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NdhP and NdhQ: Two Novel Small Subunits of the Cyanobacterial NDH-1 Complex†

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ABSTRACT: The subunit composition of the NAD(P)H dehydrogenase complex of *Thermosynechococcus elongatus* was analyzed by different types of mass spectrometry. All 15 known subunits (NdhA−NdhO) were identified in the purified NDH-1L complex. Moreover, two additional intact mass tags of 4902.7 and 4710.5 Da could be assigned after reannotation of the *T. elongatus* genome. NdhP and NdhQ are predicted to contain a single transmembrane helix each, and homologues are apparent in other cyanobacteria. Additionally, *ndhP* is present in some cyanophages in a cluster of PSI genes and exhibits partial similarity to NDF6, a subunit of the plant NDH-1 complex.

The internal membrane system of cyanobacteria and chloroplasts, the thylakoids, contains a type I NAD(P)H dehydrogenase structurally and functionally similar to complex I (NADH:ubiquinone oxidoreductase) found in mitochondria and eubacteria (*1*, *2*). The number of subunits of complex I-type enzymes varies from 14 (*Escherichia coli* complex, NuoA−NuoN) (*3*), which is the minimal set for bioenergetic function (*4*), to 45 for the bovine complex (*5*). The cyanobacterial NDH-1 complex is presently believed to consist of 15 different subunits (*6*−*9*). Of these, 11 (NdhA−NdhK) are similar to *E. coli* proteins, whereas for *E. coli* subunits NuoE, NuoF, and NuoG, no homologues could be found in cyanobacteria or plant chloroplasts (http://www.uniprot.org). These subunits contain the NADH-binding site and carry the FMN cofactor as well as several Fe−S clusters (*10*, *11*), features that are essential for the function of complex I in eubacteria and mitochondria. To date, it is still a puzzle which proteins are responsible for the import of electrons into the NDH-1 complex of cyanobacteria and plant chloroplasts. Subunits NdhL, NdhM, NdhN, and NdhO are unique to organisms performing oxygenic photosynthesis (*6*, *8*, *12*). NdhL is important for carbon uptake in cyanobacteria (*13*), but the functional roles of the others remain unclear. Genes encoding NdhD and NdhF are found in most cyanobacterial genomes in several copies. These gene families represent the basis for the functional variety of NDH-1 complexes in cyanobacteria. Four different types could be defined by reverse genetics (*14*, *15*) and functional proteomics (*6*−*9*).

NDH-1L (NdhD1/NdhF1) and NDH-1L′ (NdhD2/NdhF1) are thought to be involved in respiration and cyclic electron flow around PSI (*16*). NDH-1L is the predominating complex in the thylakoid membrane, whereas NDH-1L′ has not yet been detected on the protein level. Single-particle analysis revealed the typical "L-shaped" structure for this type of complex (*17*), and although NDH-1L is strikingly similar to the mitochondrial counterpart, there are arguments against its respiratory function in cyanobacteria. The impaired rates of plastoquinone reduction in *ndh* mutants might be related to low levels of succinate in these strains, which limit the activity of the succinate dehydrogenase, rather than the lack of NDH-1 activity (*18*). The NDH-1MS (NdhD3/NdhF3) and NDH-1MS′ (NdhD4/NdhF4) complexes are responsible for high-affinity and low-affinity CO_2 uptake, respectively (*9*, *19*), both unique functions of the cyanobacterial NDH-1 complex. NDH-1MS′ is expressed constitutively at low level, whereas NDH-1MS is induced under low-CO_2 conditions (*14*, *15*). The NDH-1MS complex has a "U-shaped" structure caused by additional proteins (CupA/CupS) bound to the cytoplasmic surface (*20*). Here we focus on the analysis of additional small proteins appearing in the cyanobacterial NDH-1L complex. In-depth mass spectrometry analysis of highly purified material revealed the presence of two new subunits that were designated NdhP and NdhQ.

The NDH-1L complex of *Thermosynechococcus elongatus* was isolated via the "natural His tag" of the NdhF1 subunit (*9*) by Ni^{2+} column chromatography (Figure 1A). In the second step, the prepurified complex was loaded on a size exclusion column (see experimental procedures of the Supporting Information for details) to separate impurities and breakdown products (Figure 1B). Fractions containing the NDH-1L complex (∼500 kDa) corresponding to peak F1 were pooled and concentrated. All further experiments were conducted with this sample. Figure 1C shows the result of two-dimensional gel electrophoresis with the purified complex. The major band in the BN-PAGE gel corresponds to the NDH-1L complex, and 12 of 15 known subunits could be identified in the spots of the second dimension (Table S1 of the Supporting Information). Additionally, the purified complex was analyzed by tandem mass spectrometry in a shotgun experiment after digestion with trypsin and/or chymotrypsin, and all 15 known subunits of the NDH-1L complex could be identified (Table S2 of the Supporting Information).

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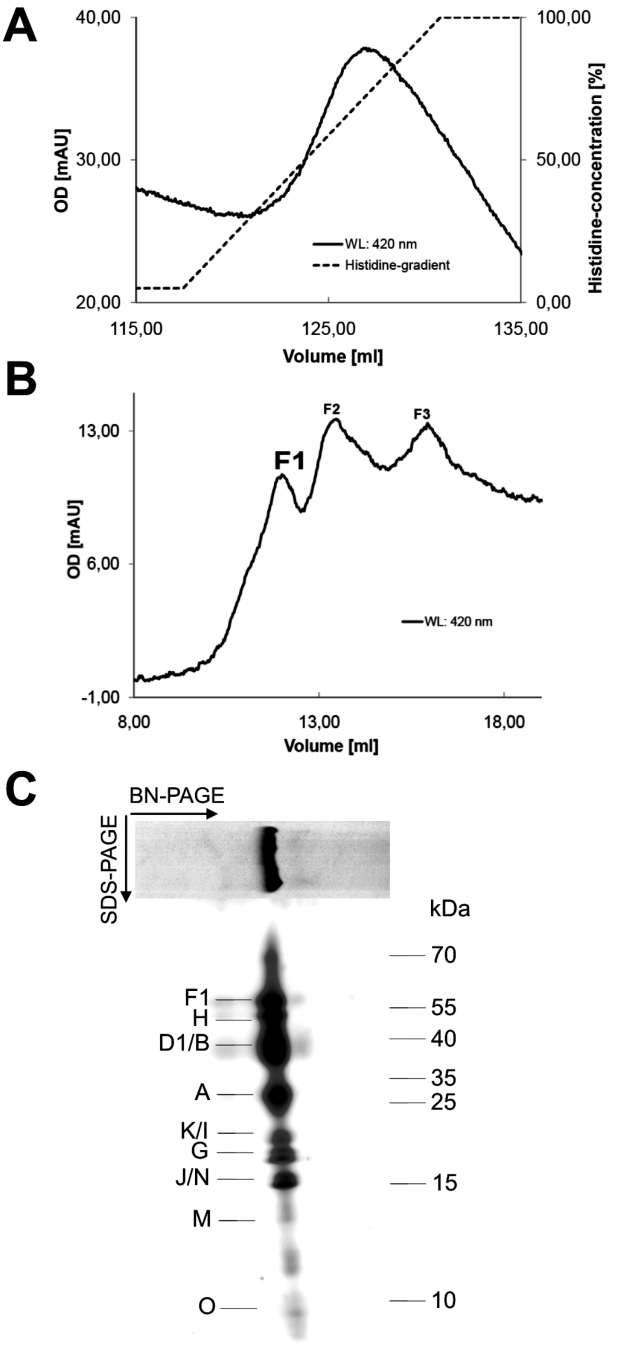

FIGURE 1: Purification of the NDH-1L complex. NDH-1L complexes were purified by a combination of (A) Ni^{2+} affinity chromatography and (B) size exclusion chromatography. The purified complexes were analyzed by (C) two-dimensional gel electrophoresis (BN- and SDS−PAGE).

Moreover, the intact complex was analyzed by liquid chromatography with electrospray ionization mass spectrometry (*21*), and two masses in the low-molecular mass region were identified, which could not be assigned to known NDH-1L subunits. Even after calculation of the theoretical molecular masses of all open reading frames (ORFs) assigned in the genome of *T. elongatus* (http://genome.kazusa.or.jp/cyanobase), no concordance was found. Additionally, known post-translational modifications of small subunits like N-terminal formylation, acetylation, and cleavage of the N-terminal methionine (*22*, *23*) have been considered. However, only after a search for unassigned ORFs in the genome of *T. elongatus* with GLIMMER (http://bioinformatics.biol.rug.nl/websoftware/orf/orf_start.php) could two new ORFs (Figure S1 of the Supporting Information) be assigned to the unknown masses, named NdhP and NdhQ. The mass of NdhP (4902.7 Da) indicates retention of the formyl group on the initiating N-terminal methionine, whereas the measured mass of NdhQ (4710.5 Da) matches the calculated mass based on the amino acid sequence after cleavage of the N-terminal methionine (Table S4 of the Supporting Information), both common post-translational modifications in cyanobacteria. MALDI-ToF mass spectrometry shows that both subunits seem to be present in rather equal amounts compared to known NDH-1L subunits (Figure S2 of the Supporting Information), although the exact stoichiometry could not be concluded from the data because of ionization effects. The translated amino acid sequence is predicted to exhibit a single transmembrane helix (TMH) in both cases (Figure S3 of the Supporting Information). In addition, the identification of the new subunits was verified on the peptide level. For that purpose, the derived amino acid sequences were included in the database for protein identification, which was done by analysis of the spectra generated in the LC−ESI-MS/MS shotgun experiment. Only one peptide of each protein was identified with a high degree of confidence, but this covers the hydrophilic domain almost completely in both cases (Table S3 of the Supporting Information and Figure 2). Other possible peptides might be not in the right mass range, or they might be lost during sample preparation because of their high level of hydrophobicity.

Moreover, the identified post-translational modifications of NdhP and NdhQ might be used to predict their orientation in the membrane. N-Terminal processing requires the accessibility of the N-terminus, which is located toward the cytoplasm in this case, whereas a remaining formyl group at the N-terminus indicates an orientation toward the lumen (*24*). These predictions were included in the model of the NDH-1L complex with NdhP and NdhQ shown in Figure 3. Sequence analysis of *ndhP* and *ndhQ* with tblastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that both genes are widespread within the phylum of cyanobacteria (Figure S4 of the Supporting Information). *ndhP* is present in all 37 cyanobacterial genomes, whereas *ndhQ* is absent in *Gloeobacter violaceus* and in some *Prochlorococcus* strains (http://genome.kazusa.or.jp/cyanobase). Moreover, a domain similar to NdhP could be revealed in the amino acid sequence of NDF6 (Figure S5 of the Supporting Information), a subunit of the NDH-1 complex present in the chloroplasts of *Arabidopsis thaliana* that was discovered recently (*25*). However, the relation to NdhP is unclear because NDF6 is much larger and only a small part of it shows some sequence similarity. Interestingly, among the similar sequences present in other organisms, a gene encoding an NdhP homologue could be found in the genome of a cyanophage (PSSM2_253, Figure S4 of the Supporting Information). It is part of an operon with PS1 genes and thought to be a phage-specific ORF with unknown function (*26*). Here we could show that this protein is part of the cyanobacterial NDH-1 complex and, in conclusion, its expression by the phage might control the amount of NDH-1 in the host cell or might support cyclic electron flow around PS1 to force the production of ATP. Although they are rather small and sometimes overlooked, single-transmembrane domain (STMD) proteins seem to play important roles during the biogenesis of membrane protein complexes in general (*27*), but this has to be proven for NdhP and NdhQ in further experiments.

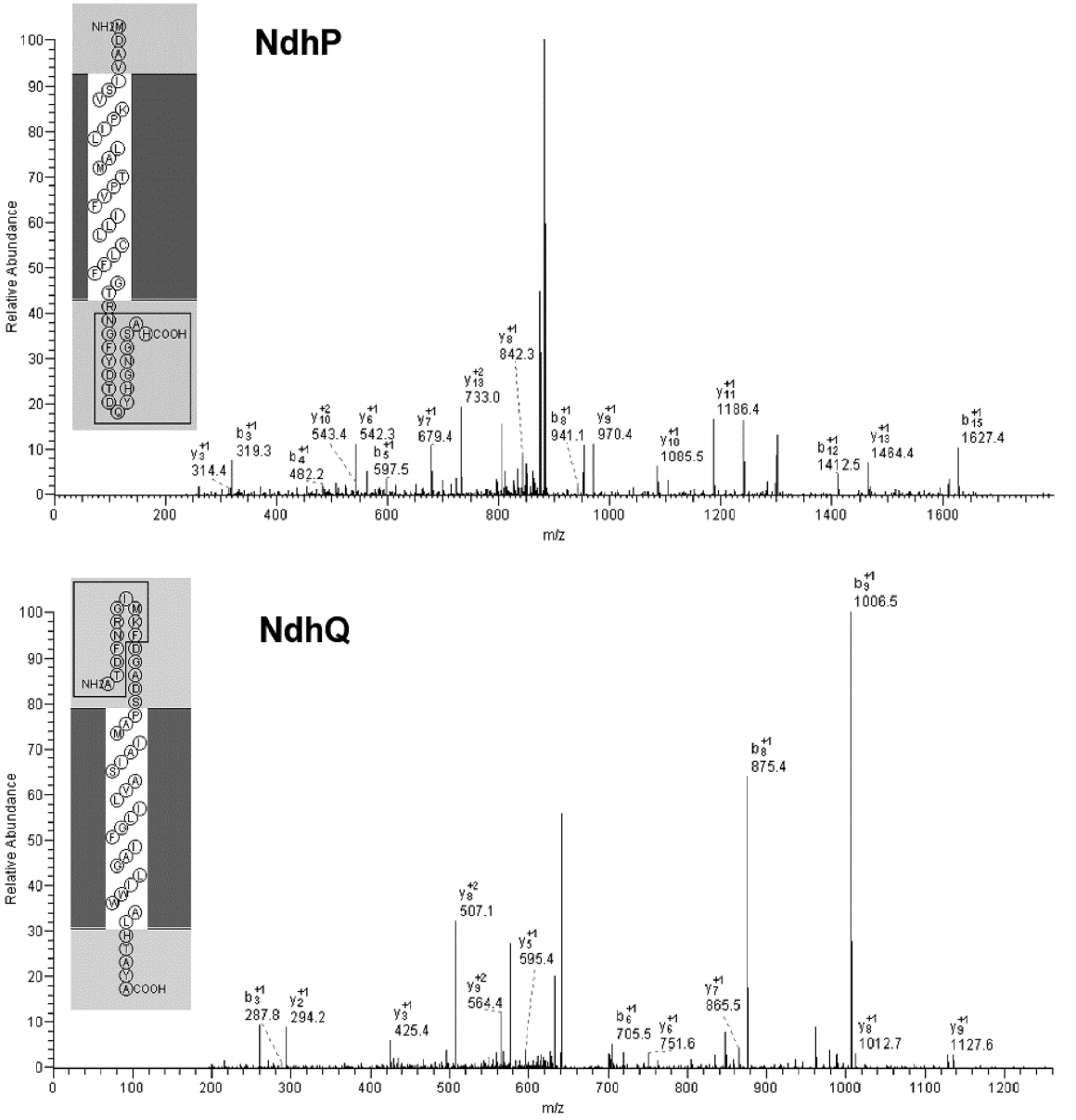

FIGURE 2: Identification of NdhP and NdhQ. Purified NDH-1L complexes were digested with trypsin or chymotrypsin, and the peptides were analyzed in a shotgun experiment by LC−ESI-MS/MS. The sequence, the predicted topology, and the location of the identified peptide (box) of NdhP (A) and NdhQ (B), as well as the corresponding spectra, are shown. The topology of NdhP and NdhQ was predicted by SOSUI (http://www.bp.nuap.nagoya-u.ac.jp/sosui/) as indicated in the left figure. TMH is denoted with a white background.

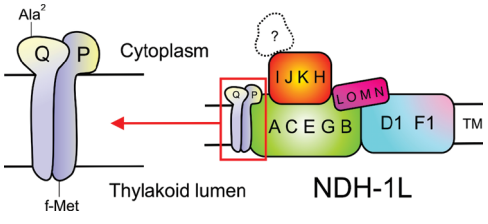

FIGURE 3: Model of the NDH-1L complex. The two new NDH-1 subunits, NdhP and NdhQ, were included in a model of the NDH-1L complex. Both proteins are predicted to span the membrane by a single TMH, and the differences in N-terminal processing suggest an unequal location of the N-termini. The unknown subunits responsible for NAD(P)H oxidation are denoted by the question mark. TM means thylakoid membrane.

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SUPPORTING INFORMATION AVAILABLE

Details for experimental procedures and additional tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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