The subunit compositions of two types of NAD(P)H dehydrogenase complexes of *Synechocystis* sp. PCC 6803, NDH-1L and NDH-1M, were studied by two-dimensional blue-native/SDS-PAGE followed by electrospray tandem mass spectrometry. Fifteen proteins were observed in NDH-1L including hydrophilic subunits (NdhH, -I, -J, -M, and -N) and hydrophobic subunits (NdhA, -B, -E, -G, -D1, and -F1). In addition, NdhL and a novel subunit, Ssl1690 (designated NdhO), were shown to be components of this complex. All subunits mentioned above were present in the NDH-1M complex except NdhD1 and NdhF1. NdhL and Ssl1690 (NdhO) were homologous to hypothetical proteins encoded by genomic DNA in higher plants, suggesting that chloroplast NDH-1 complexes contain related subunits. Diagnostic sequence motifs were found for both NdhL and NdhO homologous proteins. Analysis of ndhL deletion mutant (M9) revealed the presence of assembled NDH-1L and NDH-1M complexes, but these complexes appear to be functionally impaired in the absence of NdhL. Both NDH-1 complexes were absent in the ndhB deletion mutant (M55).

The proton-pumping NADH-ubiquinone oxidoreductase catalyzes the electron transfer from NADH to ubiquinone linked with proton translocation across the membrane (1). Subunit composition of the enzyme varies among organisms. In complex I originating from mammalian mitochondria, 45 different proteins were discovered (2, 3). In bacteria, the corresponding complex NDH-1 consists of 14 different polypeptides. Homologues of these 14 proteins are found among subunits of the mitochondrial complex I, and therefore bacterial NDH-1 might be considered as a model proton-pumping NADH dehydrogenase with a minimal set of subunits (4). *Escherichia coli* NDH-1 readily disintegrates into 3 subcomplexes: a water-soluble NADH dehydrogenase fragment (NuoE, -F, and -G), the connecting fragment (NuoB, -C, -D, and -I), and the membrane fragment (NuoA, -H, -J, -K, -L, -M, and -N) (5).

In cyanobacteria and their descendents, plast NDH-1 complexes contain related subunits. Diagnostic sequence motifs were found for both NdhL and NdhO homologous proteins. Analysis of ndhL deletion mutant (M9) revealed the presence of assembled NDH-1L and NDH-1M complexes, but these complexes appear to be functionally impaired in the absence of NdhL. Both NDH-1 complexes were absent in the ndhB deletion mutant (M55).
Isolation of Crude Thylakoid Membranes—The cells were harvested at the logarithmic phase, washed twice with 20 ml of washing buffer (50 mM Hepes-NaOH, pH 7.5, 30 mM CaCl2). The thylakoids were isolated according to Combos et al. (20) as follows. The cells were suspended in 2 ml of isolation buffer (50 mM Hepes-NaOH, pH 7.5, 30 mM CaCl2, 800 mM sorbitol, 1 mM e-aminocaproic acid), supplemented by the same volume of glass beads and disrupted by vortexing eight times at the highest speed for 1 min at 4 °C with 1–2 min cooling on ice between runs. The crude extract was centrifuged at 3000 × g for 5 min to remove the glass beads and unbroken cells. Membranes were pelleted by centrifugation at 17,000 g for 20 min and resuspended in storage buffer (50 mM Tricine-NaOH, pH 7.5, 600 mM sucrose, 30 mM CaCl2, 1 mM glycinebetaine).

Separation of NDH-1 Protein Complexes and Their Subunits—The blue-native PAGE (BN-PAGE) of Synechocystis 6803 membranes was performed basically as described earlier (21) with modifications for cyanobacteria (15). Membranes were washed with 330 mM v/v/0, 50 mM BisTris, pH 7.0, 250 µg/ml Pheloablol, and subsequently resolved at the final concentration of 20 µg of protein/ml in 20% glycerol w/v, 25 mM BisTris pH 7.0, 10 mM MgCl2, 0.002 units/ml RNase-Free DNase RQ1 (Promega, Madison, WI). The samples were incubated on ice for 10 min, and the equal volume of 3% dodecyl-β-maltoside in the same buffer was added. Solubilization was performed by incubation on ice for 10 min and followed by temperature for 20 min. Insoluble material was removed by centrifugation at 18,000 × g for 15 min. Solubilized membranes were mixed with 1:10 volume of 0.1 mM EDTA and 1:10 volume of sample buffer (5% Serva blue G, 200 mM BisTris, pH 7.0, 75% sucrose, 1 mM e-aminocaproic acid) and directly applied to 0.75-mm-thick 5–12.5% acrylamide gradient gel (Hoeffer Mighty Small mini-vertical unit) in amounts of ~150 µg of protein per well. Electrophoresis was performed at a constant current of 1000 µA for a total duration of 1 h, or 120 V for 1 h, 175 V for 20 min and 200 V for 1 h. For electrophoresis in the second dimension, a protein lane was laid onto a 1-mm thick 14% SDS-PAGE gel (12). The lane was then run at 150 V for 1 h, 100 V for 1 h, 125 V for 30 min, 150 V for 1 h, 175 V for 1 h, and 200 V for 1 h. For electrophoresis in the second dimension, a lane of the BN gel was cut out and incubated in Laemmli SDS sample buffer supplemented with 5% β-mercaptoethanol and 6 µl urea for 1 h at 23 °C. The lane was then laid onto a 1-mm thick 14% SDS-PAGE gel with 6 µl urea (22). Prestained protein markers, broad range (New England Biolabs, Beverly, MA) were used for estimation of apparent molecular masses of proteins in SDS/PAGE. The proteins were visualized by silver staining (23).

N-terminal Protein Sequencing—Proteins separated by two-dimen- sional BN/SDS-PAGE were electrotransferred to a polyvinylidene difluo- ride membrane (Immobilon P, Millipore), and visualized with Coomas- sie Brilliant Blue R-250. The corresponding protein spots from four gels were excised from the membrane, lyophilized, and subjected to digestion with trypsin autodigestion products (842.510, 1045.564, and 2211.105 Da).

Separation of NDH-1L and NDH-1M Complexes and Their Subunits from Synechocystis 6803—Membrane protein complexes isolated from Synechocystis 6803 cells were separated by BN-PAGE in the first dimension followed by denaturing SDS- PAGE in the second direction. Sections of silver-stained gels containing NDH-1L and NDH-1M complexes are presented in Fig. 1. The identification of the major photosynthetic complex, PSI, PSII, and ATP synthase, has been described in detail by Herranen et al. (15). Approximate molecular masses of NDH-1L and NDH-1M appeared to be ~490 and 350 kDa, respectively, based on the mobility of photosynthetic complexes in BN-PAGE.

In vitro, the relative amounts of NDH-1 complexes varied depending on growth conditions. The abundant NDH-1M complex was the characteristic feature of cells grown photoautotrophically under air level of CO2 (Fig. 1A). In cells grown in high CO2 (3%) the relative amount of NDH-1M drastically decreased. NDH-1L, conversely, was slightly up-regulated in high CO2-grown cells (Fig. 1B).

Protein patterns of NDH-1L and NDH-1M closely resembled each other. Fifteen protein spots were resolved in NDH-1L by BN-PAGE. The subunit composition of NDH-1M was more difficult to access in WT cells since the complex overlapped with the PSI monomer and partially with the PSI monomer. The better resolution of NDH-1M subunits was obtained using the membrane preparation from the PSI-less mutant of Synechocystis 6803 (26) despite the relatively low amount of NDH-1M compared with NDH-1L (data not shown). The diffuse spot in NDH-1L moving at the level of CP43 (PsbC) of PSI corresponded to two unresolved proteins, and the sub- units of NDH-1L were numbered as shown in Fig. 1C. The distinct difference between NDH-1L and NDH-1M was the absence of spots 2 and 8 in the NDH-1M complex.

To exclude the possibility that NDH-1L overlapped with an unknown complex, the membrane preparation of Synechocystis 6803 strain M55 (ΔndhB) (18) grown at high CO2 was analyzed under the same conditions. Both NDH-1L and NDH-1M complexes were absent in this strain (Fig. 1D), and none of the spots depicted in Fig. 1C was detected, demonstrating that all 15 proteins indeed belonged to the NDH-1L complex.

Mass Spectrometry Identification of NDH-1 Subunits—Earlier NDH-1L and NDH-1M were recognized as NDH-1 complex since MALDI-TOF analysis demonstrated the presence of NdhH, -K, -I, and -J subunits in both of them (15). Other
spots failed MALDI identification, most probably because of the scarce amounts of proteins in small subunits or because of hydrophobic properties of Ndh subunits with several transmembrane regions. Here we have used a combination of MALDI-TOF and ESI-QTOF for identification of separated proteins and results are summarized in Table I.

Spots 1, 5, 6, and 7 corresponded to NdhH, -K, -I, and -J, respectively. The proteins did not possess transmembrane regions, and they were efficiently digested by trypsin providing many peptides to ensure the reliable identification by MALDI-TOF MS alone (15). ESI MS/MS analysis was nevertheless performed to exclude the possibility of the existence of co-migrating unknown proteins. Spot 10 corresponded to one of the additional (compared with minimal bacterial complex) subunits Sll1262 (NdhN) recently identified by Prommeenate et al. (14) whereas spot 11 represented Slr1623 (NdhM), another additional subunit originally found by Berger et al. (12). Spots 3, 4, 9, 13, and 15 corresponded to NdhB, -A, -G, -E, and -L, respectively. For these subunits, only a few tryptic peptides were seen in MALDI-TOF spectra, most probably because transmembrane proteins are not effectively cleaved by trypsin and/or highly hydrophobic peptides are lost during sample preparation. However, results obtained by ESI MS/MS allowed the identification of these proteins. For NdhE and NdhL, assigned on the basis of the sequence of a single peptide, the complete series of y ions were obtained allowing unambiguous reading of the whole peptide sequence. Further, the corresponding peptides were also detected in MALDI-TOF spectra (data not shown). The MS/MS spectrum of the NdhL peptide is shown in Fig. 2, the peptide appeared to be modified (formaldehyde adduct of Trp residue) during the silver staining procedure. The NdhL was identified in spot 15 originating from the NDH-1M complex of cells grown under carbon-limiting conditions when the complex is most abundant. Under no growth conditions the NDH-1L complex was expressed as strongly as NDH-1M at low CO2, and therefore the amount of the NdhL protein from this complex was not sufficient for analysis.

There was a clear difference between the protruding spot 2/3 in NDH-1L and spot 3 in NDH-1M. Peptides of only the NdhB

![Figure 1. NDH-1L and NDH-1M complexes in Synechocystis 6803. A, B, D, and E, sections of the two-dimensional BN/SDS-PAGE of the thylakoid membrane protein complexes from cells grown under air level of CO2 (A, WT) or in high CO2 (B, WT; D, M55; E, M9). C, scheme of NDH-1L and NDH-1M subunits. Corresponding Ndh subunits are shown by arrowheads in gels. An unidentified diffuse spot that might represent the N-terminal part of NdhF is marked by an asterisk. Subunits of PSI, PSII, and ATP synthase complexes shown by arrows were described earlier (15) and are indicated only in A.](http://www.jbc.org/)}
Markers.

**Table 1**

Identification of Ndh subunits by mass spectrometry

| Spot no. | Ndh subunit | ORF | Mass app | Mass theor | MALDI-TOF peptides matched | ESI MS/MS⁴ | Observed peptide mass | [M+H]+ theor. |
|----------|-------------|-----|----------|------------|-----------------------------|------------|-----------------------|--------------|
| 4        | NdhA        | slr0519 | 29       | 40.5       | 8                           | YSLLLGLG³  | 439.79, 2+/37         | 878.51       |
|          |             |       |          |            | 756.45                      | LTFP³AFG'aff | 478.72, 2+/25         | 956.46       |
|          |             |       |          |            | 1199.6                      | IDQLNNLW³  | 600.37, 2+/35         | 1199.68      |
|          |             |       |          |            | 1205.55                     | LVFKEDVF³  | 622.89, 2+/52         | 1244.73      |
|          |             |       |          |            | 1994.10                     | GPIEGYAGPLQ³ | 997.60, 2+/36         | 1994.09      |
| 3        | NdhB        | slr0223 | 35       | 55.4       | 14                          | AAAGFAAIR³ | 438.25, 2+/85         | 875.51       |
|          |             |       |          |            | 705.39                      | EPEMQES³   | 595.27, 2+/45         | 1198.58      |
|          |             |       |          |            | 875.50                      | EPEMQES³   | 603.30, 2+/41         | 1205.57      |
|          |             |       |          |            | 1205.55                     | TSGQDSYAGLY³ | 552.26, 2+/70         | 1654.77      |
| 12       | NdhC        | slr1270 | 11       | 13.7       | 3                           | MGYYALRR³  | 443.70, 0+/47         | 880.47       |
| 2        | NdhDI       | slr0331 | 37       | 55.7       | 12                          | 2087.14    |                       |              |
|          |             |       |          |            | 880.48                      |            |                       |              |
| 13       | NdhF        | slr0522 | 8.5      | 11.2       | 3                           |            |                       |              |
| 8        | NdhF1       | slr0644 | 20       | 74.4       | 2                           |            |                       |              |
|          |             | C-term |          |            | 806.43                      |            |                       |              |
|          |             |        |          |            | 1025.50                     |            |                       |              |
|          |             |        |          |            | 1264.55                     |            |                       |              |
|          |             |        |          |            | 1309.71                     |            |                       |              |
|          |             |        |          |            | 2055.17                     |            |                       |              |
| 9        | NdhG        | slr0521 | 19       | 21.5       | 5                           |            |                       |              |
|          |             |        |          |            | 1286.66                     |            |                       |              |
|          |             |        |          |            | 1325.76                     |            |                       |              |
|          |             |        |          |            | 1442.78                     |            |                       |              |
| 1        | NdhH        | slr0281 | 42       | 45.4       | 0                           |            |                       |              |
|          |             |        |          |            | 20 peptides⁶                   |            |                       |              |
| 6        | NdhI        | slr0520 | 25       | 22.2       | 0                           |            |                       |              |
|          |             |        |          |            | 6 peptides⁶                   |            |                       |              |
| 7        | NdhJ        | slr1281 | 23       | 20.6       | 6                           |            |                       |              |
|          |             |        |          |            | 2087.14                     |            |                       |              |
| 5        | NdhK        | slr1280 | 27       | 27.3       | 0                           |            |                       |              |
|          |             |        |          |            | 14 peptides⁶                   |            |                       |              |
| 15       | NdhL        | slr1386 | 7        | 9.3        | 2                           |            |                       |              |
| 11       | NdhM        | slr1625 | 16       | 14.1       | 0                           |            |                       |              |
| 10       | NdhN        | slr1282 | 18       | 17.6       | 0                           |            |                       |              |

⁴ Tompa et al. (15).

NdhA, NdhB, and NdhD1, in spot 2/3 derived from NDH-1L. ESI-QTOF MS/MS identified spot 8 specific for the NDH-1L complex as NdhF1. To explain the discrepancy between the subunits, (NDH-1M. In contrast, both mass spectrometry techniques demonstrated the presence of two proteins,
predicted molecular mass of the NdhF1 (74.4 kDa) and the
apparent molecular mass (20 kDa), spot 8 was N-terminally
sequenced after blotting of the protein from two-dimensional
BN/SDS-PAGE onto a polyvinylidene difluoride membrane.
The sequence obtained (AMXXVXL-) demonstrated that the
NdhF1 was cleaved between Gly482 and Ala483, and spot 8 was
formed by the C-terminal fragment of NdhF1. To further in-
vestigate the occurrence of NdhF1 protein in the NDH-1L
complex, we made an attempt to diminish the probable unspe-
cific proteolysis of NdhF1 by including a mixture of protease
inhibitors (leupeptin, pepstatin, and EDTA) instead of pefab-
lock in all steps from disruption of cells to the SDS-PAGE
loading buffer during preparation of the thylakoid samples. We
could not significantly prevent the proteolysis of NdhF1 and
the C-terminal part of NdhF1 (spot 8) was still prominent.
However, an additional spot of ~60 kDa was observed under
these conditions (Fig. 3). Mass spectrometry analysis revealed
the peptides typical for spot 8 in the spectrum of this protein
(data not shown).

Spot 14 was identified as the unknown protein Ssl1690 by
both MALDI-TOF MS and ESI-MS/MS. Spot 12 failed both
techniques. However, the only peak observed in MALDI-TOF
spectrum (besides trypsin peaks) with m/z of 654.43 might
belong to NdhC subunit (marked with brackets in the Fig. 1C).
High hydrophobicity of NdhC (3 membrane-spanning regions)
might explain the lack of other tryptic peptides suitable for
mass spectrometry analysis.

Sequence Analysis of Ssl1690 and NdhL and Search for
Homologous Proteins in Other Organisms—NdhL of Syneco-
cystis 6803 encoded by ssr1386 contains 80 amino acids. Two
transmembrane helices were found in this protein by TMHMM
ver. 2.0 (27). The BLAST search performed against NCBI data
base demonstrated the presence of homologous sequences in
other cyanobacteria. The alignment of cyanobacterial NdhL
sequences (Fig. 4) revealed 16 identical and 16 conserved
amino acid residues. In order to find related proteins in green
plants, the Synechocystis 6803 NdhL sequence was compared
using the FASTA algorithm (28) to the MIPS Arabidopsis ge-
nome scaffold (29), the TIGR rice genome data base (30) and to
assembled plant unigene sequences from the Sputnik data base
(31). No unambiguous sequence matches were identified, but a
number of related plant proteins did show a weak similarity
over a restricted area of between 53 and 88 amino acids. The
candidate match sequences from plants including the unknown Arabidopsis protein, At1g70760 and the rice protein, 9633.102493, all showed sequence conservation (Fig. 4). The proteins from green plants are larger than cyanobacterial homologues. The chloroplast location was predicted for these Arabidopsis and rice nuclear-encoded proteins by Predotar (32). The alignment of the sequences using DiAlign (33) revealed that there is a motif conserved between the cyanobacteria and the plants. The sequences from the cyanobacteria, the protein sequences from both the Arabidopsis and rice genomes and the plant EST sequence-derived peptides were used to identify possible diagnostic domain description patterns using the Pratt software (34). The best descriptive domain identified was $\text{Y}_{\text{x}} \text{I}_{\text{x}} \text{L}_{\text{x}} \text{P}_{\text{a/L/P/V}} \text{V}_{\text{l/I/V/X}} \text{A/W/Y} \text{I/L/M/X}_{\text{R/W,E_X}} (- F/I/L/V/X/F/Y/X,C_{7/12})$. Multiple sequence alignment of NdhL and homologous proteins from cyanobacteria and green plants. Protein sequences were aligned using the ClustalW program (ww.ebi.ac.uk/clustalw). Sequences from the following organisms are included: Synechocystis sp. PCC 6803 (accession no. NP_488849), Synechococcus elongatus PCC 7902 (AA053559), Thermosynechococcus elongatus BP-1 (NP_681485), Nostoc sp. PCC 7120 (NP_574985), Arabidopsis thaliana (AAQ96960), and Oryza sativa (9633.102493, MIPS rice data base). N-terminal sequences of proteins from green plants are omitted from the alignment. Asterisks indicate identical amino acids; colons and dots indicate conserved amino acid substitutions. Transmembrane regions predicted by TMHMM are shown by arrows. Top, for NdhL of Synechocystis 6803, bottom, for At1g70760.

**Fig. 4** Multiple sequence alignment of NdhL and homologous proteins from cyanobacteria and green plants. Protein sequences were aligned using the ClustalW program (ww.ebi.ac.uk/clustalw). Sequences from the following organisms are included: Synechocystis sp. PCC 6803 (accession no. NP_441443), Synechococcus elongatus PCC 7902 (AA053559), Thermosynechococcus elongatus BP-1 (NP_681485), Nostoc sp. PCC 7120 (NP_574985), Arabidopsis thaliana (AAQ96960), and Oryza sativa (9633.102493, MIPS rice data base). N-terminal sequences of proteins from green plants are omitted from the alignment. Asterisks indicate identical amino acids; colons and dots indicate conserved amino acid substitutions. Transmembrane regions predicted by TMHMM are shown by arrows. Top, for NdhL of Synechocystis 6803, bottom, for At1g70760.

The occurrence of NDH-1 complexes in cyanobacteria and the plastidial NDH-1 complex of green plants function not only as a respiratory/chlororespiratory complex like in a majority of other organisms but also participate in cyclic electron flow around photosystem I (PSI) (35–37). Based on genome studies, it was predicted that eleven subunits are similar between NDH-1 of E. coli and the enzymes located in thylakoid membranes of photosynthetic organisms. Since the complexity of the enzyme varies between organisms, it was plausible to presume that additional subunits exist in NDH-1 of cyanobacteria and chloroplasts compared with minimal NDH-1 of E. coli.

**Fig. 2** Comparison of this pattern against the SwissProt and TrEMBL databases using the Prosite tools resulted in identification of representative sequences from Synechocystis 6803, Arabidopsis, and Prochlorococcus marinus. By utilizing EST data we have characterized this domain within arsides, rosids, caryophyllids, and monocots. Thus, the pattern was present within both cyanobacterial and plant sequences, and no evidence for this domain was found within other organisms.

**Fig. 3** The novel Ndh subunit, Ssl1690, consists of 72 amino acids. Topology prediction recognized Ssl1690 as a hydrophilic protein, At1g70760 and the rice protein, 9633.102493, all showed sequence conservation (Fig. 4). The proteins from green plants are larger than cyanobacterial homologues. The chloroplast location was predicted for these Arabidopsis and rice nuclear-encoded proteins by Predotar (32). The alignment of the sequences using DiAlign (33) revealed that there is a motif conserved between the cyanobacteria and the plants. The sequences from the cyanobacteria, the protein sequences from both the Arabidopsis and rice genomes and the plant EST sequence-derived peptides were used to identify possible diagnostic domain description patterns using the Pratt software (34). The best descriptive domain identified was $\text{Y}_{\text{x}} \text{I}_{\text{x}} \text{L}_{\text{x}} \text{P}_{\text{a/L/P/V}} \text{V}_{\text{l/I/V/X}} \text{A/W/Y} \text{I/L/M/X}_{\text{R/W,E_X}} (- F/I/L/V/X/F/Y/X,C_{7/12})$. Multiple sequence alignment of NdhL and homologous proteins from cyanobacteria and green plants. Protein sequences were aligned using the ClustalW program (ww.ebi.ac.uk/clustalw). Sequences from the following organisms are included: Synechocystis sp. PCC 6803 (accession no. NP_441443), Synechococcus elongatus PCC 7902 (AA053559), Thermosynechococcus elongatus BP-1 (NP_681485), Nostoc sp. PCC 7120 (NP_574985), Arabidopsis thaliana (AAQ96960), and Oryza sativa (9633.102493, MIPS rice data base). N-terminal sequences of proteins from green plants are omitted from the alignment. Asterisks indicate identical amino acids; colons and dots indicate conserved amino acid substitutions. Transmembrane regions predicted by TMHMM are shown by arrows. Top, for NdhL of Synechocystis 6803, bottom, for At1g70760.

### Discussion

The NDH-1 complex of cyanobacteria and the plastidial NDH-1 complex of green plants function not only as a respiratory/chlororespiratory complex like in a majority of other organisms but also participate in cyclic electron flow around photosystem I (PSI) (35–37). Based on genome studies, it was predicted that eleven subunits are similar between NDH-1 of E. coli and the enzymes located in thylakoid membranes of photosynthetic organisms. Since the complexity of the enzyme varies between organisms, it was plausible to presume that additional subunits exist in NDH-1 of cyanobacteria and chloroplasts compared with minimal NDH-1 of E. coli.

Traditional chromatographic methods used for purification of NDH-1 from Synechocystis 6803 have been unsuccessful since the enzyme containing both hydrophilic and hydrophobic components appeared to be extremely fragile and present in thylakoid membranes in a low quantity. Moreover, it has been difficult to discriminate between the new subunits of the complex and contaminating proteins (12). Only recently, Prommeen et al. (14) isolated the NDH-1 complex of a high purity using His-tagged NdhJ. They confirmed that Slr1623 (NdhM), discovered by Berger et al. (12), indeed belongs to the Synechocystis NDH-1 complex, and revealed a novel subunit, Sli1262 (NdhN). However, NdhL has never been found before as a component of isolated NDH-1 complexes.

In our previous proteome studies (15) we described four complexes containing ndh gene products. Among them NDH-1L and NDH-1M showed a multiple Ndh subunit composition, but the majority of protein subunits remain unknown. These two complexes seem to have distinct functions in cyanobacterial cells. To clarify the structural difference, we took a systematic approach to identify the subunits comprising NDH-1L and NDH-1M using ESI MS/MS analysis supplemented by MALDI-TOF mass spectrometry.

### Fifteen Subunits of the Synechocystis NDH-1 Complex

In the NDH-1L complex we identified 10 of 11 subunits of Synechocystis NDH-1 (NdhA, -B, -D1, -E, -F1, -H, -I, -J, -K) that are homologous to the E. coli NDH-1 complex. The 11th subunit, NdhC, was most probably represented by the unidentified spot 12 since the relative mobility of the protein corresponded well to the NdhC subunit identified by Prommeen et al. (14) by N-terminal sequencing. NDH-1M contained the known NdhL and Ssl1690 in NDH-1 of Synechocystis 6803.

Intriguingly, we found four additional subunits of Synechocystis NDH-1 that do not have homologues in the bacterial “minimal” complex. The unknown protein, Ssl1690, appeared to be a novel component of NDH-1. We also found NdhL...
The presence in the NDH-1L complex of the intact NdhF1 (75/7120 (NP_488361), Trichodermium erythraeum IMS101 (ZP_00325793), P. marinus (AAP28747), and O. sativa (BX922826)). Proteins were aligned with ClustalW. N-terminal sequences of proteins from green plants are omitted. Asterisks indicate amino acids as described in the legend to Fig. 4.

### Table II

| Ci supply | WT | M55 | M9 |
|-----------|----|-----|----|
| High CO₂ | +a | +a | ++ |
| Low CO₂  | +b | −   | −   |
| Glucose   | +c | −   | −   |

*a, +, effective growth.  
b, +, moderate growth.  
c, −, no growth.  
d, BL, cells bleached.

(75/7120 (NP_488361), Trichodermium erythraeum IMS101 (ZP_00325793), P. marinus (AAP28747), and O. sativa (BX922826)). Proteins were aligned with ClustalW. N-terminal sequences of proteins from green plants are omitted. Asterisks indicate amino acids as described in the legend to Fig. 4.

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apparent pl values (4.8 and 7.9) indicating the presence of a post-translational modification. In contrast to NdhO, the NdhL subunit containing 2 transmembrane helices most probably belongs to the membrane fragment of the enzyme. The hypothesis scheme of cyanobacterial NDH-1 is shown in Fig. 6. It should be noted that the three catalytically active subunits homologous to NuoE, -F, and -G and comprising the NAD dehydrogenase fragment of E. coli NDH-1 still remain unknown for cyanobacterial or chloroplast enzyme. Therefore, it is still uncertain what is the electron donor for cyanobacterial NDH-1. Alternatively to NADH, NADPH (13, 35) or ferredoxin (1) might be the electron donor. The situation is similar for the plastidial NDH-1 complex of green plants (36, 40).

At present it is not clear what roles NdhM, -N, -O, and -L, the “extra” components of cyanobacterial NDH-1, play in the complex. No known sequence motifs have been found in sequences of these subunits that could provide an insight into their function.

Functional studies of M9, the NdhL deletion mutant, demonstrated that the presence of this protein is essential to inorganic carbon acquisition and phototrophic growth of Synechocystis 6803 (17, 41). The phenotype of M9 is similar to that of M55, the NdhB deletion mutant (18). Both strains grow photoautotrophically in high CO2 conditions and do not grow at pH 7.5, air level of CO2. Moreover, in both strains the cyclic electron flow around PSI was suppressed (41). The effect of the NdhL mutation was, however, slightly less profound compared with that of NdhB. In low CO2 conditions, M9 cells did not grow but remained alive whereas M55 cells died. Further, cyclic electron flow was almost completely suppressed in M55 whereas in M9 a rather low cyclic PSI activity could be observed (41). Our results demonstrated that NdhL is not essential to the in vivo integrity of the NDH-1 complex because both NDH-1L and NDH-1M were present in M9. In contrast, NdhB is crucial for complex formation. Because in the absence of NdhL all physiological functions so far demonstrated for Synechocystis NDH-1 were severely impaired, it can be concluded that NdhL is a critical subunit for the function but not for the assembly of NDH-1 complexes in Synechocystis 6803. Therefore, it is likely to be located to the periphery of the membrane fragment of NDH-1 yet in a close proximity to the soluble subcomplex (Fig. 6). The exact role of this small subunit for the activity of NDH-1 remains to be elucidated.

Homologues of Ssl1690 and NdhL Exist in Higher Plants—The results of BLAST searches demonstrated the presence of Ssl1690 and NdhL homologues in cyanobacteria, but the presence of homologous subunits in higher plants remained ambiguous. This was an important question particularly for NdhL because this protein was shown to be involved in Ci transport (11). In order to distinguish between two hypotheses: (a) NdhL and Ssl1690 are specific for cyanobacterial species, and (b) these subunits have relatives in higher plants despite the lack of significant similarity, we performed a profound bioinformatic analysis of several plant databases. The results showed that for both subunits homologous unknown proteins are present in various plant species. For example, At1g70760 from Arabidopsis and 9633.102493 from rice are homologous to NdhL, and At1g74880 and 9629.106877 are homologous to Ssl1690. Moreover, corresponding diagnostic sequence motifs were found for both NdhL and Ssl1690 homologues that are conserved among cyanobacteria and plants but absent in other organisms.

Comparison of cyanobacterial sequences with green plant counterparts showed that proteins considerably differ in size. Plant proteins have N-terminal extensions, and At1g70760 extended also at C termini (the C terminus of the rice protein is not certain because of the splicing ambiguity). A significant part of N-terminal extensions should be cleaved in mature proteins during translocation into chloroplasts, thus increasing similarity with cyanobacterial sequences. It is consistent that At1g74880 and 9629.106877 are hydrophilic proteins, likewise Ssl1690, while At1g70760 was predicted to have three transmembrane helices (Fig. 4). The second and third helices match the corresponding hydrophobic regions of NdhL. It seems probable that during evolution some additional feature(s) was/were encountered in NdhL homologous sequences of green plants in parallel with functionally important preservation of the cyanobacterial-like domain.

Regarding green plants, it is necessary to consider two complexes, the mitochondrial complex I and the NDH-1 complex of chloroplasts. Heazlewood et al. (16) studied the subunit composition of mitochondrial complexes I from Arabidopsis and rice and described several subunits specific for the complex. Importantly, proteins homologous to Ssl1690 and NdhL were not found among them. This is consistent with Predorat results for the plastidial location of these proteins. We conclude that in green plants unknown proteins homologous to Ssl1690 and NdhL might be the components of the poorly studied NDH-1 complex located in chloroplasts. Together with NdhM and NdhN, it makes four nuclear-encoded Ndh subunits discovered in plastidial NDH-1. NdhM has been reported to be homologous to the B13 subunit of the bovine complex I (12, 14). The other three proteins seem to be specific for thylakoid-located NDH-1 of photosynthetic organisms.
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Identification of NdhL and Ssl1690 (NdhO) in NDH-1L and NDH-1M Complexes of Synechocystis sp. PCC 6803

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