Co-ordination of NDH and Cup proteins in CO₂ uptake in cyanobacterium Synechocystis sp. PCC 6803

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Abstract

High and low affinity CO₂-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 CO₂ 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% CO₂. 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% CO₂. 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 CO₂-uptake systems under high CO₂ 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 CO₂ to HCO₃⁻. In turn, deletion of cup genes lowered the transthylakoid membrane proton gradient and deletion of ndhDs decreased the CO₂ hydration. Our results suggest that NDH-1M provides an alkaline region to activate Cup proteins involved in CO₂ uptake.

Key words: CO₂ uptake, CupA, CupB, NDH-1MS, NDH-1MS', Synechocystis sp. PCC 6803.

Introduction

Cyanobacteria possess a CO₂-concentrating mechanism (CCM) that enables the accumulation of inorganic carbon (HCO₃⁻ and CO₂, collectively called Ci) at the carboxylation site to a level for efficient CO₂ fixation despite the low affinity of their Rubisco for CO₂ (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 HCO₃⁻ transporters (BicA and SbtA), one ATPase-dependent HCO₃⁻ transporter (BCT1), and two CO₂-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
of the CO₂ uptake complexes, the NDH-1MS’ complex, consists of Ndhd4, Ndhd4 and CupB (ChpX) and is a constitutive system with weaker uptake affinity for CO₂; another one, the NDH-1MS complex, consists of Ndhd3, Ndhd3 and CupA (ChpY), and is inducible at limiting Ci conditions and has a higher uptake affinity for CO₂ (Okahara et al., 2000b; 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. PC 7002 cells grown under low CO₂ condition (Okahara et al., 2000b; 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 CO₂ conditions even when NDH-1S was present, suggesting that the normal operation of the CO₂-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 NdH 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 with single particle electron microscopy after purification from the thylakoid membranes of Thermosynechococcus 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 Thermosynechococcus 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 CO₂ 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 NdH 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 CO₂ uptake systems, NDH-1MS and NDH-1MS’, in which NdhdS function in proton pumping (Battchikova et al., 2011). Cups function in CO₂ hydration as carbonic anhydrase (CA)-like proteins (Kaplan and Reinhold, 1999; Price, 2011). However, whether NDH-1MS induced by low CO₂ exists or functions under high CO₂ 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 CO₂ uptake using reverse genetics and biochemical methods. Our results suggest that NDH-1MS and NDH-1MS’ are essential for efficient CO₂ uptake especially under changed pH conditions. We proposed a model for the function of CO₂ uptake systems in Synechocystis 6803.

Materials and methods

Cell culture conditions

Wild type (WT) and mutant cell strains 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 CO₂ 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 (OD₆₃₀=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; Okahara et al., 2000a; 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). Those 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 hx1114/hx1115 and hx1118/1119, with the CM resistance cassette using the primers pair hx1116/hx1117 (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 electro-transferred 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 NdH, NdH, NdH, and YFP were raised in our laboratory and were used in previously published work (Hu et al., 2013).
Function of NDH and Cup proteins in CO₂ uptake

Wild type and mutants of *Synechocystis* 6803 were grown in BG11 medium bubbled with 3% v/v CO₂ in air until logarithmic phase. Cells were harvested by centrifugation at 5000 g for 10 min and suspended in BG11 buffer, pH 8.0, to a final concentration of OD₇₃₀nm 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 coverslip. After that, CO₂ uptake was measured by an Li-6400 XT portable photosystem with the concentration of CO₂ controlled at 2%, 1% or 0.04% (v/v in air) as described previously (He and Mi, 2016). Three independent measurements were performed and CO₂ uptake activities were calculated from concentration of chlorophyll. Light intensity and temperature 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., 2013). 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 CO₂-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 MgCl₂ 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 5 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 CO₂-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 CO₂ 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 10⁻¹(t₀−t₁)/t₀, where t₀ and t₁ are the times required for the pH change in the control and the sample, respectively (Jiang et al., 2013).

Oxygen exchange

The rate of O₂ evolution in the cells of wild type and mutants was determined using a Clark-type O₂ electrode at 30 °C as described previously (He and Mi, 2016). The cell at logarithmic phase was broken by vortexing five times at the highest speed for 20 s at 0.5 mM phenylmethylsulfonyl fluoride) at a final chlorophyll concentration of 1 mg ml⁻¹. Then the thylakoid membranes were separated 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.

Results

**Growth and CO₂-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% CO₂. 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 CO₂. The results indicate that CO₂ is not supplied by diffusion even under 3% CO₂ in the absence of the CO₂-uptake systems.

Measurement of the rate of CO₂ uptake in the WT and mutants under various CO₂ 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% CO₂ but had no effect at 0.04% CO₂ (Fig. 1D). The ΔcupA/B and M55 mutants were unable to take up CO₂ even at 2% CO₂, consistent with the inability of these mutants to grow at pH 7.0. Since the expression of *cupA* is induced by low CO₂, the growth of cells is mainly supported by CupB under high CO₂ conditions below pH 7, where the contribution of HCO₃⁻ transporters is limited. Taken together, these data suggest that CupB is the key component under high CO₂ condition and the CO₂ uptake ability of the CupB-containing complex is dependent on the *ndh* and *cup* 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 O₂ 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% CO₂ at pH 8.0 were solubilized by n-dodecyl-β-D-maltoside and subjected to
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 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 NdhF4-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 ΔndhD4 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 CO2 conditions but was induced under low CO2 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′ and NDH-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.
Function of NDH and Cup proteins in CO₂ uptake

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

It has been suggested that cyanobacterial NDH-1 provides ATP for CO₂ 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 $\Delta pH$ 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%), $\Delta ndhD3/4$ (by 84%), $\Delta ndhD4$ (by 72%), $\Delta ndhD1/D2$ (by 69%), $\Delta ndhD1/D2$ (by 67%), $\Delta cupB$ (by 49%), partly in $\Delta ndhD3$ (by 16%) and slightly in $\Delta cupA$ (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 CO₂ to HCO₃⁻, we measured the CA activity of the membrane proteins isolated from the wild type, $\Delta ndhD1/D2$, $\Delta ndhD3$, $\Delta ndhD4$, $\Delta ndhD3/D4$, $\Delta cupA$, $\Delta cupB$, $\Delta cupA/B$, and M55 (Fig. 4B). Compared with wild type, CA activity was most greatly lowered in both $\Delta cupA/B$ (23%) and M55 (33%), more significantly suppressed in $\Delta cupB$ (43%), and $\Delta ndhD3/D4$ (47%), slightly in $\Delta ndhD4$ (62%), $\Delta cupA$ (63%) and $\Delta ndhD3$ (78%) compared with wild type, but almost not affected in $\Delta ndhD1/D2$. Those results demonstrate that the Cup proteins are involved in the conversion of CO₂ to HCO₃⁻ 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 ΔndhD1/D2, D3, D4, D3/D4, ∆cupA, ∆cupB, ∆cupA/B, and M55. (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 ∆ndhM. (D) Comparison of the accumulation of CupA and CupB and their assembly into NDH-1MS' in wild type, ∆cupA, and ∆ndhD3, and NDH-1MS in wild type, ∆cupB, and ∆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, ΔndhD1/D2, D3, D4, D3/D4, ΔcupA, ΔcupB, ΔcupA/B, and M55. (A) Intact cells of WT, ΔndhD1/D2, ΔndhD3, ΔndhD4, and M55 were harvested at midlogarithmic phase (OD730=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 (∆F/∆F0) of the decreased fluorescence intensity (∆F) to the background fluorescence intensity (∆F0). (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 CO₂-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 CO₂ 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 CO₂ is severely compromised (Fig. 1D), indicating that NDH-1S′ alone is not functional in CO₂ uptake.

**Co-ordination of NdhDs and Cup proteins in CO₂ 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; Vinokhkhom 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 CO₂ 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 (Okawa et al., 1998) and lesser amount (Fig. 2D) under the high CO₂ 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 CO₂ to HCO₃⁻ (Fig. 5).

**The possible role of CupA/CupB and NDH-1M in regulation of CO₂ 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 CO₂ and HCO₃⁻ (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 CO₂ into HCO₃⁻. On the other hand, in the NDH-1M-Δ1s degradation mutants, including ΔndhD4, ΔndhD3/D4, and M55 (Fig. 2), not only the 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 CO₂ into HCO₃⁻ 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 HCO₃⁻ after CO₂ has diffused into the cytosol. After HCO₃⁻ enters the carboxysome, it is converted into CO₂ by CA for carbon assimilation by Rubisco. (This figure is available in color at JXB online.)

**The relationship of CupB and CupA in the CO₂ 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 CO₂ concentration, while the expression of CupA is induced by low CO₂ and is involved in CO₂ uptake under low CO₂ conditions. In this work, we found that in addition to the main function of CupB, CupA also functioned at high CO₂ conditions (Figs 1 and 2), in
accordance with the results of the isolated NDH-1S from high CO₂-cultured cells (Wulhorst et al., 2014), suggesting CupA was also expressed under high CO₂ conditions. The mutant defective in both CupA and CupB hardly grew on the agar plate at pH lower than 7.0 even at 3% CO₂ (Fig. 1B), and the photosynthetic oxygen evolution was also evidently suppressed (Fig. 1E), suggesting that diffusion of CO₂ through cells to the carboxylation site is insignificant in the absence of CO₂-uptake systems, and that both CupA and CupB are required for efficient CO₂ uptake (Fig. 1C).

In conclusion, using reverse genetics and biochemical methods, we investigated the function of NDH-1MS and NDH-1MS in CO₂ 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 CO₂ into HCO₃⁻ 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 ndhD4Δstrept.

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

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

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References
Arteni AA, Zhang P, Batetchikova N, Ogawa T, Aro EM, Boekema EJ. 2006. Structural characterization of NDH-1 complexes of Thermus thermophilus HB8 by single particle electron microscopy. Biochimica et Biophysica Acta 1757, 1469–1475.

Batetchikova N, Eisenhut M, Aro EM. 2011. Cyanobacterial NDH-1 complexes: novel insights and remaining puzzles. Biochimica et Biophysica Acta 1807, 935–944.

Birungi M, Folea M, Batetchikova N, Xu M, Mi H, Ogawa T, Aro EM, Boekema EJ. 2010. Possibilities of subunit localization with fluorescent protein tags and electron microscopy exemplified by a cyanobacterial NDH-1 study. Biochimica et Biophysica Acta 1797, 1681–1686.

Chen X, He Z, Xu M, Peng L, Mi H. 2016. NdhV subunit regulates the activity of type-1 NAD(P)H dehydrogenase under high light conditions in cyanobacterium Synechocystis sp. PCC 6803. Scientific Reports 6, 28361–28361.

Efremov RG, Baradaran R, Sazanov LA. 2010. The architecture of respiratory complex I. Nature 465, 441–445.

Folea IM, Zhang P, Nowaczky MM, Ogawa T, Aro EM, Boekema EJ. 2008. Single particle analysis of thylakoid proteins from Thermosynechococcus elongatus and Synechocystis 6803: localization of the CupA subunit of NDH-1. FEBS Letters 582, 249–254.

He Z, Mi H. 2016. Functional characterization of the subunits N, H, J, and O of the NAD(P)H dehydrogenase complexes in Synechocystis sp. strain PCC 6803. Plant Physiology 171, 1320–1332.

He Z, Xu M, Wu Y, Lv J, Fu P, Mi H. 2016. NdhM subunit is required for the stability and the function of NAD(P)H dehydrogenase complexes involved in CO₂ uptake in Synechocystis sp. strain PCC 6803. The Journal of Biological Chemistry 291, 5902–5912.

Hu P, Lv J, Fu P, Hualing M. 2013. Enzymatic characterization of an active NDH complex from Thermosynechococcus elongatus. FEBS Letters 587, 2340–2345.

Jiang HB, Cheng HM, Gao KS, Qiu BS. 2013. Inactivation of Ca²⁺/H⁺ exchanger in Synechocystis sp. strain PCC 6803 promotes cyanobacterial calcification by upregulating CO₂-concentrating mechanisms. Applied and Environmental Microbiology 79, 4048–4055.

Kaplan A, Reinhold L. 1999. CO₂ concentrating mechanisms in photosynthetic microorganisms. Annual Review of Plant Physiology and Plant Molecular Biology 50, 539–570.

Kupriyanova EV, Pronina NA. 2011. Carbonic anhydrase: enzyme that has transformed the biosphere. Russian Journal of Plant Physiology 58, 197–209.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Li L, Nelson CJ, Carrie C, Gawryluk RM, Solheim C, Gray MW, Whelan J, Millar AH. 2013. Subcomplexes of ancestral respiratory complex I subunits rapidly turn over in vivo as productive assembly intermediates in Arabidopsis. The Journal of Biological Chemistry 288, 5707–5717.

Maeda S, Badger MR, Price GD. 2002. Novel gene products associated with NdhD3/D4-containing NDH-1 complexes are involved in photosynthetic CO₂ hydration in the cyanobacterium, Synechococcus sp. PCC7942. Molecular Microbiology 43, 425–435.

Meyer EH, Solheim C, Tansz SK, Bonnard G, Millar AH. 2011. Insights into the composition and assembly of the membrane arm of plant complex I through analysis of subcomplexes in Arabidopsis mutant lines. The Journal of Biological Chemistry 286, 26081–26092.

Ogawa T. 1991. A gene homologous to the subunit-2 gene of NADH dehydrogenase is essential to inorganic carbon transport of Synechocystis PCC6803. Proceedings of the National Academy of Sciences, USA 88, 4275–4279.

Ogawa T, Kaplan A. 2003. Inorganic carbon acquisition systems in cyanobacteria. Photosynthesis Research 77, 105–115.

Ogawa T, Mi H. 2007. Cyanobacterial NADPH dehydrogenase complexes. Photosynthesis Research 93, 69–77.

Ohkawa H, Pakrasi HB, Ogawa T. 2000a. Two types of functionally distinct NAD(P)H dehydrogenases in Synechocystis sp. strain PCC6803. The Journal of Biological Chemistry 275, 31630–31634.

Ohkawa H, Price GD, Badger MR, Ogawa T. 2000b. Mutation of ndhF genes leads to inhibition of CO₂ uptake rather than HCO₃⁻ uptake in Synechocystis sp. strain PCC 6803. Journal of Bacteriology 182, 2591–2596.

Ohkawa H, Sonoda M, Katoh H, Ogawa T. 1998. The use of mutants in the analysis of the CO₂-concentrating mechanism in cyanobacteria. Canadian Journal of Botany-Revue Canadienne De Botanique 76, 1035–1042.

Price GD. 2011. Inorganic carbon transporters of the cyanobacterial CO₂ concentrating mechanism. Photosynthesis Research 109, 47–57.

Royer WE Jr, Sharma H, Strand K, Knapp JE, Bhryavbhatal B. 2006. Lmbicus erythrocraurin at 3.5 angstrom resolution: architecture of a megadalton respiratory complex. Structure 14, 1167–1177.

Shibata M, Ohkawa H, Kaneko T, Fukuzawa H, Tabata S, Kaplan A, Ogawa T. 2001. Distinct constitutive and low-CO₂-induced CO₂ uptake systems in cyanobacteria: Genes involved and their phylogenetic relationship with homologous genes in other organisms. Proceedings of the National Academy of Sciences, USA 98, 11789–11794.
Teuber M, Rögner M, Berry S. 2001. Fluorescent probes for non-invasive bioenergetic studies of whole cyanobacterial cells. Biochimica et Biophysica Acta 1506, 31–46.

Vinothkumar KR, Zhu J, Hirst J. 2014. Architecture of mammalian respiratory complex I. Nature 515, 80–84.

Wulfforst H, Franken LE, Wessinghage T, Boekema EJ, Nowaczyk MM. 2014. The 5 kDa protein NdhP is essential for stable NDH-1L assembly in Thermosynechococcus elongatus. PLoS ONE 9, e103584.

Wydro MM, Sharma P, Foster JM, Bych K, Meyer EH, Balk J. 2013. The evolutionarily conserved iron-sulfur protein INDH is required for complex I assembly and mitochondrial translation in Arabidopsis. The Plant Cell 25, 4014–4027.

Xu M, Ogawa T, Pakrasi HB, Mi H. 2008. Identification and localization of the CupB protein involved in constitutive CO₂ uptake in the cyanobacterium, Synechocystis sp. strain PCC 6803. Plant & Cell Physiology 49, 994–997.

Xu M, Shi N, Li Q, Mi H. 2014. An active supercomplex of NADPH dehydrogenase mediated cyclic electron flow around Photosystem I from the panicle chloroplast of Oryza sativa. Acta Biochimica et Biophysica Sinica 46, 757–765.

Zhang P, Battchikova N, Jansen T, Appel J, Ogawa T, Aro EM. 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. The Plant Cell 16, 3326–3340.

Zhang P, Battchikova N, Paakkarinen V, Katoh H, Iwai M, Ikeuchi M, Pakrasi HB, Ogawa T, Aro EM. 2005. Isolation, subunit composition and interaction of the NDH-1 complexes from Thermosynechococcus elongatus BP-1. The Biochemical Journal 390, 513–520.