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Single particle analysis of thylakoid proteins from *Thermosynechococcus elongatus* and *Synechocystis* 6803: Localization of the CupA subunit of NDH-1

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Abstract **The larger protein complexes of the cyanobacterial photosynthetic membrane of *Thermosynechoccus elongatus* and *Synechocystis* 6803 were studied by single particle electron microscopy after detergent solubilization, without any purification steps. Besides the "standard" L-shaped NDH-1L complex, related to complex I, large numbers of a U-shaped NDH-1MS complex were found in both cyanobacteria. In membranes from *Synechocystis* Δ*cupA* and Δ*cupA*/*cupB* mutants the U-shaped complexes were absent, indicating that CupA is responsible for the U-shape by binding at the tip of the membrane-bound arm of NDH-1MS. Comparison of membranes grown under air levels of CO_2 or 3% CO_2 indicates that the number of NDH-1MS particles is 30-fold higher under low-CO_2.**

Keywords: Electron microscopy; Single particle analysis; Membrane protein; NDH-1; CupA; *Thermosynechococcus elongatus*; *Synechocystis* 6803

1. Introduction

Over the last 20 years, single particle analysis of electron microscopy (EM) projections has become a well-established technique to obtain structural information about large biomacromolecules at a resolution of 10–20 Å [1]. In some favourable cases a much higher resolution has been achieved on ice-embedded virus particles or other complexes with a high symmetry [2]. Membrane proteins are more difficult to process by cryo-EM at high resolution, because single complexes are surrounded by a detergent layer to keep them in a monodisperse state. Single particle averaging on negatively stained specimens, however, offers the possibility to process thousands of projections within a day, yielding 2D projection maps of at least 20 Å resolution. The method includes statistical analysis and classification, and is able to sort and average 2D projection maps of membrane proteins from heterogeneous samples, which can be semi-purified samples. In this way, a set of projection maps is achieved, which may contain the object of interest, but also maps specific contaminants. In a recent study of photosystem II (PSII) complexes from cyanobacteria two different particles, obtained by different purification schemes, were compared by single particle analysis, SDS gel analysis and mass spectrometry [3]. Mass spectrometry indicated that the two particles differed by the absence or presence of PsbZ, a small peripheral subunit of 6.8 kDa. By EM analysis the location within the structure could be determined [3]. In addition, a contaminating L-shaped protein was found, which was suggested to be NDH-1, the cyanobacterial counterpart of Complex I. This tentative assignment of NDH-1 could be confirmed after comparison with purified NDH-1 [4].

Arteni and coworkers suggested that screening for membrane protein structures by single particle EM, in combination with biochemical analysis, might be an interesting approach to find novel protein structures [3]. To further test the idea of finding novel transient membrane protein complexes by single particle EM we applied the method to a complete set of non-purified complexes from solubilized photosynthetic membranes of two different cyanobacteria: *Thermocynechococcus elongatus* and *Synechocystis* 6803 (see below). One of the aims was to find novel structures, for instance of NDH-1, because this is a very fragile, heterogeneous complex, see [5] for a review.

Proteomic studies revealed that the cyanobacterium *Synechocystis* 6803 has several functionally distinct NDH-1 complexes [5,6]. The two largest complexes, NDH-1L and NDH-1MS, have a mass of about 500 kDa. The NDH-1L complex participates in respiration and cyclic electron flow around PSI. It is composed of 15 subunits, of which eight belong to the membrane domain (NdhA, NdhB, NdhC, NdhD1, NdhE, NdhF1, NdhG and NdhL) and seven to the hydrophilic domain (NdhH-J, NdhM–NdhO) [7]. NDH-1L appeared relatively unaffected by different growth conditions such as high- and low-CO_2, iron deficiency, salt stress. The NDH-1MS complex functions as a high-affinity CO_2 uptake system and converts CO_2 to HCO_3^- within the cell. It is strongly expressed in cells cultured under air levels of CO_2, but the expression is drastically reduced under increased CO_2 levels [8]. The two NDH-1 complexes differ in subunit composition. In the

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Abbreviations: HC, high-CO_2 (3% or 5%); LC, low-CO_2 (air level)

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NDH-1MS complex the D1 subunit of 56 kDa and the F1 subunits of 72 kDa have been replaced by homologous but slightly smaller D3 (54 kDa) and F3 (66 kDa) subunits, which are essential for active CO_2 uptake [9]. In addition, NDH-1MS has two water-soluble proteins attached. One is CupA, a 51 kDa protein, which was found to be a component of NDH-1 involved in CO_2 uptake by reverse genetics studies [10,11] and the other is a small subunit recently named CupS [12] and encoded by *sll1735* in *Synechocystis* 6803 and *tll0221* in *T. elongatus*. There is in addition a second NDH-1 particle functionally in CO_2 uptake; it has been designated NDH-1MS′ [5]. This particle of 440 kDa has subunits D1 and F1 replaced by D4 and F4 and has CupB attached, which is a homologue of CupA, and CupS [10,11]. It appears that the NDH-1MS′ particle shows low affinity to CO_2 and is constitutively expressed, in contrast to the high-CO_2 affinity NDH-1MS complex, which is induced under low-CO_2 [10].

In this paper, we analyzed the larger membrane- and membrane associated complexes from *T. elongatus* and *Synechocystis* 6803 by single particle EM analysis directly after solubilization, without a purification step, to omit potential loss of transiently bound subunits. We focussed on NDH-1 complexes, which can be easily detected in EM images, because of the unique L-shape. In addition to L-shaped NDH-1, we found U-shaped particles, which had a similar hydrophilic domain as the L-shaped NDH-1 complex named NDH-1L [4]. To establish the presence of CupA in the extra domain of the U-shaped particles, we made a comparison to particles solubilized from the membranes of CupA and CupA/B mutants and here we show that the loosely attached density at the tip of the membrane arm of NDH-1 particles is identical to the CupA protein.

2. Materials and methods

2.1. Cultivation of cyanobacteria and preparation of thylakoid membranes

Synechocystis sp. PCC 6803 WT, Δ*cupA* and Δ*cupA*/*cupB* strains were grown in BG-11 medium under continuous light of 50 μmol photons m^{-2} s^{-1} at 3% CO_2 (HC), and then transferred to air level of CO_2 (LC). The thylakoid membranes of *Synechocystis* WT and mutant strains were isolated from 100 ml cell culture according to Ref. [13].

Thermosynechococcus elongatus WT was cultured at 45 °C in BG-11 media at 5% CO_2 (HC) or air level of CO_2 (LC) and illuminated with white light of increasing intensity (50–250 μmol photons m^{-2} s^{-1}). Thylakoid membranes were prepared according to Ref. [14].

2.2. Solubilization of membranes from T. elongatus and Synechocystis 6803

For preparing EM specimens, membranes from *T. elongatus* grown at high (5%)-CO_2 and low-CO_2 were solubilized with 1% (or 2%) digitonin at a 0.5 mg/ml final chlorophyll concentration. The suspension was stirred for 30 min using a small magnet stirrer, at 4 °C and followed by 15 min centrifugation at 15000 g. The unsolubilized material was discarded and the supernatant used for electron microscopy analysis. Thylakoid membranes from *Synechocystis* WT and mutants were solubilized using digitonin at 3% final concentration and 0.3 μg/μl chlorophyll final concentration for all samples (WT, ΔcupA, ΔcupA/B grown in low-CO_2 and high-CO_2).

For blue-native (BN)/SDS–PAGE, the thylakoid membranes of *Synechocystis* WT, Δ*cupA* and Δ*cupA*/*cupB* (7.5 μg chlorophyll) were pelleted down and resuspended at a chlorophyll concentration of 0.6 μg/μl. Then an equal volume of detergent solution was added to the thylakoid suspensions to final detergent concentration of 1.5% β-DM or 3% digitonin (the final chlorophyll concentration 0.3 μg/μl), and the gels were run for 5 h and stained with silver as described earlier [13]. For immunoblotting, thylakoids equivalent to 3 μg chlorophyll were solubilized and separated in SDS–PAGE containing 6 M urea [15]. The proteins were electrotransferred to a PVDF membrane and immunodetected by specific antibodies against CupA, NdhD3 and AtpA/B proteins [13]. The AtpA/B antibody was kindly provided by Dr. Hundal.

2.3. Electron microscopy and single particle analysis

Samples were made from solubilized membranes by dilution in buffer with detergent and subsequent negative staining using 2% uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120 equipped with a LaB_6 tip operating at 120 kV. The "GRACE" system for semi-automated specimen selection and data acquisition [16] was used to record 2048 × 2048 pixel images at 66850× calibrated magnification (3.75 Å) with a Gatan 4000 SP 4K slow-scan CCD camera. A total of 15000 particle projections were collected. Single particle analysis was performed using Groningen Image Processing ("GRIP") software packages on a PC cluster. The best 70–80% of the class members was taken for the final class-sums.

3. Results

3.1. Structural analysis of solubilized membranes

Thylakoid membranes of wild-type *T. elongatus* and *Synechocystis* 6803, grown at low-CO_2 and high-CO_2 conditions were solubilized with 1–3% digitonin and directly applied to electron microscopy grids without further purification steps. A typical EM image of negatively stained particles is presented in Fig. 2. From such images we selected all large recognizable single particle projections. They were analyzed by single particle averaging, which includes classification of projections and the averaging of class-members of homogenous subsets of particles into 2D maps. A gallery of selected 2D maps is presented in Fig. 2. First, we found a novel U-shaped complex in top- and side-view position (Figs. 2A and B). The upper left part strongly resembles the hydrophilic domain of purified NDH-1 from *T. elongatus* in side-view position (Fig. 2C) [4], which indicates that this U-shaped particle is a NDH-1 complex.

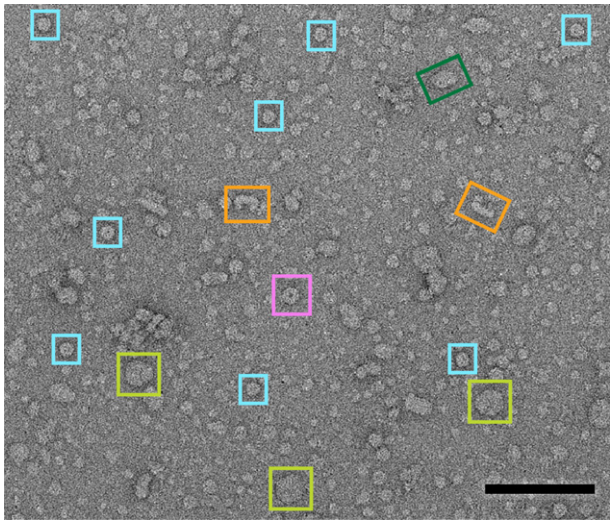

Fig. 1. EM frame showing an overview of solubilized membranes from *Thermocynechococcus elongatus*, grown on high-CO_2. Boxes mark projections of trimeric Photosystem I (bright green); dimeric photosystem II (dark green), phycobilisome fragments (blue), NDH-1 (orange), and an unknown hexagonal particle (purple). The space bar equals 100 nm.