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Enzymatic characterization of an active NDH complex from *Thermosynechococcus elongatus*

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ABSTRACT

Although type-1 NAD(P)H dehydrogenase (NDH) complex subunit constituents and physiological functions have been reported in plants and cyanobacteria, the biochemical properties of this enzyme are not clear. We used chromatographic isolation to purify and characterize a NADPH-active NDH from the cyanobacterium *Thermosynechococcus elongatus*. Ferredoxin (Fd) and ferredoxin-NADP$^+$ oxidoreductase (FNR) were co-eluted with NDH, implying the electron donation from NADPH to NDH via the interaction with FNR. We investigated the enzymatic properties of the complex. Furthermore, the activity is competitively inhibited by rotenone, suggesting that it possesses a quinone binding site, similar to mitochondria complex I.

1. Introduction

Cyanobacterial type-1 NAD(P)H dehydrogenase (NDH) [1] complexes are involved in a variety of bioenergetic reactions, including respiration, cyclic electron transport around photosystem I (PS I) [2] and CO_2 uptake [3]. Of the 14 subunits of complex I in *Escherichia coli*, only 11 *ndh* subunits were identified in cyanobacteria and in chloroplasts [4], three subunits (NuoE, NuoF and NuoG) involved in accepting electrons from NADH in *E. coli* are missing from cyanobacterial and chloroplastic NDH. On other hand, based on proteiomics study with several NDH mutants, Battchikova et al. speculate that NdhL-O comprise a domain of unknown function specific for cyanobacteria and chloroplasts, and propose to designate it as the OPS (Oxygenic Photosynthesis-Specific) domain [5]. However, so far, the electron donor and electron entry for cyanobacterial and chloroplastic NDH are still debatable. Using thylakoid membranes isolated from wild type and NdhB defective mutant M55 of *Synechocystis* PCC 6803, it was demonstrated that NADPH but not NADH donates electrons to the plastoquinone (PQ) pool via NDH [6]. In addition, a reconstitution of NADPH- and ferredoxin (Fd)-dependent cyclic electron flow around PS I has been achieved [6,7]. After then, several NADPH-active NDHs were identified using activity staining for NADPH-NBT oxidoreductase combined with Western blot [8–10]. The activity of a supercomplex of NDH is found to be proportional to cyclic PSI electron flow [11]. To study the biochemical properties of these complex enzymes, active NDH must be purified. However, this presents a considerable technical challenge due to the instability of the complex. Berger et al. [12] first described the isolation of an NDH subcomplex from Triton X-100 treated *Synechocystis* 6803 cell extracts by immunoaffinity chromatography using an NdhK-specific antibody coupled to Protein A Sepharose. The authors monitored the purification with NdhK- and NdhJ-specific antibodies and measurements of NADH-oxidising activity and concluded that the subcomplex was functionally inactive. Later, Matsuo et al. [13] described an active hydrophilic NDH subcomplex of 380 kDa from a CHAPS-treated *Synechocystis* 6803 cell extract. The enzyme appeared to be substrate specific for NADPH but was

Abbreviations: NDH, type-1 NAD(P)H dehydrogenase; Fd, ferredoxin; FNR, ferredoxin-NADP$^+$ oxidoreductase; PBS, phycobilisome protein; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; DM, *n*-dodecyl-β-D-maltoside; PS I, photosystem I; DCPIP, 2,6-dichloroindophenol sodium salt dydrate; Q_0, ubiquinone-0; Q_{10}, ubiquinone-10

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not inhibited by rotenone, a classic inhibitor of Complex I in mitochondria. Deng et al. used conventional chromatographic methods to purify another NADPH-active NDH-1 subcomplex of approximately 230–250 kDa from cells grown under low CO_2 conditions [14]. These authors further showed that a complex containing the hydrophobic subunit NdhA specifically oxidised NADPH [9]. NDH complexes have been isolated from both *Synechocystis* 6803 [15] and *Thermosynechococcus elongatus*[16] using His-tag techniques. A major complex of approximately 460 kDa was isolated from *Synechocystis* 6803, and two complexes of approximately 450 and 490 kDa were isolated in *T. elongatus*, but none of those complexes displayed any NADPH-$K_3Fe(CN)_6$ oxidoreductase activity. Nowaczyk et al. [17] isolated NDH-1L complex and identified two small novel subunits, NdhP and Q from *Thermosynechococcus elongatus*. To date, because all of the isolation approaches described above have been insufficient to obtain either a single purified complex or active NDH, the biochemical properties of NDH remain to be elucidated. Recently, it's reported that knocking out a new identified NdhS in *Arabidopsis thaliana* (also known as CRR31) and in *Synechocystis* 6803 caused inactivation of the activity of NDH pathway [18,19]. According to the structure similarity of NdhS with PsaE, the authors suggested that NdhS might bind with Fd which might be an electron donor for NDH. Here, we report the enzymatic properties of a purified active NDH complex of approximately 170 kDa that contains both hydrophilic and hydrophobic subunits. We found that Fd and Fd-$NADP^+$ oxidoreductase (FNR) were co-eluted with the active NDH, providing biochemical evidence for interaction of Fd with cyanobacteria NDH and donation of electron from NADPH to NDH via FNR. Furthermore, the activity is competitively inhibited by rotenone.

Table 1
Purification of NDH complex from *T. elongatus*[a].

Purification step	Total protein (mg)	Total activity[b] (IU)	Specific activity IU (mg protein)$^{-1}$	Purification (fold)	Yield (%)
Cell extracts (DM treated)	1170.00	1815.00	1.552	1	100
Q Sepharose Fast flow	117.47	569.73	4.85	3.13	31.39
Hiload 26/60 Superdex 200	18.21	112.36	6.17	3.98	6.19

[a] From 10 L of *T. elongatus* cell culture in BG-11 medium.
[b] Activity was determined with 100 μM NADPH, 100 μM $K_3Fe(CN)_6$ in 20 mM Tris–Cl, pH 8.0.

2. Materials and methods

2.1. Organisms and conditions

Thermosynechococcus elongatus-BP1 cells were cultured at 50 °C in BG-11 medium [20] buffered with Tris–HCl (5 mM, pH 8.0) bubbled with 2% (v/v) CO_2 in air under continuous illumination by fluorescent lamps (60 μEm^{-2} s^{-1}).

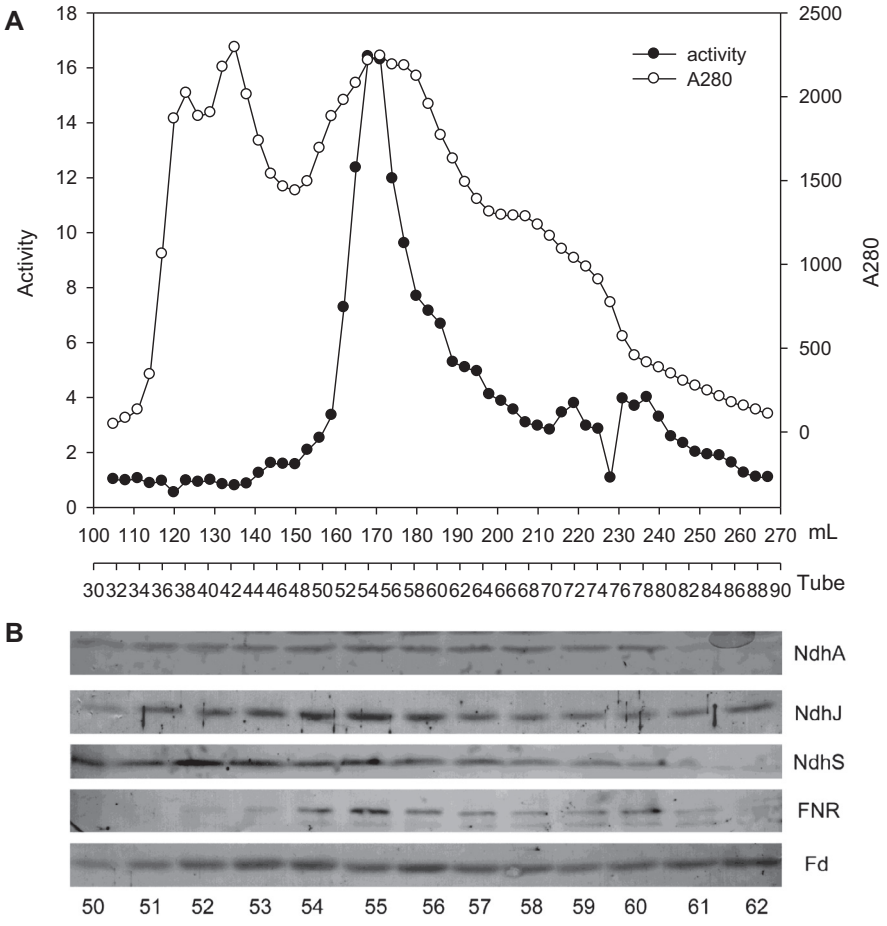

Fig. 1. Size exclusion chromatography. The factions from the ion exchange step with NADPH oxidation activity were concentrated and applied to Superdex 200 prep grade column. Protein concentration was monitored by absorbance at 280 nm and the activity was detected with a decrease in absorbance at 340 nm using 100 μM NADPH as electron donor and 100 μM $K_3Fe(CN)_6$ as an electron acceptor. The same fractions were separated by SDS/PAGE and probed with antibodies specific for NdhA, NdhJ, NdhS, Fd, FNR.

2.2. Protein purification

Cells cultured for 4 days (A_{730} = 0.6–0.8) were harvested by centrifugation (5000×*g* for 5 min at 4 °C). Cells from 10 l of culture were suspended in 40 ml of medium A (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH, 5 mM sodium phosphate (pH 7.5), 10 mM $MgCl_2$ and 10 mM NaCl) supplemented with 25% glycerol and then stored at −80 °C. The thylakoids were isolated as described by Gombos et al. [21], with some modifications. The suspension was mixed with glass beads at a ratio of 1:1 (v/v) and disrupted by 5 pulses of 20 s with a Biospec Beadbeater (Biospec, Japan) followed by a 3 min incubation on ice. The homogenate was centrifuged at 5000×*g* for 5 min at 4 °C to remove unbroken cells and debris, and the supernatant was ultracentrifuged at 150,000×*g* for 40 min. The membranes in the pellet were resuspended and solubilised in 1% (w/v) *n*-dodecyl-β-D-maltoside (DM) with magnetic stirring on ice for 1 h and then further ultracentrifuged at 150,000×*g* for 40 min. The supernatant was immediately subjected to chromatographic separation.

2.3. Chromatographic separations

Membrane protein extracts were applied to a Q Sepharose Fast Flow column (Pharmacia, Sweden), equilibrated in buffer A (20 mM Tris–HCl pH 8.0 and 0.05% DM). Elution was performed with buffer B (1 M NaCl, 20 mM Tris–HCl pH 8.0, and 0.05% DM; flow rate 2 ml/min; fraction size 10 ml). Chromatographic fractions with NADPH oxidation activity were concentrated to 2 ml by ultrafiltration (YM 100 membrane; Amicon) at 4 °C. This extract was loaded onto a HiLoad 26/60 Superdex 200 column (Pharmacia, Sweden) connected to an AKTA system and previously equilibrated in buffer C (150 mM NaCl, 20 mM Tris–HCl pH 8.0, and 0.05% DM). The enzyme was eluted with buffer C (flow rate 1 ml/min; fraction size 1 ml). The fractions that exhibited NADPH oxidation activity were collected for further investigation.

2.4. Electrophoresis and protein identification

Native-PAGE was conducted on 7% polyacrylamide gels at 4 °C and a low constant current of 5 mA as described by Davis [22]. SDS–PAGE was carried out on 15% polyacrylamide gels at room temperature as described by Laemmli [23]. Protein bands were detected by Coomassie Brilliant Blue or $AgNO_3$. Protein bands on SDS gels were transferred to 0.2 mm nitrocellulose membranes. Immunoblot patterns were visualised with the Alkaline Phosphatase (AP) Assay Kit (Bio-Rad).

2.5. Kinetics assays

NADPH oxidase activity was measured spectrophotometrically as a decrease in absorbance at 340 nm (6.22 mM^{-1} cm^{-1}) at room temperature, as described by Matsuo [13]. The standard assay mixtures containing 1, 10, or 100 μM NADPH and 20 mM Tris–HCl (pH 8.0) were measured with a spectrophotometer (UV-3000, Shimadzu) to determine the effects of various pH values, electron acceptors and inhibitors.

2.6. Mass spectrometry

The purified complex was analysed by mass spectroscopy. The samples were prepared according to the manufacturer's instructions. In brief, bands were excised from the native gels, destained, dehydrated, vacuum-dried and incubated overnight with methylated porcine trypsin (Trypsin Gold, Promega). Peptides were analysed with MALDI-TOF using a Voyager-DE-STR mass spectrometer (Thermo Finnigan). Database searches were performed against a database of *T. elongatus* proteins supplemented with the sequences of common protein contaminants.

3. Results

3.1. Purification of the NDH complex

A summary of the purification of the protein complex responsible for the NADPH-oxidation activity in *T. elongatus* is shown in Table 1. Approximately 6 per-cent of the total NADPH-oxidation activity detected in the crude cell extraction was recovered in the fractions eluted from the HiLoad 26/60 Superdex 200 column as a single peak (Fig. 1A). Western blot analysis showed that the content of the NDH subunits NdhA, J, S (Fig. 1B) were proportioned to the NADPH oxidizing activity (Fig. 1), indicating the purified complex is an NADPH-active NDH. In addition, Fd and FNR were co-eluted with the NDH (Fig. 1B), suggesting that the interaction of Fd with the NDH and the electron donation from NADPH might be via FNR. The active protein eluted from the HiLoad 26/60 Superdex 200 column appeared as multiple bands after SDS–PAGE (Fig. 2A) and a single band with an apparent molecular weight about 170 kDa after native PAGE (Fig. 2B). In addition to NdhA, J, S, other NDH subunits NdhB, F, H, and I were identified by Western blot (Fig. 2C), and NdhA, H, and N were identified by mass spectrometric analysis (Table2) in the complex. Several phycobilisome

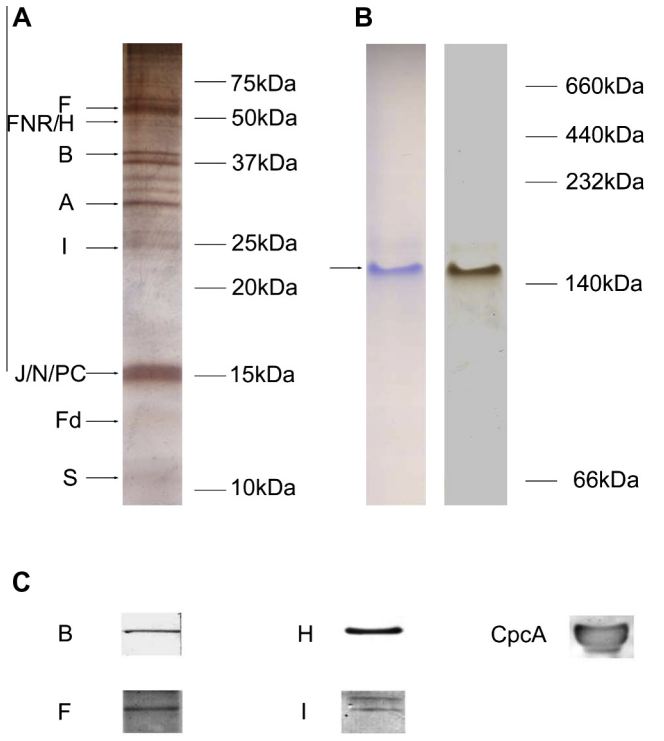

Fig. 2. Profiling of the NDH complex purified from *T. elongatus*. (A) 15% SDS–PAGE of the NDH complex. (B) Coomassie-stained (left) and $AgNO_3$-stained (right) 7% native PAGE of the NDH complex. (C) Western blot of the NDH complex with antibodies against various NDH subunits.

Table 2
Identification of NDH subunits from purified NDH complex of *T. elongatus* by mass-spectrometry analysis.

Pep count	Identified name
3	tel:tlr1288; NADH dehydrogenase I subunit H
2	tel:tlr1130; NADH dehydrogenase I subunit N
2	tel:tlr0667; NADH dehydrogenase I subunit A

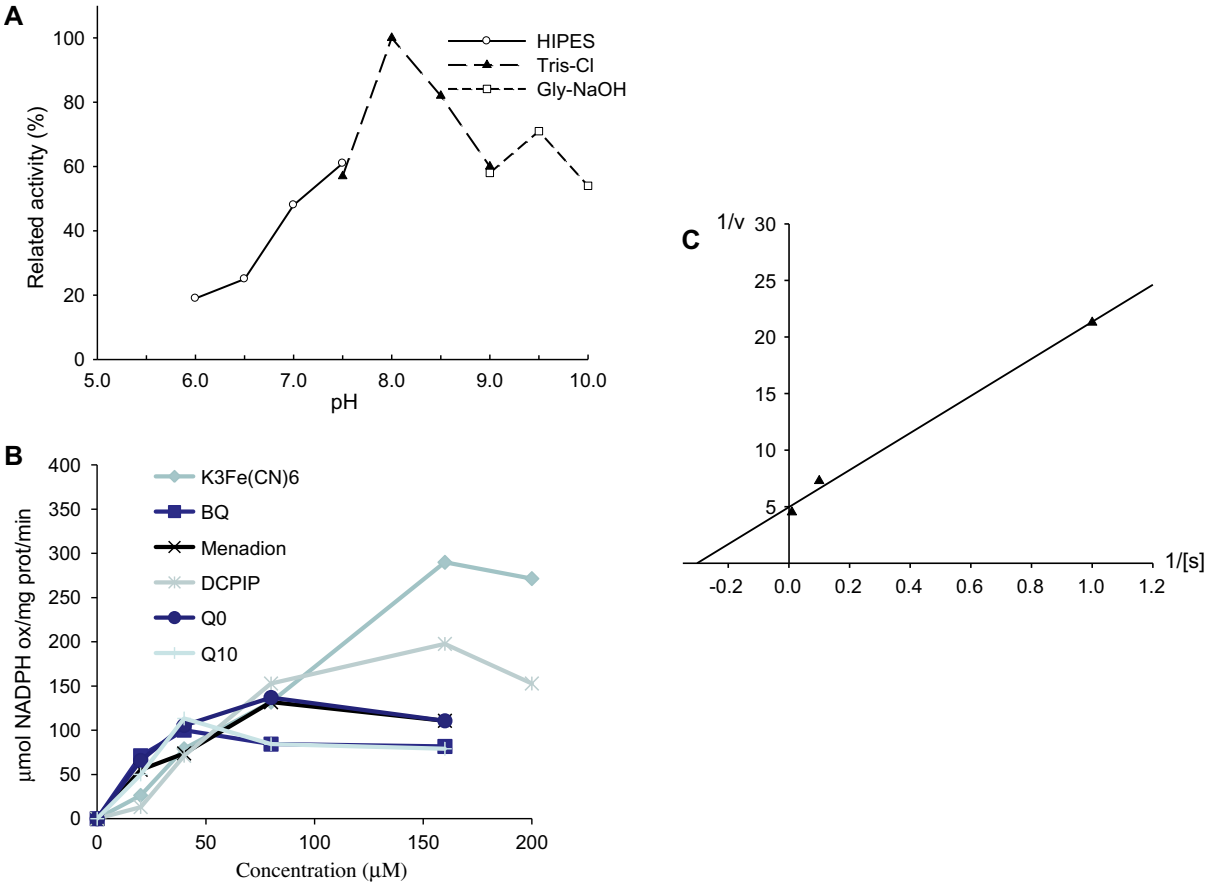

Fig. 3. Enzymatic properties of the purified NDH complex. (A) The effect of pH on the NADPH-$K_3Fe(CN)_6$ oxidoreductase activity of NDH complex. The NADH oxidation activity of the NDH complex was measured with 100 μM $K_3Fe(CN)_6$ as an electron acceptor at pH values ranging from 6.0 to 10.0 (6.0–7.5 buffered with 20 mM HEPES, 7.5–9.0 buffered with 20 mM Tris–HCl, and 9.0–10.0 buffered with 20 mM Gly-NaOH, respectively). (B) NADPH oxidation activity of the NDH complex with different electron acceptors, buffered with 20 mM Tris–HCl pH 8.0. The following electron acceptors $K_3Fe(CN)_6$, DCPIP, menadione (VK_3), ubiquinone-0 (Q_0), ubiquinone-10 (Q_{10}) were applied. (C) Apparent kinetics of the NADPH-$K_3Fe(CN)_6$ oxidoreductase activity of the NDH complex. NADPH-$K_3Fe(CN)_6$ oxidoreductase activity assays were performed with 0.36 mg/ml purified NDH complex and various NADPH concentrations (1, 10, 100 μM). The k_m for NADPH and V_{max} for NADPH-$K_3Fe(CN)_6$ oxidoreductase activity were calculated according to a Lineweaver–Burk plot.

proteins (PBSs) were also detected by Western blot (Fig. 2C), indicating that PBSs may bind to NDH subunits via interaction with FNR to accomplish some biological function.

3.2. Optimal pH, electron donors and electron acceptors of the purified active NDH

The dependence of NDH complex activity on pH and electron acceptors was investigated; the results of these investigations are shown in Fig. 3. The optimal pH of the NADPH oxidation of the complex was determined to be 8.0 in Tris–HCl buffer, which is the pH that *T. elongatus* is grown (Fig. 3A). The NDH complex had a higher affinity for PQ analogues than for $K_3Fe(CN)_6$ and 2,6-dichloroindophenol sodium salt dydrate (DCPIP) (Fig. 3B), implying that PQ is a suitable electron acceptor from NDH. We did not observe any NADH-$K_3Fe(CN)_6$ oxidoreductase activity in the complex with NADH as substrate (Supplementary Fig. 2), indicating that the active NDH specifically oxidises NADPH.

3.3. Kinetic properties of the active NDH

Apparent kinetic parameters (k_m and V_{max}) of the NDH complex were calculated using the Lineweaver–Burk plot method. At pH 8.0 and with $K_3Fe(CN)_6$ as the electron acceptor, experiments with different concentrations of NADPH (1, 10, 100 μM) as substrate showed that the k_m for NADPH was 3.30 μM and the V_{max} for NADPH-$K_3Fe(CN)_6$ oxidoreductase activity was 1.01 μmol min^{-1} (mg protein)$^{-1}$ (Fig. 3C).

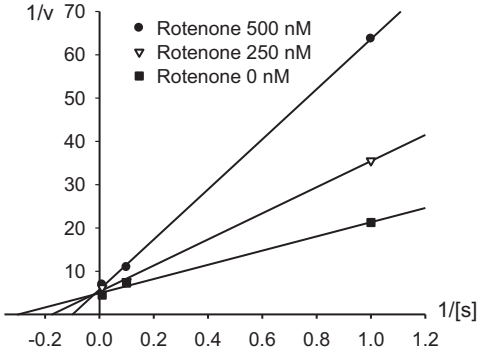

Fig. 4. The effect of rotenone on NADPH-$K_3Fe(CN)_6$ oxidoreductase activity. NADPH-$K_3Fe(CN)_6$ oxidoreductase activity was assayed as described in Fig. 2C except that 0 nM (control), 250 nM or 500 nM rotenone was added. The Lineweaver–Burk plot demonstrates that rotenone competitively inhibited the activity of the NDH complex.

3.4. The effect of rotenone on NDH activity

Because rotenone, a specific inhibitor of Complex I [24], competitively inhibited the NADPH oxidation activity of a cyanobacterium NDH complex [6], we investigated the effect of rotenone on

the NDH complex purified here. Lineweaver–Burk plot analysis showed that rotenone competitively inhibited the NADPH-oxidation activity of the complex with a K_i value of 0.26 μM (Fig. 4).

Taken together, the above results strongly suggested that the purified complex responsible for the NADPH-oxidation activity in vitro is identical to the NDH complex in *T. elongatus*.

4. Discussion

There have been reports describing the enzymatic properties of cyanobacterial NDH complexes. Matsuo calculated the k_m for NADPH of a hydrophilic NDH complex isolated from *Synechocystis* 6803 as 5.1 μM [13], and Deng reported the NADPH k_m of a hydrophobic NDH complex from *Synechocystis* 6803 as 6.5 μM [14]. Here, we showed that an NDH complex from *T. elongatus* has a k_m for NADPH of 3.30 μM (Fig. 3C). Although these NDH complexes are different, the values of their k_m are similar, implying they have common activity domain. We further found that the active NDH had a higher affinity for PQ analogues (Fig. 3B), suggesting that PQ is a suitable electron acceptor for the NDH.

Several distinct NDH subcomplexes, such as NDH-1L, NDH-1MS, NDH-1M, NDH-1S, were discovered by proteomics approaches [25–27]; these complexes are characterised by their distinct NDH subunit compositions. Although the active NDH purified in this report contained NdhA, B, F, H, I, S and N (Figs. 1 and 2), whose were found in NDH-1L, but its molecular weight was lower than that of NDH-1L and it contained FNR and Fd, indicating that it might be another NDH-1 complex. Furthermore, the previously isolated NDH was not inhibited by rotenone (Matsuo et al., 1998); this observation suggests that the previously isolated NDH was a hydrophilic subcomplex that had lost the subunit containing the quinone binding site. In contrast, the NDH purified in this study was sensitive to rotenone (Fig. 4), suggesting that it might contain a quinone binding site similar to that of mitochondria complex I, one of which crystal structure has been resolved very recently [28]. The active NDH also contained NdhS (Fig. 1), the new subunit of NDH identified in *Arabidopsis thaliana*[18]and in *Synechocystis* 6803 [19] responding to the activity of NDH pathway. According to the structure similarity of NdhS with PsaE, the authors suggested that NdhS might bind with Fd which might be an electron donor for NDH. We further detected Fd and FNR co-eluted with the active NDH (Fig. 1), indicating the donation of electron from NADPH to NDH via FNR, as suggested in chloroplast NDH [29]. It has been reported that Fd and FNR form a 1:1 complex, which is stabilized by electrostatic interactions between acidic residues of Fd and basic residues of FNR [30]. Therefore, the low purification fold (Table 1) in this work might be attributive to the dissociation of FNR from the NDH complex during ion exchange isolation and ultrafiltration. However, solid evidence to support the interaction of NDH with Fd and FNR is still lacking in the present study. Such a possibility warrants further investigation. Ndh genes in cyanobacterial genomes show a high homology to those in chloroplast genomes [31]. Therefore, both the NDHs might have similar activity site. It has been indicated that different plastids, derived from proplastids, may have bioenergetic functions and redox chemistry even in the absence of photosynthesis, in which case the NAD(P)H dehydrogenase genes may be retained for the same reason as respiratory complex I in mitochondria of both plant and animal cells [32].

The photosynthetic machinery regulates the distribution of excitation energy between PSI and PSII under fluctuating light conditions. This dynamic and rapid achievement of energy balance is called "state transition" [33,34]. Because PBSs movement is a prerequisite for cyanobacterial state transitions [35–39], "mobile PBSs" are believed to play key roles in allowing state transitions in cyanobacteria. Previous studies have shown that light-induced state transitions is fully [40] or partially [41] dependent on "mobile PBS" in cyanobacteria. Another study further showed that the movement of PBSs between PSII and PSI affects both cyclic and respiratory electron transport [42]. The inactivation of *ndh*B locked the *Synechocystis* PCC 6803 in state 1 [43]. PBSs such as CpcA were observed in our purified NDH complex through western blot (Fig. 2), probably due to the interaction of the co-eluted FNR (Fig. 1) with PBS [44]. Based on these results, we suggest that PBS and NDH subunits might functionally bind to and be involved in the redistribution of excitation energy in cyanobacteria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.05.040.

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