trypsin, and 50 ppm mass accuracy.

Determination of Protein Concentration

Protein was determined using a DC (detergent-compatible) protein assay kit (Bio-Rad, Hercules, CA).

Measurement of P700⁺ Rereduction Rate

P700⁺ reduction was measured as an absorption change at 820 nm as described by Appel et al. (2000). The first order kinetics of P700⁺

reduction was determined in the dark after illumination of cells with 26.5 W m⁻² of far red light with a maximum at 715 nm.

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