Short Communication

Active NDH-1 Complexes from the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

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We identified eight bands by staining native gels for NADPH-nitroblue tetrazolium oxidoreductase activity after electrophoresis of n-dodecyl-β-D-maltoside-treated membranes of *Synechocystis* sp. strain PCC 6803. Among them, bands A, C, D and E were attributed to the activity of NDH-1 dehydrogenase (NDH-1). Band A is a highly active supercomplex of NDH-1 (about 1,000 kDa) that was absent in the ΔndhD1/D2 mutant and was suppressed under low CO2. Band C was induced under low CO2 or in the ΔndhD1/D2 mutant and was converted to bands D and E. Bands A and C appear to be an NDH-1L dimer and NDH-1M, respectively, with subunits essential for the activity.

Keywords: Active NDH-1 complexes — Activity staining — *Synechocystis* sp. strain PCC 6803.

Abbreviations: DM, n-dodecyl-β-D-maltoside; FNR, ferredoxin-NADP⁺ oxidoreductase; H-cells, high CO2-grown cells; L-cells, low CO2-grown cells; NBT, nitroblue tetrazolium; NDH-1, type-1 NAD(P)H dehydrogenase; *Synechocystis* 6803, *Synechocystis* sp. strain PCC 6803; WT, wild-type.

In cyanobacteria, the type-1 NAD(P)H dehydrogenase (NDH-1) contains at least 15 subunits (NdhA-O; Herranen et al. 2004, Prommeenate et al. 2004, Zhang et al. 2004, Battchikova et al. 2005), which are encoded by genes homologous to the chloroplast and mitochondrial *ndh* genes (Ohyama et al. 1986, Kaneko et al. 1996). A hydrophilic subcomplex with a molecular mass of about 380 kDa active in NADPH oxidation has been isolated from the cyanobacterium *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis* 6803) by chromatography of the cell homogenate treated with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Matsuo et al. 1998). By improving their method, Deng et al. (2003c) identified a low CO2-inducible NDH-1 complex of about 380 kDa that contains the NdhA subunit and is active in NADPH oxidation. Recently, three NDH-1 complexes have been identified in *Synechocystis* 6803 (Herranen et al. 2004, Prommeenate et al. 2004, Zhang et al. 2004) and *Thermosynechococcus elongatus* (Zhang et al. 2005) by blue native (BN)-PAGE. These NDH-1 complexes, named NDH-1L, NDH-1M and NDH-1S, showed apparent molecular masses of approximately 460, 330 and 190 kDa, respectively. The NDH-1 complex with a molecular mass of about 550 kDa in maize chloroplasts forms dimers of 1,000–1,100 kDa and splits into 300 and 250 kDa subcomplexes as analyzed by BN-PAGE combined with mass spectrometry (Darie et al. 2005). However, none of these complexes in cyanobacteria and chloroplasts showed NADPH oxidation activity.

In this work, we identified NDH-1 complexes of *Synechocystis* 6803 active in NADPH oxidation. We compare the wild-type (WT) and mutant strains of *Synechocystis* 6803 for the presence of these active complexes and the response of their activity to high or low CO2. A supercomplex of NDH-1 (about 1,000 kDa) was first identified and was suppressed under low CO2. A possible requirement for active NDH-1 complexes is discussed.

Fig. 1A and B shows the profiles of native gels stained for NADH- and NADPH-nitroblue tetrazolium (NBT) oxidoreductase activities, respectively, after electrophoresis of n-dodecyl-β-D-maltoside (DM)-treated thylakoid membranes isolated from high CO2-grown (H)-cells of WT *Synechocystis* 6803. Eight bands (A–H) were identified for the activity of NADPH-NBT-oxidoreductase, with the most active band (A) at the apparent molecular mass of about 1,000 kDa (Fig. 1B). There was no evident active band of NADH-NBT-oxidoreductase (Fig. 1A). Western analysis indicated that the antibody against NdhI cross-reacted with bands A, B, C, D and E. The antibody cross-reacted much more strongly with bands C and E than with bands A and B, although band A was the most densely stained for the NADPH-NBT-oxidoreductase activity (Fig. 1B, C). The activity of band A is thus much higher than that of the other bands on a protein basis. Western analysis using the antibody against

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ferredoxin-NADP$^+$ oxidoreductase (FNR) showed that the antibody cross-reacted only with band H (Fig. 1D), indicating that this active band is derived from the activity of FNR but other bands are not.

In order to see if bands A, B, C, D and E represent the activity of NDH-1 complexes, similar activity staining was carried out on H-cells of two mutant strains, ΔndhD1/D2 and M55 (ΔndhB) (Fig. 2). As described later, band E is derived from band C under certain conditions but band E was not detected in this experiment even in the WT. The results indicated that bands A, C and D were absent in M55 (lane C in Fig. 2) but band B was present in both M55 and ΔndhD1/D2 strains (lanes B and C in Fig. 2). It has been reported that H-cells of M55 do not contain any NDH-1 complexes and that the ΔndhD1/D2 mutant does not contain NDH-1L similar in size to band B (Zhang et al. 2004). Thus, bands A, C and D represent the activity of NDH-1. This was confirmed by Western analysis using the antibodies against five subunits of NDH-1 showing the presence of the membrane and peripheral subunits of NDH-1 in these bands (Fig. 3). Western analysis with the same antibodies also indicated the presence of all these subunits in the position of band B in the WT (Fig. 3) but not in ΔndhD1/D2 and M55 (data not shown). The presence of band B in these mutants indicates that the band does not represent the activity of the NDH-1 complex but shows the activity of some unknown protein complex. Band C was present in ΔndhD1/D2, indicating that this band does not contain NdhD1 or NdhD2. Band C was more strongly stained in ΔndhD1/D2 than in the WT. The result can be explained by the fact that deletion of NdhD1 from NDH-1L led to the formation of NDH-1M (Zhang et al. 2004). Band C could be attributed to the activity of an NDH-1M-like complex that does not contain NdhD1 or NdhD2, and the increase of band C in ΔndhD1/D2 could be the result of conversion of band A to band C by deletion of NdhD1. The absence of band A in ΔndhD1/D2 indicates that band A contains NdhD1 and/or NdhD2 and is an NDH-1L-like complex (Herranen et al. 2004, Zhang et al. 2004).
However, the molecular mass of band A is more than twice that of NDH-1L, suggesting that it could be a dimer of NDH-1L with additional unknown subunits. The complex at the position of band B was similar in size to NDH-1L but its NADPH oxidation activity was hardly detectable. The results suggested that the complex at the position of band B is NDH-1L that is a broken product of the supercomplex and has lost the subunits essential to the activity.

Matsuo et al. (1998) and Deng et al. (2003c) have isolated NDH-1 complexes active in NADPH oxidation activity, using CHAPS and DM, respectively. These complexes were similar in size to band C (Fig. 1). Band b1 identified by Deng et al. (2003c) was induced under low CO₂ and could be identical to band C in this study. They also identified band b2 that is smaller than band b1 and suggested that band b2 was derived from band b1 especially at high temperature (Deng et al. 2003c).
Probably band b2 is identical to band D or E in this study, which was derived from band C.

The activity staining used in this study reflects the diaphorase activity and, therefore, also shows the activity of FNR. Western analysis using the antibody against FNR indicates that only band H contains FNR (Fig. 1D). Judging from the molecular mass, band H is considered to be dimeric FNR. Since no other bands contained FNR, this enzyme is not associated with any of the NDH-1 complexes to accept electrons from NADPH.

Band C does not contain NdhD1 and/or NdhD2 but shows NADPH oxidation activity (Fig. 2). NdhF might also be absent in band C, since this subunit is present next to NdhD on the outer side of the complex (Casano et al. 2004). This indicates that NdhD and NdhF are not essential for the NADPH oxidation activity. A dimeric structure may be important to achieve the high activity of band A but is not prerequisite for the activity because of band C (monomeric). The absence or low activity of the NDH-1 complex at the position of band B indicates that this complex lacks the subunit(s) essential for the activity. The fact that bands D and E still possess the activity indicates that the complexes in these bands still possess the subunit(s) needed for the activity. It is not known what subunits were deleted when band C was degraded to bands D and E. These subunits are not NdhA, NdhB, NdhH, NdhF or NdhK (see Fig. 3). They may be other peripheral subunits already identified or those not yet identified.

**Materials and Methods**

Cells of WT *Synechocystis* 6803 and its specific *ndh* gene knockout mutants M55 (Δ*ndhB*) and ΔndhD1/ΔndhD2 (Ogawa 1991, Ohkawa et al. 2000) were cultured at 30°C in BG-11 medium (Allen 1968) buffered with Tris–HCl (5 mM, pH 8.0) and bubbled with 2% (v/v) CO2 in air, under continuous illumination by fluorescent lamps (40 μE(m²·s)⁻¹). WT cells were also bubbled with air. The mutant strains were grown in the presence of appropriate antibiotics. Cells cultured for 4d (A₅₇₀m=0.6–0.8) that showed the highest light-dependent NADPH oxidation activity (Ma and Mi 2005) were harvested by centrifugation (5,000 g for 5 min at 4°C). Cells from 11 of culture were suspended in 5 ml of medium A [10 mM HEPES-NaOH, 5 mM sodium phosphate (pH 7.5), 10 mM MgCl₂ and 10 mM NaCl] supplemented with 25% (v/v) glycerol, and the suspension was mixed with 13 g of glass beads and kept on ice for 1h. Cells were then disrupted by 10 pulses of 10 s with a Bead-beater (BioSpec, Japan) followed by 5 min incubation on ice. The activity dropped sharply as the duration of the pulses increased. Almost no activity of the supercomplex (band A) was found when the duration of the pulses was 30 s. The homogenate was centrifuged at 5,000 g for 5 min at 4°C to remove unbroken cells and debris. The supernatant was stored at −30°C for about 2 years without losing the NADPH oxidation activity. Membranes in the supernatant were solubilized with 1.2% (w/v) DM while shaking on ice for 1h. The samples were then immediately subjected to native-PAGE.

Native-PAGE was run overnight using 7.0% polyacrylamide gels at 0°C and low constant current of 3 mA according to the method of Davis (1964). The NADPH-specific enzyme activity was incubated as described elsewhere (Deng et al. 2003a) with some modifications. Briefly, following native-PAGE, gels were incubated in 20 mM Tris–HCl (pH 7.5) and 0.1% (w/v) NBT for 20 min, and then supplemented with 1 mM NAD(P)H in the dark at room temperature to stain the NAD(P)H-NBT oxidoreductase.

The active bands were excised from the native gels and incubated in medium B [10 mM Tris–HCl (pH 8.0), 0.1 g l⁻¹ SDS and 0.1% (w/v) DM] overnight at 30°C with shaking. The mixture was then centrifuged at 10,000 g for 10 min. The supernatant was concentrated by 75% (v/v) cool acetone and then subjected to SDS-PAGE for Western blot.

SDS-PAGE was carried out on 12% polyacrylamide gels according to the method of Laemmli (1970). Immunoblotting was performed with an ECL assay kit (Amersham Pharmacia), according to the manufacturer’s protocol. The antibodies against NdhH, NdhI, NdhK and FNR of *Synechocystis* 6803 were raised in our laboratory (Ma and Mi 2005; this study). The antibodies against NdhA and NdhB of *Synechocystis* 6803 were kindly provided by Professor Asada (Department of Biotechnology, Faculty of Engineering, Fukuyama University).

**Acknowledgments**

This work was partially supported by grants to H.M. from the National Natural Science Foundation of China (Nos. 30470151 and 90306013) and in part by a grant to T.O. from the Membrane Biology EMSL Scientific Grand Challenge Project at the W. R. Wiley Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the US Department of Energy Office of the Biological and Environmental Research program located at Pacific Northwest National Laboratory.

Pacific Northwest National Laboratory is operated for the Department of Energy by Battelle. The authors are grateful to Professor Asada (Department of Biotechnology, Faculty of Engineering, Fukuyama University) for kindly providing the antibodies against NdhA and NdhB.

**References**


(Received July 27, 2006; Accepted September 11, 2006)