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RESEARCH PAPER

Co-ordination of NDH and Cup proteins in CO_2 uptake in cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract

High and low affinity CO_2-uptake systems containing CupA (NDH-1MS) and CupB (NDH-1MS′), respectively, have been identified in *Synechocystis* sp. PCC 6803, but it is yet unknown how the complexes function in CO_2 uptake. In this work, we found that deletion of *cupB* significantly lowered the growth of cells, and deletion of both *cupA* and *cupB* seriously suppressed the growth below pH 7.0 even under 3% CO_2. The rate of photosynthetic oxygen evolution was decreased slightly by deletion of *cupA* but significantly by deletion of *cupB* and more severely by deletion of both *cupA* and *cupB*, especially in response to changed pH conditions under 3% CO_2. Furthermore, we found that assembly of CupB into NDH-1MS′ was dependent on NdhD4 and NdhF4. NDH-1MS′ was not affected in the NDH-1MS-degradation mutant and NDH-1MS was not affected in the NDH-1MS′-degradation mutants, indicating the existence of independent CO_2-uptake systems under high CO_2 conditions. The light-induced proton gradient across thylakoid membranes was significantly inhibited in *ndhD*-deletion mutants, suggesting that NdhDs functions in proton pumping. The carbonic anhydrase activity was suppressed partly in the *cupA*- or *cupB*-deletion mutant but severely in the mutant with both *cupA* and *cupB* deletion, indicating that CupA and CupB function in conversion of CO_2 to HCO_3^-. In turn, deletion of *cup* genes lowered the transthylakoid membrane proton gradient and deletion of *ndhD*s decreased the CO_2 hydration. Our results suggest that NDH-1M provides an alkaline region to activate Cup proteins involved in CO_2 uptake.

Key words: CO_2 uptake, CupA, CupB, NDH-1MS, NDH-1MS′, *Synechocystis* sp. PCC 6803.

Introduction

Cyanobacteria possess a CO_2-concentrating mechanism (CCM) that enables the accumulation of inorganic carbon (HCO_3^- and CO_2, collectively called Ci) at the carboxylation site to a level for efficient CO_2 fixation despite the low affinity of their Rubisco for CO_2 (Kaplan and Reinhold, 1999; Ogawa and Kaplan, 2003). The CCM requires the coordination of two systems, an inorganic carbon transporter system and the carboxysome containing Rubisco. To date, five inorganic carbon transporters have been found, including two Na^+-dependent HCO_3^- transporters (BicA and SbtA), one ATPase-dependent HCO_3^- transporter (BCT1), and two CO_2-uptake NDH-1 complexes, in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) and other cyanobacterial strains (Ogawa and Kaplan, 2003; Ogawa and Mi, 2007; Price, 2011). One

Abbreviations: CCM, CO_2-concentrating mechanism; CET, cyclic electron flow; NDH, NAD(P)H dehydrogenase; PQ, plastoquinone; PS, photosystem.

of the CO_2 uptake complexes, the NDH-1MS′ complex, consists of NdhD4, NdhF4 and CupB (ChpX) and is a constitutive system with weaker uptake affinity for CO_2; another one, the NDH-1MS complex, consists of NdhD3, NdhF3 and CupA (ChpY), and is inducible at limiting Ci conditions and has a higher uptake affinity for CO_2 (Ohkawa *et al.*, 2000*b*; Shibata *et al.*, 2001; Maeda *et al.*, 2002). According to the mutant phenotype, the expression of the *ndhF3-ndhD3-cupA-sll1735* operon is induced in both *Synechocystis* 6803 and *Synechococcus* sp. PCC 7002 cells grown under low CO_2 condition (Ohkawa *et al.*, 2000*b*; Shibata *et al.*, 2001; Maeda *et al.*, 2002). Further research showed that the proteins encoded by *ndhF3-ndhD3-cupA-sll1735* form a small complex, NDH-1S, in which CupA and a small protein, CupS, were identified as subunits by proteomic analysis (Zhang *et al.*, 2004, 2005). The *ndhB*-defective mutant M55 was shown to be unable to grow under low CO_2 conditions even when NDH-1S was present, suggesting that the normal operation of the CO_2-uptake system requires both NDH-1M and NDH-1S. A complex (NDH-1MS) containing both NDH-1S and NDH-1M has been isolated from a *Thermosynechococcus elongatus* strain in which the C-terminus of NdhL has been tagged with 6xHis. This complex is easily dissociated into NDH-1M and NDH-1S complexes (Zhang *et al.*, 2005; Battchikova *et al.*, 2011). NDH-1MS has been characterized as a U-shape structure by analysis by single particle electron microscopy after purification from the thylakoid membranes of *Thermosynechoccus elongates* (Arteni *et al.*, 2006). CupA is responsible for the U-shape by binding at the tip of the membrane-bound arm of NDH-1MS in *Thermosynechoccus elongatus* and *Synechocystis* 6803 (Folea *et al.*, 2008). Although the constitution and the function of NDH-1MS have been studied, the underlying mechanism explaining the functional link between NDH-1M and NDH-1S or NDH-1S′ still remains to be resolved.

Reverse genetic studies have indicated an essential role for the *cupB* gene, a homolog of *cupA*, in constitutive CO_2 uptake and suggest that the proteins encoded by *ndhF4-ndhD4* and *cupB* form the small complex, NDH-1S′ (Shibata *et al.*, 2001; Maeda *et al.*, 2002). In a previous study, we reported that the CupB protein in *Synechocystis* resides in the thylakoid membrane but is missing from the *ndhD4* deletion mutant (Xu *et al.*, 2008). Based on the purification of a 450 kDa complex containing both NdhH and CupB proteins, we suggested that the complex is NDH-1MS′ residing in the thylakoid membranes. However, the function of NDH-1MS′ still needs elucidation.

It has been suggested that there exist two independent CO_2 uptake systems, NDH-1MS and NDH-1MS′, in which NdhDs function in proton pumping (Battchikova *et al.*, 2011), Cups function in CO_2 hydration as carbonic anhydrase (CA)-like proteins (Kaplan and Reinhold, 1999; Price, 2011). However, whether NDH-1MS induced by low CO_2 exists or functions under high CO_2 conditions, and whether or how it relates to the constitutive type NDH-1MS′ still remain to be clarified.

In this work, we investigated the function of NDH-1MS and NDH-1MS′ in CO_2 uptake using reverse genetics and biochemical methods. Our results suggest that NDH-1MS and NDH-1MS′ are essential for efficient CO_2 uptake especially under changed pH conditions. We proposed a model for the function of CO_2 uptake systems in *Synechocystis* 6803.

Materials and methods

Cell culture conditions

Wild type (WT) and mutant cells of *Synechocystis* 6803 were grown at 30 °C in 50 ml liquid BG11 medium buffered with 5 mM Tris-HCl (pH 8.0) and bubbled with 3% v/v CO_2 in air at 3 ml min^{-1}. The mutant strains were grown in liquid BG11 medium with appropriate antibiotics, and cell cultures were harvested at the logarithmic phase (OD_{730}=0.6–0.8). Solid medium was BG11 supplemented with 1.5% agar. Continuous illumination was provided by fluorescent lamps, generating 50 µmol of photons m^{-2} s^{-1}.

Construction and isolation of mutants

Construction of single mutants, such as *ΔcupA*, *ΔcupB*, *ΔndhD1*, *ΔndhD2*, *ΔndhD3*, *ΔndhD4*, *ΔndhL*, *ΔndhK*, and M55, has been described in previous studies (Ogawa, 1991; Ohkawa *et al.*, 2000*a*; Shibata *et al.*, 2001). BHM is a *Synechocystis* 6803 mutant with CupB tagged with 6xHis-cMyc at the C-terminus, which has also been described before (Xu *et al.*, 2008). Those constructions of single mutants were used to transform various appropriate mutants to generate the double mutants or triple mutants, i.e. *ΔcupA*/*B*, *ΔndhD1*/*D2*, *ΔndhD3*/*D4*, and *ΔndhD1*/*D2*/*D3*. The *ΔndhF4*/BHM and *ndhL-YFP-6His* were also described in previous studies (Xu *et al.*, 2008; Birungi *et al.*, 2010). To construct the *ΔndhF1* mutant, its coding region was inserted by a chloramphenicol (CM) resistance cassette as follows. The *ndhF1* upstream and downstream regions were amplified using primer pairs hxl108/hxl109 and hxl112/113, with CM resistance cassette using primers hxl110/hxl111 (see Supplementary Table S1 at *JXB* online). These three fragments were used as the template to synthesize an Up-CM-Dn fragment through overlap PCR, and this was ligated into the T-vector to make the construct for transforming the wild type of *Synechocystis* 6803 (Supplementary Fig. S1A). The *ΔndhF3* mutant was made with the same strategy, using primer pairs hxl114/hxl115 and hxl118/119, with the CM resistance cassette using the primers pair hxl116/hxl117 (see Supplementary Table S1). The plasmids were separately transformed into BHM to generate the double mutants, *ΔndhF1*/BHM and *ΔndhF3*/BHM. The mutated genes in the transformants were segregated to homogeneity (by successive streak purification) as determined by PCR amplification (see Supplementary Fig. S1B).

Isolation of soluble fractions and total membrane fractions

Soluble fractions and total membrane fractions of *Synechocystis* 6803 cells were isolated as described previously with slight modifications (He and Mi, 2016).

Electrophoresis and immunoblotting

SDS-PAGE of thylakoid membranes from *Synechocystis* 6803 was carried out on a 1.0 mm thick, 12% polyacrylamide gel (Laemmli, 1970). Blue native (BN)-PAGE of *Synechocystis* 6803 membranes was performed as described previously with modifications from He *et al.* (2016). After electrophoresis, the proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes and detected with specific antibodies. Finally, an ECL assay kit was used according to the manufacturer's protocol. The CupB antibody was prepared against 156 amino acids of the C-terminus, which was expressed in a pET-51b(+) vector and isolated with a His tag in our lab (see Supplementary Fig. S2); it was made by the Shanghai Immune Biotech Co. Ltd (China). Antibodies against NdhA, NdhB, NdhK, and YFP were raised in our laboratory and were used in previously published work (Hu *et al.*, 2013).

CO_2 uptake measurements

Wild type and mutants of *Synechocystis* 6803 were grown in BG11 medium bubbled with 3% v/v CO_2 in air until logarithmic phase. Cell cultures were harvested by centrifugation at 5000 *g* for 10 min and suspended in BG11 buffer, pH 8.0, to a final concentration of OD_{730nm} of 100. Then, 30 µl of the cells were spotted on agar plates containing BG11 buffer at pH 8.0. A slice (1 cm×1 cm) of solid BG11 medium containing the cells was cut off and put on a piece of microscope coverglass. After that, CO_2 uptake was measured by an Li-6400 XT portable photosystem with the concentration of CO_2 controlled at 2%, 1% or 0.04% (v/v in air) as described previously (Chen *et al.*, 2016). Three independent measurements were performed and CO_2 uptake activities were calculated from concentration of chlorophyll. Light intensity and temperate were 100 µmol photons m^{-2} s^{-1} and 30 °C, respectively.

Quinacridine fluorescence quenching

Fluorescence of quinacridine (QA) at 503 nm was measured using the PAM chlorophyll fluorometer (Maxi-version, Walz, Effeltrich, Germany) attached to a US-370 emitter with an emission peak at 375 nm and a PM-101/D detector as described previously (Xu *et al.*, 2014; Chen *et al.*, 2016). Cells were harvested at logarithmic phase and suspended in reaction mixture of fresh BG11 medium with 5 µM QA at a final chlorophyll concentration of 10 µg ml^{-1}. The quenching of QA fluorescence was induced by illuminating the cells with actinic light (60 µmol photons m^{-2} s^{-1}) after the background fluorescence became stable after about 2 min.

Carbonic anhydrase activity measurement

The carbonic anhydrase assay was based on the rate of change in pH value after the injection of a standard amount of CO_2-saturated water, as described previously with modification (Jiang *et al.*, 2013). The cell cultures (1 l) were harvested at the logarithmic phase and were broken by vortexing five times at the highest speed for 20 s at 4 °C with a beadbeater (Biospec) followed by 3 min cooling on ice. Then the thylakoid membranes were separated and suspended in assay buffer (20 mM sodium barbital, pH 8.2, 10 mM $MgCl_2$ and 0.5 mM phenylmethylsulfonyl fluoride) at a final chlorophyll concentration of 1 mg ml^{-1}. Then the sample corresponding to 100 µg chlorophyll was added to 8 ml 20 mM sodium barbital, pH 8.2 with a pH electrode inserted into the assay solution. After the temperature equilibrated at 4 °C, 4 ml ice-cold CO_2-saturated water was injected, and then the time for the pH to change from 8.0 to 7.0 was recorded. The buffer without membrane fraction was used as the control. CA activity was calculated as the difference in the initial rate of CO_2 hydration between the control and the samples. CA activity is expressed in Wilbur–Anderson units (WAU) per mg of chlorophyll. One WAU is defined as $10\times(t_0–t)/t$, where t_0 and t are the times required for the pH change in the control and the sample, respectively (Jiang *et al.*, 2013).

Oxygen exchange

The rate of O_2 evolution in the cells of wild type and mutants was determined using a Clark-type O_2 electrode at 30 °C as described previously (He and Mi, 2016). The cell at logarithmic phase was used for the measurement in the presence of 10 µM $NaHCO_3$ and the light intensity used to induce the photosynthetic oxygen evolution was 1000 µmol photons m^{-2} s^{-1}.

Results

Growth and CO_2-uptake characteristics

Figure 1A–C shows the growth of the WT, *ΔcupA* (deleted *cupA*), *ΔcupB* (deleted *cupB*), *ΔcupA*/*B* (both *cupA* and *cupB* deleted) and M55 (deleted *ndhB*) strains of *Synechocystis* 6803 on agar plates containing BG11 medium buffered at pH 8.0, 7.0 and 6.5, respectively, under 3% CO_2. Five days after inoculation, there was no significant difference between the WT and *ΔcupA* strain in growth under the given conditions. The growth of *ΔcupB*, *ΔcupA*/*B*, and M55 mutants was slightly slower than the WT at pH 8.0. However, the growth of these mutants was severely suppressed at pH 7.0 and they hardly grew at all at pH 6.5, where Ci is predominantly, if not totally, supplied as CO_2. The results indicate that CO_2 is not supplied by diffusion even under 3% CO_2 in the absence of the CO_2-uptake systems.

Measurement of the rate of CO_2 uptake in the WT and mutants under various CO_2 concentrations revealed that inactivation of *cupA* had little effect on the activity, being consistent with the growth characteristics of the *ΔcupA* mutant (Fig. 1D). In contrast, inactivation of *cupB* decreased the activity to less than half that of the WT at 1% and 2% CO_2 but had no effect at 0.04% CO_2 (Fig. 1D). The *ΔcupA*/*B* and M55 mutants were unable to take up CO_2 even at 2% CO_2, consistent with the inability of these mutants to grow at pH 7.0. Since the expression of *cupA* is induced by low CO_2, the growth of cells is mainly supported by CupB under high CO_2 conditions below pH 7, where the contribution of HCO_3^- transporters is limited. Taken together, these data suggest that CupB is the key component under high CO_2 condition and the CO_2 uptake ability of the CupB-containing complex is dependent on the *ndh* and *cupB* genes.

Photosynthetic oxygen evolution was decreased in Cup-deletion mutants in response to different pH values

To confirm the function of both CupA and CupB, we further compared photosynthetic capacities in response to different pH values between the cells of wild type and *cup*-deletion mutants (Fig. 1E). By comparison with the value of 250 µmol O_2 mg^{-1} Chl h^{-1} in wild type at pH 8.0, the rate of photosynthetic oxygen evolution was suppressed slightly in *ΔcupA* (92%), significantly in *ΔcupB* (71%), more evidently in *ΔcupA*/*B* (65%), and most severely in M55 (42%) under the same growth condition. The suppression of photosynthetic oxygen evolution was less in *ΔcupA* at pH 6.5 (96%) but more evidently at pH 10.0 (86%), severely in *ΔcupB* (68%) and in *ΔcupA*/*B* (63%), and almost completely in M55 at pH 6.5 or pH 10.0. The results indicate that Cup proteins as well as NdhB contribute to the photosynthetic capacity especially under changed pH conditions.

Identification of large and small complexes containing CupB

To study CupB-containing complexes in more detail, we made polyclonal CupB antibody, which cross-reacted specifically with CupB but not with CupA (see Supplementary Fig. S2). Total membrane fractions isolated from the WT and BHM cells grown under 3% CO_2 at pH 8.0 were solubilized by *n*-dodecyl-β-D-maltoside and subjected to

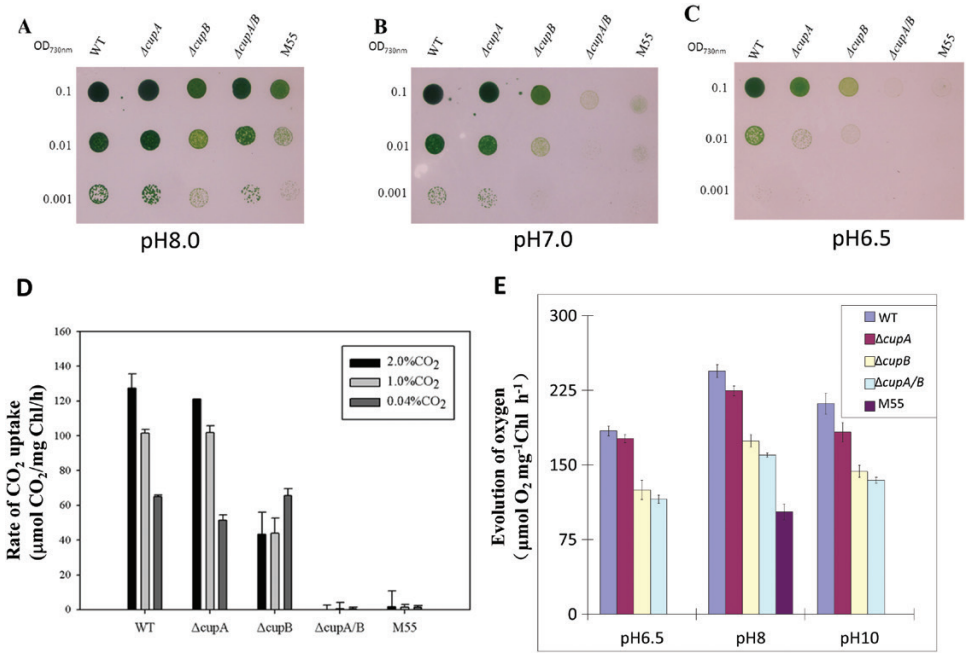

Fig. 1. Effects of pH on the growth of wild-type and mutants on agar plates, their rates of CO_2 uptake under various CO_2 concentrations and their rates of photosynthetic oxygen evolution. (A–C) Five microliters of the cell suspensions with the OD_{730nm} values of 0.1, 0.01, and 0.001 were spotted on agar plates containing BG11 buffer at pH 8.0 (A), pH 7.0 (B), and pH 6.5 (C) and grown in a CO_2 concentration of 3% for 5 days. (D) A portable photosynthesis system capable of recording the rate of CO_2 uptake was used for measurement of CO_2 uptake of wild-type, Δ*cupA*, Δ*cupB*, Δ*cupA/B*, and M55 under 2%, 1% and 0.04% CO_2 concentrations on agar plates. (E) The rate of photosynthetic oxygen evolution was compared among wild type and the mutants under different pH values in the presence of 10 μM $NaHCO_3$.

BN-PAGE followed by SDS-PAGE in the second dimension. Immunoblotting of the proteins electrotransferred to a PVDF membrane with antibody against CupB or α-Myc revealed three CupB-containing bands: a large band of about 500 kDa and a small one of about 200 kDa, as well as free CupB (Fig. 2A, B). The large and small bands correspond to the NDH-1MS′ and NDH-1S′ complexes, respectively.

Assembly of CupB to NDH-1MS′ was dependent on NdhD4 and NdhF4

Genetic studies have suggested that CupB might be associated with NdhD4 and NdhF4 (Xu *et al.*, 2008). To test this possibility, thylakoid membranes of the WT and mutants were subjected to two-dimensional BN-PAGE/SDS-PAGE analysis. The immunoblot profiles of the *ΔndhD1/D2* and *ΔndhD3* mutants showed the profile of NDH-1MS′, NDH-1S′ and free CupB bands to be essentially similar to that of WT (Fig. 2A). To the contrary, none of these bands was found in the thylakoid membrane of the *ΔndhD4* mutant. Similar results were obtained with the *ndhF*-deletion mutants: deletion of *ndhF1* and *ndhF3* had no effect on the CupB-containing bands but deletion of *ndhF4* completely abolished these bands in the thylakoid membrane (Fig. 2B). It is evident that CupB is associated with NdhD4 and NdhF4 in the CupB-containing complexes. The NDH-1S′ complex is similar to NDH-1S in size and the NDH-1MS′ complex is similar to NDH-1MS in size. Analysis of the membranes and soluble fractions by western blot indicated that CupB was localized in the thylakoid membrane in the WT whereas in the *ΔndhF4* mutant it was present only in the soluble fraction (Fig. 2C), indicating that NdhF4 is essential for attachment of CupB to the thylakoid membranes. The absence of NDH-1MS′ in M55 and *ΔndhB*/BHM suggests that the assembly of NDH-1MS′ requires NdhB (Fig. 2A, B). The amount of CupA expressed was less than CupB grown under high CO_2 conditions but was induced under low CO_2 conditions (Fig. 2D), consistent with a previous observation of transcript levels (Shibata *et al.*, 2001).

NDH-1MS and NDH-1MS′ independently exist

To distinguish NDH-1MS′ from NDH-1MS, thylakoid membranes of the WT and *ΔndhD1/D2/D3* strains were subjected to two-dimensional BN/SDS-PAGE analyses. Immunoblotting with antibodies against CupB, NdhK, and NdhI confirmed that CupB-containing complexes (NDH-1MS′ andNDH-1S′) appeared in *ΔndhD1/D2/D3* strains as well as in wild type where CupB co-localized with Ndh subunits such as NdhI and NdhK (Fig. 3A). In NdhB deletion mutant M55, both NDH-1MS and NDH-1MS′ were degraded, but NDH-1S and NDH-1S′ were still detected in *ndhB* deletion mutant M55 (Fig. 3B) and in *ndhM* deletion mutant (Fig. 3C). Furthermore, to know whether NDH-1MS and NDH-1MS′ are associated, we checked the localization of the complexes in different background mutants. As shown in Fig. 3D, detection of NDH-1MS′ and NDH-1S′ in *ΔndhD3* and *ΔcupA* was unchanged from the wild type (Zhang *et al.*, 2004). This is also true for detection of the NDH-1MS and NDH-1S complexes in *ΔndhD4* and *ΔcupB*. The NDH-1MS′ and NDH-1MS complexes were degraded in the mutants *ΔndhD4* and *ΔndhD3*, respectively (Fig. 2A). These results indicate that both NDH-1MS and NDH-1MS′ exist independently.

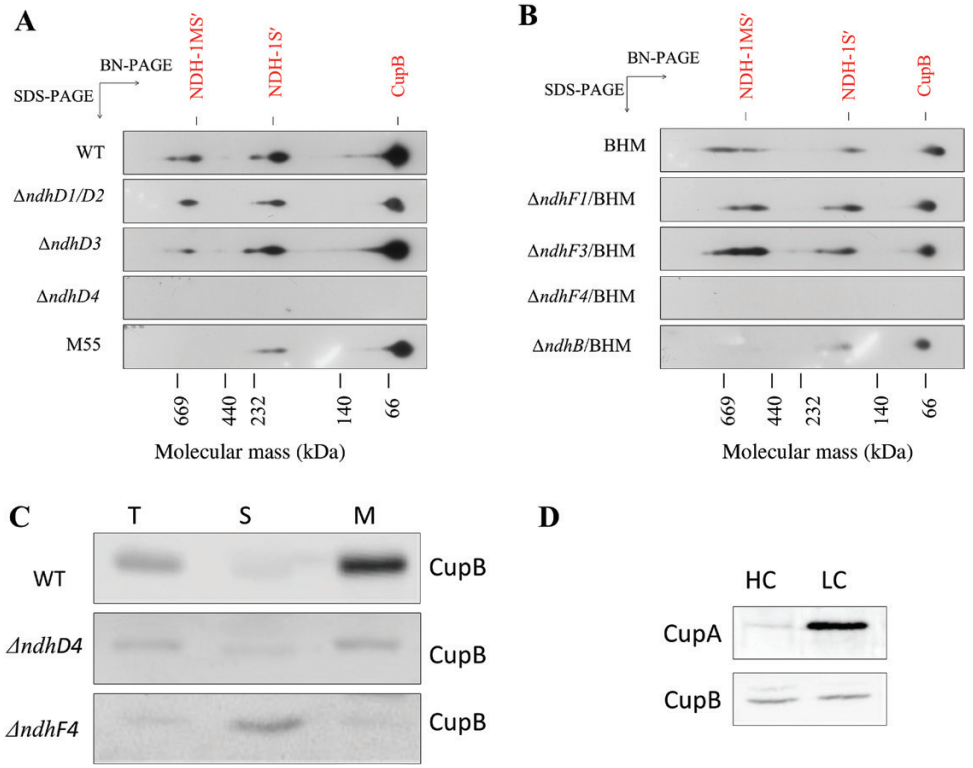

Fig. 2. Assembly of CupB-containing complexes in different NDH-1 mutant backgrounds and localization and expression of CupB in WT, Δ*ndhD4*, and Δ*ndhF4*. (A) Immunodetection of CupB-containing complexes using antibody of CupB in the wild type, Δ*ndhD1/D2*, Δ*ndhD3*, Δ*ndhD4*, Δ*ndhD3/D4*, and M55 backgrounds. Total membranes complexes were separated by BN-PAGE at the first dimension and further subjected to SDS-PAGE at the second dimension. Then, immunodetections were performed with antibody of CupB. (B) Immunodetection of CupB-containing complexes in the BHM, Δ*ndhF1*/BHM, Δ*ndhF3*/BHM, and Δ*ndhF4*/BHM backgrounds. (C) Comparison of the amount of CupB in different fractions among wild type, Δ*ndhD4*, and Δ*ndhF4*. The supernatant and membranes were separated and immunodetected with antibody against CupB. M, the thylakoid membrane proteins; S, the supernatant proteins; T, total proteins. (D) Accumulation of CupA and CupB in different fractions from cells of wild type grown under high CO_2 (HC) and low CO_2 (LC). Proteins were loaded on an equal chlorophyll basis. (This figure is available in color at *JXB* online.)

Analysis of proton gradient across thylakoid membranes in the NDH-1 mutant backgrounds

It has been suggested that cyanobacterial NDH-1 provides ATP for CO_2 uptake (Ogawa, 1991). To confirm whether the NDH-1 complex contributes to the proton gradient across thylakoid membranes, a driving force for synthesis of ATP, light-induced quenching of quinacridine (QA) fluorescence for the determination of ΔpH across the thylakoid membrane for intact cells of *Synechocystis* 6803 (Teuber *et al.*, 2001) was measured in different NDH-1 mutants backgrounds. As shown in Fig. 4A, the quenching of QA fluorescence was remarkably suppressed in M55 (by 97%), Δ*ndhD3/4* (by 84%), Δ*cupA/B* (by 72%), Δ*ndhD4* (by 69%), Δ*ndhD1/D2* (by 67%), Δ*cupB* (by 49%), partly in Δ*ndhD3* (by 16%) and slightly in Δ*cupA* (by 8%) compared with wild type. Those results suggested that both NDH and Cup proteins are involved in building up the proton gradient across thylakoid membrane and NdhD4 is a key component in this process.

Carbonic anhydrase activity was suppressed in the NDH- and Cup-deletion mutants

To further investigate the mechanism of NDH-1MS and NDH-1MS′ in the conversion of CO_2 to HCO_3^-, we measured the CA activity of the membrane proteins isolated from the wild type, Δ*ndhD1/D2*, Δ*ndhD3*, Δ*ndhD4*, Δ*ndhD3/D4*, Δ*cupA*, Δ*cupB*, Δ*cupA/B*, and M55 (Fig. 4B). Compared with wild type, CA activity was most greatly lowered in both Δ*cupA/B* (23%) and M55 (33%), more significantly suppressed in Δ*cupB* (43%), and Δ*ndhD3/D4* (47%), slightly in Δ*ndhD4* (62%), Δ*cupA* (63%) and Δ*ndhD3* (78%) compared with wild type, but almost not affected in Δ*ndhD1/D2*. Those results demonstrate that the Cup proteins are involved in the conversion of CO_2 to HCO_3^- as CA-like proteins.

Discussion

NdhD4 and NdhF4 are essential for the assembly of CupB-containing complexes

Although NDH-1MS′ was identified, its assembly is still unclear. In this work, we show evidence that NdhD4 and NdhF4 are crucial for the assembly of NDH-1MS′, based on the result that when *ndhF4* was knocked out, the CupB was completely missing from the thylakoid membrane, but was found soluble in the cytoplasm (Fig. 2C), probably resulting in no detection of NDH-1MS′ or NDH-1S′ (Fig. 2B). On the other hand, when *ndhD4* was deleted, this significantly decreased the accumulation of CupB in the thylakoid membrane (Fig. 2C), as reported in our previous study (Xu *et al.*, 2008), and also resulted in degradation of NDH-1MS′ or NDH-1S′ (Fig. 2A). We further show that NdhD4 is

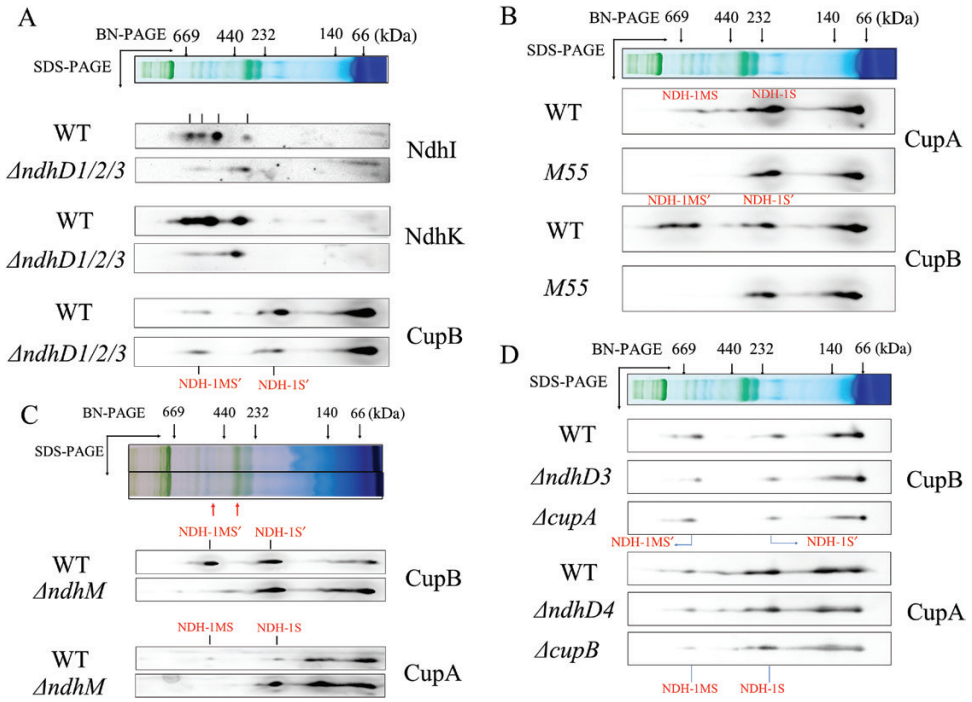

Fig. 3. The localization of NDH-1MS and NDH-1MS′ complexes in wild type and different mutant backgrounds. The thylakoid membrane proteins from the wild type and indicated mutant strains were separated by BN-PAGE and further subjected to 2-D/SDS-PAGE. Then the proteins were immunodetected with the indicated antibodies against the Ndh subunits or Cup proteins. The co-localization of Ndh subunits and CupA in a larger molecular size band is defined as NDH-1MS while that with smaller molecular size is NDH-1S; the co-localization of Ndh subunits and CupB in the larger molecular band is NDH-1MS′ while that with smaller molecular size is NDH-1S′. (A) Comparison of accumulation of Ndh subunits, CupB and their assembly into NDH-1MS′ in wild type and Δ*ndhD1/D2D3*. (B) Comparison of accumulation of CupA and CupB and their assembly into NDH-1MS and NDH-1MS′ in wild type and M55. (C) Comparison of accumulation of CupA and CupB and their assembly into NDH-1MS and NDH-1MS′ in wild type and Δ*ndhM*. (D) Comparison of the accumulation of CupA and CupB and their assembly into NDH-1MS′ in wild type, Δ*cupA*, and Δ*ndhD3*, and NDH-1MS in wild type, Δ*cupB*, and Δ*ndhD4*. The red arrow which indicates the higher molecular site is NDH-1L and the arrow that indicates the lower one is NDH-1M. (This figure is available in color at *JXB* online.)

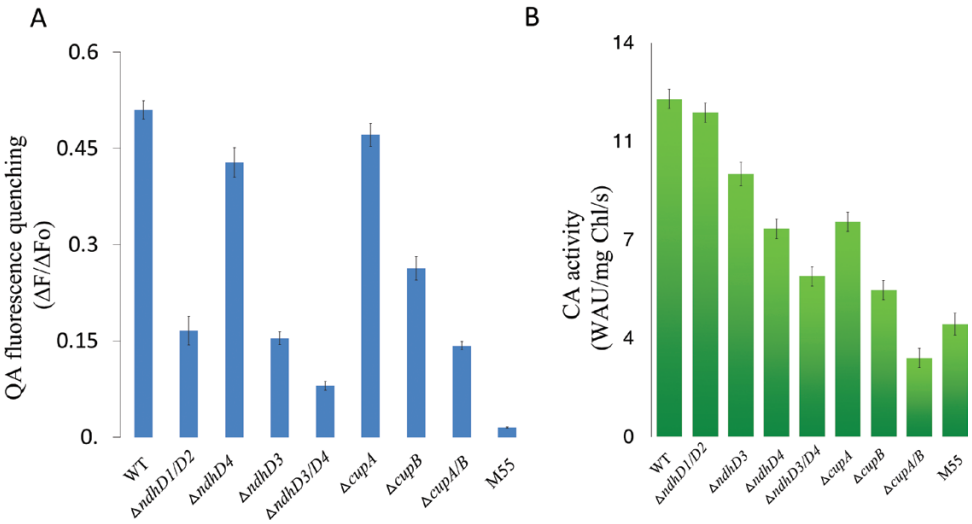

Fig. 4. Comparison of light-induced proton gradient across thylakoid membranes and the carbonic anhydrase activities among WT, Δ*ndhD1/D2*, Δ*ndhD3*, Δ*ndhD4*, Δ*ndhD3/D4*, Δ*cupA*, Δ*cupB*, Δ*cupA/B*, and M55. (A) Intact cells of WT, Δ*ndhD1/D2*, Δ*ndhD3*, Δ*ndhD4*, and M55 and Δ*cupA*, Δ*cupB*, and Δ*cupA/B* were harvested at midlogarithmic phase (OD_{730}=0.4) and then suspended at a final chlorophyll concentration of 10 μg ml^{-1} in fresh BG11 medium with 5 μM quinacridine (QA). The quenching of QA fluorescence was induced by illumination with actinic light (60 μmol photos m^{-2} s^{-1}) after starting measurement. The QA fluorescence quenching was calculated as the ratio ($\Delta F/\Delta F_o$) of the decreased fluorescence intensity (ΔF) to the background fluorescence intensity (ΔF_o). (B) The thylakoid membranes from these strains were suspended in 20 mM Tricine buffer at a final chlorophyll concentration of 1 mg ml^{-1}. Then, samples containing 100 μg of chlorophyll were added to 8 ml 20 mM sodium barbital, pH 8.2 with a pH electrode inserted into the assay solution. After the temperature equilibrated, 4 ml ice-cold CO_2-saturated water was injected, and the time for the pH to change from 8.0 to 7.0 was recorded. The buffer without membrane fraction was used as the control. CA activity was calculated as the difference in the initial rate of CO_2 hydration between the control and the samples. Values are the averages of four independent measurements. Standard errors are indicated by the vertical bars. (This figure is available in color at *JXB* online.)

co-located with CupB, evidenced by detecting the strep tag fused to the C-terminus of NdhD4 (see Supplementary Figs S3 and S4A). The recovery of the assembly of CupB-containing complexes by complementing *ndhF4* gene to *ΔndhF4* mutant (Supplementary Figs S3 and S4B) provided further evidence for the crucial role of NdhF4 in the NDH-1S′ complex. Recently an NDH-1S′ complex containing NdhD4, NdhF4, and CupB has been isolated from a *Thermosynechoccus elongatus* with twin-strep tagged to NdhL (Wulfhorst *et al.*, 2014). We also found that when *ndhB* is deleted, NDH-1S′ can still be normally assembled (Figs 2A and 3B), but the ability to take up CO_2 is severely compromised (Fig. 1D), indicating that NDH-1S′ alone is not functional in CO_2 uptake.

Co-ordination of NdhDs and Cup proteins in CO_2 uptake activity

By resolution of mitochondrial complex I structure, it has been suggested that complex I regulates the transmembrane proton gradient by its conformation change during electron transfer (Royer *et al.*, 2006; Efremov *et al.*, 2010; Vinothkumar *et al.*, 2014). The similar function has been proposed in NDH-1 previously (Battchikova *et al.*, 2011). In this work, we further found that the proton gradient across the thylakoid membrane was significantly suppressed in *ΔndhD4* and even more in *ΔndhD3/D4*, *ΔndhD1/D2*, and M55 (Fig. 4A), suggesting NdhD subunits, mainly NdhD4, function as a proton pump to provide a proton gradient across the thylakoid membranes, as it is homologous to subunit M in complex I, which drives the ATPase, to synthesize ATP for active CO_2 uptake and for regulation of pH in the cytosol. The lesser contribution of NdhD3 to proton pumping than NdhD4 might be attributable to its lesser expression (Ohkawa *et al.*, 1998) and lesser amount (Fig. 2D) under the high CO_2 culture condition. The partial suppression of QA fluorescence in *ΔcupB* suggests that CupB is also involved in building up a sufficient proton gradient across the thylakoid membrane (Fig. 4A). Although the formation of the transthylakoid membrane ΔpH was only slightly affected in *ΔcupA*, it was significantly decreased in *ΔcupA/B* (Fig. 4A), which led us to conclude that the formation of ΔpH by NDH-1MS and NDH-1MS′ complexes requires the coordination of Cup proteins for providing protons in the hydration of CO_2 to HCO_3^- (Fig. 5).

The possible role of CupA/CupB and NDH-1M in regulation of CO_2 uptake

There are four kinds of carbonic anhydrase forming a small complex located in Complex I of Arabidopsis that are suggested to be crucial for the balance of CO_2 and HCO_3^- (Meyer *et al.*, 2011; Li *et al.*, 2013; Wydro *et al.*, 2013). Kaplan *et al* also suggested that a similar structure of the carbonic anhydrase might exist in cyanobacteria (Kaplan and Reinhold, 1999). The low carbonic anhydrase activity in *ΔcupA/B* (Fig. 4B) indicates that Cup proteins, mainly CupB, function as a carbonic anhydrase to convert CO_2 into HCO_3^-. On the other hand, in the NDH-1MS′ degradation mutants, including *ΔndhD4*, *ΔndhD3/D4*, and M55 (Fig. 2), not only the

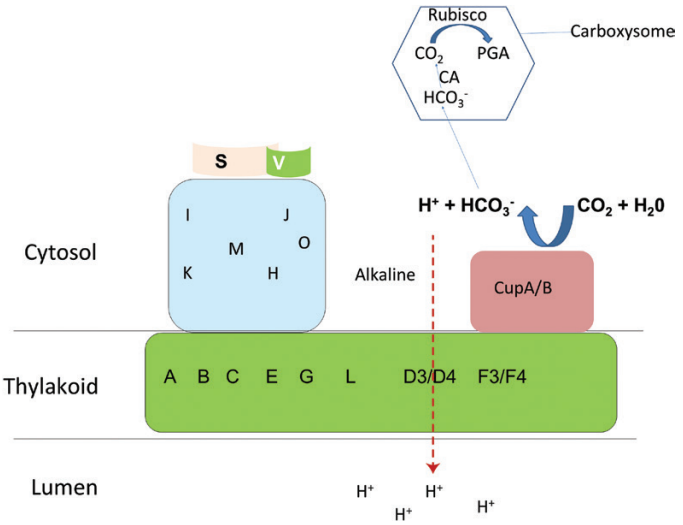

Fig. 5. A model of the proposed function of CO_2 uptake systems in *Synechocystis* sp. strain PCC6803. CupA or CupB converts CO_2 into HCO_3^- under alkaline conditions, while the conversion is reversed under acidic conditions. Under light conditions, photosynthetic electron transfer couples to the formation of a transthylakoid membrane proton gradient, subsequently forming a strong alkaline region inside the U-type structure for CupA or CupB activity, which leads to the accumulation of HCO_3^- after CO_2 has diffused into the cytosol. After HCO_3^- enters the carboxysome, it is converted into CO_2 by CA for carbon assimilation by Rubisco. (This figure is available in color at *JXB* online.)

building up of a transthylakoid membrane proton gradient (Fig. 4A) but also the activity of carbonic anhydrase (Fig. 4B) was suppressed. This allows us to conclude that Ndh subunits are required for the activation of the carbonic anhydrase. The function of carbonic anhydrase depends on the environment. Carbonic anhydrase converts CO_2 into HCO_3^- under alkaline conditions while the conversion is reversed under acidic conditions (Kupriyanova and Pronina, 2011). CupA or CupB might have a similar function as they also display CA activity (Fig. 4B). In cyanobacterial cytosol, the inorganic carbon source exists as HCO_3^- whose accumulation might require an alkaline environment. Based on our results in Fig. 4, we suggest that the transthylakoid membrane proton gradient through NdhDs coupled with the cyclic electron flow around PS I mediated by NDH-1M (He *et al.*, 2016; He and Mi, 2016) might create a strong alkaline region in the cytosol (lumen becomes more acidic and cytosol become more alkaline) as suggested previously (Kaplan and Reinhold, 1999), suitable for Cup proteins to hydrate CO_2 into HCO_3^- in the cytosol (Fig. 5).

The relationship of CupB and CupA in the CO_2 uptake pathway

CupB is a homologous protein of CupA with 40% similarity. Xu *et al.* (2008) found that the expression of CupB is constitutive and not affected by CO_2 concentration, while the expression of CupA is induced by low CO_2 and is involved in CO_2 uptake under low CO_2 conditions. In this work, we found that in addition to the main function of CupB, CupA also functioned at high CO_2 conditions (Figs 1 and 2), in

accordance with the results of the isolated NDH-1S from high CO_2-cultured cells (Wulfhorst *et al.*, 2014), suggesting CupA was also expressed under high CO_2 conditions. The mutant defective in both CupA and CupB hardly grew on the agar plate at pH lower than 7.0 even at 3% CO_2 (Fig. 1B), and the photosynthetic oxygen evolution was also evidently suppressed (Fig. 1E), suggesting that diffusion of CO_2 through cells to the carboxylation site is insignificant in the absence of CO_2-uptake systems, and that both CupA and CupB are required for efficient CO_2 uptake (Fig. 1C).

In conclusion, using reverse genetics and biochemical methods, we investigated the function of NDH-1MS′ and NDH-1MS in CO_2 uptake. Based on our results, we propose that the transthylakoid membrane proton gradient coupled by the electron transport mediated by NDH-1M might create an alkaline region, suitable for CupA or CupB to convert CO_2 into HCO_3^- in the cytosol (Fig. 5). However, further experimental data to support this hypothesis are still needed.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Construction and segregation of *ndhF* and *ndhD* mutants.

Fig. S2. Polyclonal antibody of CupB preparation and immunological characterization of CupB in mutants.

Fig. S3. Construction and segregation check of *ndhD4HA*/BHM, *ndhD4HA*/*ΔndhF4*/BHM, *ndhF4HA*/BHM, and *ndhF4HA*/*ΔndhF4*/BHM, and *ndhD4*strep.

Fig. S4. Association of CupB with NdhD4 and NdhF4.

Table S1. Primer details used in construction and isolation of mutants.

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