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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 NADPH dehydrogenase (NDH-1). Band A is a highly active supercomplex of NDH-1 (about 1,000 kDa) that was absent in the Δ*ndhD1/D2* mutant and was suppressed under low CO_2. Band C was induced under low CO_2 or in the Δ*ndhD1/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 CO_2-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 CO_2-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 CO_2. A supercomplex of NDH-1 (about 1,000 kDa) was first identified and was suppressed under low CO_2. 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 CO_2-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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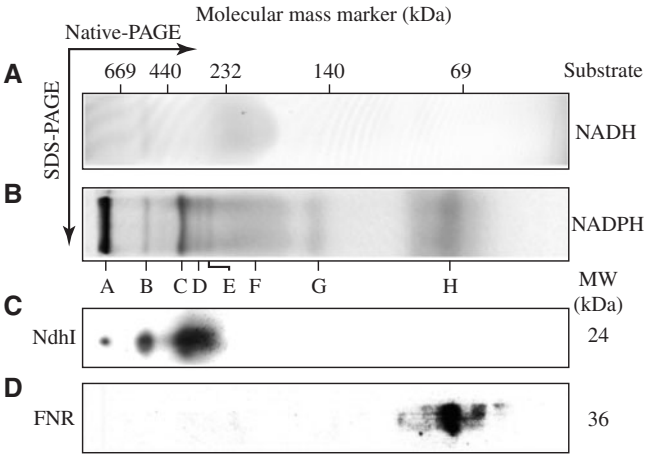

Fig. 1 The profiles of native gels stained for the activities of NADH-NBT oxidoreductase (A) and NADPH-NBT oxidoreductase (B) after electrophoresis of DM-treated thylakoid membranes isolated from H-cells of WT *Synechocystis* 6803 and Western analyses with the antibodies against NdhI (C) and FNR (D) after native- and SDS–PAGE, respectively.

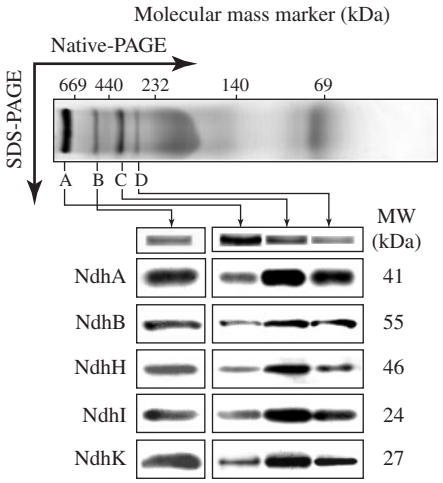

Fig. 3 Western blot analysis of proteins in bands A, B, C and D using the antibodies raised against hydrophobic subunits (NdhA and NdhB) and hydrophilic subunits (NdhH, NdhI and NdhK).

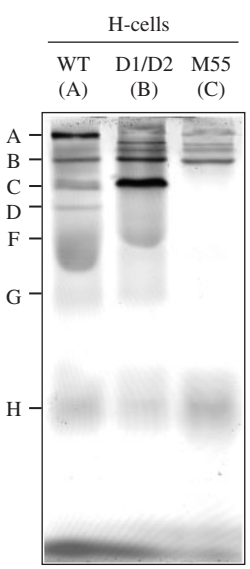

Fig. 2 The profiles of the native gel stained for the NADPH-NBT oxidoreductase activity after electrophoresis of DM-treated thylakoid membranes isolated from H-cells of WT (lane A), Δ*ndhD1/D2* (lane B) and M55 (lane C) mutants of *Synechocystis* 6803.

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, Δ*ndhD1*/*D2* and M55 (Δ*ndhB*) (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 Δ*ndhD1*/*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 Δ*ndhD1*/*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 Δ*ndhD1*/*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 Δ*ndhD1*/*D2*, indicating that this band does not contain NdhD1 or NdhD2. Band C was more strongly stained in Δ*ndhD1*/*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 Δ*ndhD1*/*D2* could be the result of conversion of band A to band C by deletion of NdhD1. The absence of band A in Δ*ndhD1*/*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).

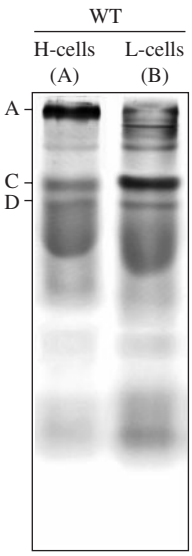

Fig. 4 Effects of CO_2 concentration on the activity of bands A, C and D.

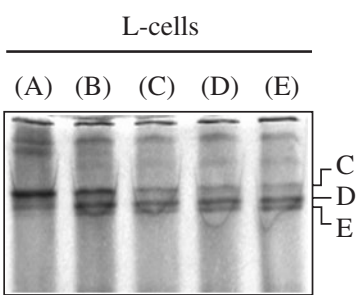

Fig. 5 Effect of temperature and incubation time on the activity of bands C, D and E. Thylakoid membranes isolated from L-cells of WT *Synechocystis* 6803 were treated with DM on ice for 1 h (A) or at 30°C for 0.5 h (B), 1 h (C), 1.5 h (D) and 2 h (E).

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 and could be a monomer of this complex, although the activity was low, if any.

The activity of NDH-1 complexes and the amount of their subunits are affected by the CO_2 concentration (Deng et al. 2003a, Deng et al. 2003b, Zhang et al. 2004). We have examined the effect of the CO_2 concentration on the activity of NDH-1 complexes. Lanes A and B in Fig. 4 show the profiles of an activity stained native gel obtained for the thylakoid membranes of high CO_2-grown (H)-cells and low CO_2-grown (L)-cells, respectively. The activity of band A was almost completely suppressed in L-cells, whereas that of band C was remarkably increased. It has been reported that the amount of NDH-1L is high in H-cells but low in L-cells (Zhang et al. 2004). These previous results are consistent with our conclusion that bands A and C represent the activities of NDH-1L-like and NDH-1M-like complexes, respectively. Furthermore, band C appears to be identical to band I isolated from L-cells of *Synechocystis* 6803 and identified by activity staining (Deng et al. 2003c). Fig. 5 shows the profiles of activity staining for the membranes isolated from L-cells that had been treated with DM at room temperature for various periods. The profiles indicate that bands D and E increased and band C decreased as the incubation time was prolonged. The result suggested that bands D and E were derived from band C.

Cyanobacteria possess multiple NDH-1 complexes that are essential to respiration, cyclic electron transport and CO_2 uptake. Recent proteomics analysis identified three NDH-1 complexes named NDH-1L, NDH-1M and NDH-1S (Herranen et al. 2004, Zhang et al. 2004). However, none of these complexes showed NADPH oxidation activity. The present study demonstrates for the first time the presence of an NDH-1 supercomplex (band A) that is highly active in NADPH oxidation in *Synechocystis* 6803 cells. The absence of band A in the Δ*ndhD1*/*D2* mutant indicates that this complex contains NdhD1 and/or NdhD2, and is similar in composition to NDH-1L. The size of this complex was, however, more than twice that of NDH-1L and was similar to the supercomplex of NDH-1 found in maize chloroplasts (Darie et al. 2005). NDH-1 complexes of similar size highly active in NADPH oxidation have been identified in spinach and tobacco chloroplasts (H. Mi unpublished data). Thus, the presence of a highly active supercomplex of NDH-1 appears to be common in cyanobacteria and chloroplasts. Recent electron microscopy analysis of the cyanobacterium *T. elongates* showed the presence of a low abundance type of U-shaped NDH-1, in addition to the normal L-shaped NDH-1 complexes (Arteni et al. 2006). It is possible that this U-shaped complex is a supercomplex of NDH-1.

The NDH-1 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 CO_2 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, NdhI 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 Δ*ndhD1*/*ndhD2* (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) CO_2 in air, under continuous illumination by fluorescent lamps (40 $\mu Em^{-2}s^{-1}$). WT cells were also bubbled with air. The mutant strains were grown in the presence of appropriate antibiotics.

Cells cultured for 4 d ($A_{730} = 0.6$–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 1 l of culture were suspended in 5 ml of medium A [10 mM HEPES-NaOH, 5 mM sodium phosphate (pH 7.5), 10 mM $MgCl_2$ and 10 mM NaCl] supplemented with 25% glycerol, and the suspension was mixed with 13 g of glass beads and kept on ice for 1 h, 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 1 h. 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 measured 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).

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References

Allen, M.M. (1968) Simple conditions for growth of unicellular blue-green algae on plates. *J. Phycol.* 4: 1–4.

Arteni, A.A., Zhang, P., Battchikova, N., Ogawa, T., Aro, E.M. and Boekema, E.J. (2006) Structural characterization of NDH-1 complexes of *Thermosynechococcus elongatus* by single particle electron microscopy. *Biochim. Biophys. Acta* In press.

Battchikova, N., Zhang, P., Rudd, S., Ogawa, T. and Aro, E.M. (2005) Identification of NdhL and Ssl1690 (NdhO) in NDH-1L and NDH-1M complexes of *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* 280: 2587–2595.

Casano, L.M., Lascano, H.R., Martin, M. and Sabater, B. (2004) Topology of the plastid Ndh complex and its NDH-F subunit in thylakoid membranes. *Biochem. J.* 382: 145–155.

Darie, C.C., Biniossek, M.L., Winter, V., Mutschler, B. and Haehnel, W. (2005) Isolation and structural characterization of the Ndh complex from mesophyll and bundle sheath chloroplasts of *Zea mays*. *FEBS J.* 272: 2705–2716.

Davis, B.J. (1964) Disc electrophoresis. II. Method and application to human serum proteins. *Ann. NY Acad. Sci.* 121: 404–427.

Deng, Y., Ye, J. and Mi, H. (2003a) Effects of low CO_2 on NAD(P)H dehydrogenase, a mediator of cyclic electron transport around photosystem I in the cyanobacterium *Synechocystis* PCC 6803. *Plant Cell Physiol*. 44: 534–540.

Deng, Y., Ye, J., Mi, H. and Shen, Y. (2003b) Response of NAD(P)H dehydrogenase complex to the alteration of CO_2 concentration in the cyanobacterium *Synechocystis* PCC 6803. *J. Plant Physiol*. 160: 967–970.

Deng, Y., Ye, J., Mi, H. and Shen, Y. (2003c) Separation of hydrophobic NAD(P)H dehydrogenase subcomplexes from cyanobacterium *Synechocystis* PCC6803. *Acta Biochim. Biophys. Sinica* 35: 723–727.

Herranen, M., Battchikova, N., Zhang, P.P., Graf, A., Sirpio, S., Paakkarinen, V. and Aro, E.M. (2004) Towards functional proteomics of membrane protein complexes in *Synechocystis* sp. PCC 6803. *Plant Physiol*. 134: 470–481.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., et al. (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res*. 3: 109–136.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Ma, W. and Mi, H. (2005) Expression and activity of type-1 NAD(P)H dehydrogenase at different growth phases of cyanobacterium, *Synechocystis* PCC 6803. *Physiol. Plant*. 125: 135–140.

Matsuo, M., Endo, T. and Asada, K. (1998) Properties of the respiratory NAD(P)H dehydrogenase isolated from the cyanobacterium *Synechocystis* PCC6803. *Plant Cell Physiol*. 39: 263–267.

Ogawa, T. (1991) A gene homologous to the subunit-2 gene of NADH dehydrogenase is essential to inorganic carbon transport of *Synechocystis* PCC 6803. *Proc. Natl Acad. Sci. USA* 88: 4275–4279.

Ohkawa, H., Pakrasi, H.B. and Ogawa, T. (2000) Two types of functionally distinct NAD(P)H dehydrogenases in *Synechocystis* sp. strain PCC 6803. *J. Biol. Chem*. 275: 31630–31634.

Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umersono, K., Inokuchi, H. and Ozeki, H. (1986) Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. *Nature* 322: 572–574.

Prommeenate, P., Lennon, A.M., Markert, C., Hippler, M. and Nixon, P.J. (2004) Subunit composition of NDH-1 complexes of *Synechocystis* sp. PCC 6803: identification of two new ndh gene products with nuclear-encoded homologues in the chloroplast Ndh complex. *J. Biol. Chem*. 279: 28165–28173.

Zhang, P., Battchikova, N., Jansen, T., Appel, J., Ogawa, T. and Aro, E.M. (2004) Expression and functional roles of the two distinct NDH-1 complexes and the carbon acquisition complex NdhD3/NdhF3/CupA/Sll1735 in *Synechocystis* sp. PCC 6803. *Plant Cell* 16: 3326–3340.

Zhang, P., Battchikova, N., Paakkarinen, V., Katoh, H., Iwai, M., Ikeuchi, M., Pakrasi, H.B., Ogawa, T. and Aro, E.M. (2005) Isolation, subunit composition and interaction of the NDH-1 complexes from *Thermosynechococcus elongatus* BP-1. *Biochem. J*. 390: 513–520.

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