Identification and Characterization of the ictA/ndhL Gene Product Essential to Inorganic Carbon Transport of Synechocystis PCC6803

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ABSTRACT

The ictA gene, renamed ndhL in this paper, essential to inorganic carbon transport of Synechocystis PCC6803, was expressed in Escherichia coli as a fusion protein with glutathione S-transferase. An antibody was raised against this fusion protein. Western analysis of the thylakoid membrane of wild-type (WT) Synechocystis revealed that a protein with an apparent molecular mass of 6.7 kilodaltons cross-reacted with this antibody. No immunoreactive protein was present in the thylakoid membranes of the Synechocystis mutants, RKb and M9, which have defects in the ictA/ndhL gene, or in the cytoplasmic membranes of the WT and mutant cells. Thus, the protein reacted with the antibody is the ictA gene product (IctA) and is localized in the thylakoid membrane of WT cells. IctA was absent in the thylakoid membranes of the M55 mutant, in which the ndhB gene is inactivated, and was poorly immunostained in the membranes of the mutants (M-ndhC and M-ndhK) constructed by inactivating the ndhC and ndhK genes of WT Synechocystis, respectively. The carbon dioxide uptake activity was nearly zero in M-ndhK and was about 40% of the activity of WT cells in M-ndhC. The RKb, M-ndhC, and M-ndhK mutants were unable to grow or grew very slowly under photoheterotrophic conditions. These results indicated that NADH dehydrogenase is essential to inorganic carbon transport and photoheterotrophic growth of Synechocystis and that IctA is one of the subunits of this enzyme.

Recently, there has been much progress in the molecular analysis of the "CO2-concentrating mechanism" in cyanobacteria (3-6). The isolation of the mutants defective in C3 transport is one of the current topics in this research field (9). Molecular analyses of these mutants of Synechocystis PCC6803 revealed that the RKa and RKb mutants have defects in the ndhB and ictA genes, respectively (10, 11). Insertional inactivation of either of these genes in WT Synechocystis led to the loss of the ability of the cells to transport external C3 into the intracellular C3 pool. The ndhB gene of Synechocystis encodes a protein of 521 amino acids, which showed more than 50% sequence homology with the product of the corresponding gene in liverwort chloroplasts (10). Based on this homology, it was assumed that the ndhB gene encodes subunit 2 of NADH dehydrogenase. The ictA gene encodes a hydrophobic protein consisting of 80 amino acids (11). No homologous gene has been found in the database. In addition to the ndhB gene, several ndh genes have recently been cloned from Synechocystis PCC6803 (1, 21). The previous study on the ndhB gene (10) suggested that inactivation of other ndh genes might also have significant effect on the C3-transporting activity of the cells. The present study was undertaken in an attempt to identify and characterize the ictA gene product. For this purpose, an antibody was raised against the product of the ictA gene expressed in Escherichia coli. In addition, Synechocystis mutants (M-ndhC and M-ndhK) were constructed by inactivating the ndhC and ndhK genes of WT cells, respectively. Western analyses of the membranes from WT and mutant cells obtained in the present and previous studies enabled me to identify the ictA gene product and to characterize it as a component of the cyanobacterial NADH dehydrogenase. Based on the evidence presented here, I propose to rename the ictA gene ndhL. A possible role of NADH dehydrogenase in C3 transport is discussed.

MATERIALS AND METHODS

Growth Conditions

The high CO2-requiring mutant defective in C3 transport (RKb) was isolated from Synechocystis PCC6803 following mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine and ampicillin enrichment (9). M9 and M55 mutants were constructed after insertional inactivation of the ictA and ndhB genes, respectively (10, 11). WT and mutant cells were grown at 30°C in BG 11 medium (20) buffered with 10 mM Tes-KOH (pH 8) under aeration with 3% CO2/97% air in the presence or absence of kanamycine (20 μg/ml). Continuous illumination was provided at 120 μmol PAR m^-2 s^-1 by incandescent lamps.

Expression of the ictA Gene

To introduce the cloning sites, the ictA gene was synthesized by the PCR method (18) using the primers that contain the BamHI and EcoRI sites, respectively, at the end of each primer. The BamHI/EcoRI digest of the PCR product was cloned into the expression vector, pGEX-2T (19), and was used to transform E. coli. After 3 h of expression induced by...
adding isopropyl-β-D-thiogalactoside, cells were harvested by centrifugation, suspended in 10 mL of PBS (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, pH 7.3) containing 5 mM DTT and 1 mg/mL of lysozyme, incubated on ice for 10 min, and then disrupted by sonication (twice for 30 s and another 30 s after addition of 1 mL of 10% Triton X-100). Inclusion bodies, which contain most of the expressed protein (the ictA gene product, IctA, fused to GSH S-transferase), was recovered as a precipitate after 15 min of centrifugation (12,000g) of the sonicated cell suspension. The protein was purified by SDS-PAGE and was injected into a rabbit to raise the antibody.

Inactivation of the ndhC and ndhK Genes

These genes were synthesized by the PCR method (18) using Vent polymerase (New England Biolabs, Beverly, MA), according to the sequence reported by Steinmüller et al. (21) and cloned into pUC119. The PCR DNA template used was genomic DNA of WT Synechocystis PCC6803. The cloned ndhC and ndhK genes were interrupted at the ArrI site, or between the two NcoI sites, by the gene for aminoglycoside 3′-phosphotransferase (Km' cartridge) that originated from the bacterial transposon Tn903 (14). The WT cells of Synechocystis were transformed with the plasmid containing the modified DNA fragment according to the method reported by Williams and Szalay (22) as described previously (10). Unless otherwise stated, standard techniques were used for DNA manipulation (9).

Preparation of Cytoplasmic and Thylakoid Membranes

Cytoplasmic and thylakoid membranes were prepared from WT and mutant cells by the method of Omata and Murata (15) after modifications (16). Cells were broken using a French pressure cell at 1400 kg/cm². Total membranes were prepared by breaking cells in 10 mM Tes-NaOH, pH 7. The broken cell suspension was centrifuged at 10,000g for 15 min and the supernatant was centrifuged at 144,000g for 1 h. The pellet was washed once with the same buffer and was used as total membrane.

Electrophoresis and Immunostaining

SDS-PAGE was performed in the system of Laemmli (7) modified by Ikeuchi and Inoue (5). The modified system utilizes a gradient gel of 16 to 22% polyacrylamide containing 7 M urea, which gives a good separation of low mol wt proteins. Polypeptides were electrotransferred to nitrocellulose and reacted with the antibody. Goat anti-rabbit immunoglobulin G/alkaline phosphatase conjugate was used as the second antibody to detect the reacting polypeptide.

Gas-Exchange Measurements

The mutant and WT cells of Synechocystis were suspended in 20 mM Hepes-NaOH buffer (pH 7.0) containing 15 mM NaCl at a Chl concentration of 5.5 μg/mL and their CO₂ exchange was measured at 30°C using the open gas-analysis system described previously (12).

Other Measurements

Growth curves were determined from the rise in the absorbance at 730 nm using a Shimadzu Recording Spectrophotometer (UV200). Pigments in the cells were extracted by methanol and Chl concentrations in the extract were determined as previously described (13).

RESULTS

Identification and Localization of IctA

Western analysis of the thylakoid membrane of WT Synechocystis PCC6803 revealed that a protein with an apparent molecular mass of 6.7 kD cross-reacted with the antibody raised against the fusion protein of IctA and glutathione S-transferase expressed in E. coli (Fig. 1, lane a). No other proteins in the thylakoid membrane cross-reacted with the antibody. The absence of a reacting band at 27 kD, the molecular mass of GSH S-transferase, indicates either that the membrane does not contain this enzyme or that the

![Figure 1. Electrophoretic profiles showing Coomassie brilliant blue staining patterns (lanes A–H) of polypeptides and immunoblots (lanes a–h) of IctA in the thylakoid membranes (lanes A–D, a–d) and cytoplasmic membranes (lanes E–H, e–h) of WT (lanes A, E, a, e), Rkb (lanes B, F, b, f), M9 (lanes C, c, g), and M55 (lanes D, H, d, h) mutants. Samples (40 μg of protein each for Coomassie brilliant blue staining and 20 μg of protein each for immunoblotting) were solubilized at room temperature and were run in a 16 to 22% gradient SDS-PAGE containing 7 M urea. Immunoblotting profiles were obtained by using the antibody raised against the fusion protein of IctA and GSH S-transferase. Molecular standards were phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactoalbumin (14.4 kD). The band of PSI-H plus PSI-K (6.5 kD) is also indicated as an internal standard (4).](https://www.plantphysiol.org/doi/abs/10.1104/0032-0221(92)80189-4)
reactivity of the antibody to the enzyme is very weak. Thylakoid membranes prepared from the mutants RKb and M9, in which the ictA/ndhL gene is inactivated, did not contain reactivity protein at 6.7 kD (Fig. 1, lanes b and c). Thus, the reacting protein is IctA. The molecular mass of the protein calculated from the amino acids encoded by the ictA/ndhL gene is 9.2 kD, which is larger than the value estimated from the SDS-PAGE. This could be attributed to the ambiguity of the value estimated from SDS-PAGE or to a processing of the gene product. The 6.7-kD band was not detected on the Coomassie brilliant blue staining profile (Fig. 1, lane A). The amount of IctA appears to be low.

The immunoblot of the cytoplasmic membrane of WT Synechocystis showed a band at 6.7 kD that cross-reacted with the antibody (Fig. 1, lane e). However, the staining was much weaker than that with the band in the thylakoid membrane. It is possible that the immunostained band in the cytoplasmic membrane arose from contaminated thylakoid membrane. To assess this possibility, the purity of the cytoplasmic membrane preparation was analyzed by determining Chl content. The absorption spectrum of an ether extract of cytoplasmic membrane preparation showed a Chl band at 662 nm, which was as low as 8.9% of the height of a carotenoid band at 475 nm. The Chl content of the cytoplasmic membrane preparation, however, was as high as 32 μg/mg protein, which was 24% of the content in the thylakoid membrane (133 μg/mg protein). This indicated that about one-fourth of the proteins in the cytoplasmic membrane preparation originated from contaminated thylakoid membrane. The contamination is high enough to account for the reacting band in the cytoplasmic membrane preparation (Fig. 1, lane e). Based on this result, I concluded that IctA is absent in the cytoplasmic membrane and is confined in the thylakoid membrane. As expected, none of the cytoplasmic membrane preparations from RKb and M9 mutants showed the cross-reacting band at 6.7 kD (Fig. 1, lanes f and g).

**Absence of IctA in M55 Mutant**

No cross-reacting protein was found at 6.7 kD in the thylakoid membrane or cytoplasmic membrane of M55, a defined mutant of Synechocystis PCC6803 in which the ndhB gene was inactivated (Fig. 1, lanes d and h). Thus, the synthesis of the ndhB gene product is essential for IctA to be assembled to the membrane. The result suggested that IctA is one of the subunits of NADH dehydrogenase. It is presumed that the ictA gene is transcribed and translated in M55, but not properly assembled into the complex of NADH dehydrogenase.

**M-ndhC and M-ndhK Mutants**

Mutants M-ndhC and M-ndhK were constructed by inactivating the ndhC and ndhK genes, respectively, of WT Synechocystis PCC6803 (the sites of insertion of the Km' cartridge are indicated in Fig. 2). Figure 3 shows the CO2 exchange profiles of the mutants and indicates that the activity of CO2 uptake in the light was completely abolished in M-ndhK and was decreased to 40% of the activity of WT in M-ndhC. The significant effect on CO2 transport of inactivation of these genes encoding the subunits of NADH dehydrogenase clearly demonstrates that this enzyme is involved in C3 transport in Synechocystis. Figure 4 shows the Coomassie brilliant blue staining profiles (upper lanes) and immunoblots (lower lanes) of the total membranes of WT, M-ndhC, and M-ndhK. The IctA band in the membranes of M-ndhC and M-ndhK was very poorly immunostained (Fig. 4, lanes b and c), in contrast with the strong staining of the band in the membranes of WT (Fig. 4, lane a). The result supports the hypothesis that IctA is a subunit of NADH dehydrogenase and is not properly assembled into the complex of this enzyme in the absence of other ndh gene products.

**Growth Characteristics**

The growth curves of the WT and mutant cells under high CO2, low CO2, and photoheterotrophic conditions are shown in Figure 5. M-ndhK was unable to grow under air levels of CO2 presumably due to the absence of C3-transport activity, whereas M-ndhC grew under low CO2 conditions (open

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**Figure 2.** Map of the ndhC-ndhK-ndhJ operon of Synechocystis PCC6803 (19) and of the constructs where a Km' cartridge was inserted at the AvrII site or between the two Ncol sites.

**Figure 3.** Changes in CO2 concentration in the gaseous phase of a cell suspension upon illumination with white light (1380 μE m$^{-2}$ s$^{-1}$). Cells of WT, M-ndhC, and M-ndhK, which were grown at 3% CO2 and then incubated in the light for 15 h under aeration with air, were suspended in 20 mm Hepes-NaOH, pH 7.0, containing 15 mm NaCl and then placed in a reaction vessel for gas-exchange measurement.
circles in the middle columns). Although the C\textsubscript{3}-transport activity is low in mutant under low-C\textsubscript{3} conditions. The mutants were unable to grow (M-ndhK) or grew very slowly (RKb and M-ndhC) under phototrophic conditions. M55 was also unable to grow under phototrophic conditions (data not shown). The results show that NADH dehydrogenase is essential to phototrophic growth of Synechocystis. The low growth rate of the RKb mutant under these conditions also supports the hypothesis that IctA is a subunit of NADH dehydrogenase and is required for phototrophic growth of the cells.

Changes in Other Proteins in the Mutants

The Coomassie brilliant blue staining profiles of the thylakoid membranes of RKb and M9 (Fig. 1, lanes B and C) were similar and showed a band at 28 kD that was stained much stronger than the corresponding band in the WT (lane A). Thus, the inactivation of the \textit{ictA/ndhL} gene appears to stimulate the synthesis of the 28-kD protein. The increase of the 28-kD band was also observed in the Coomassie brilliant blue staining profiles of the cytoplasmic membranes of RKb and M9 (Fig. 1, lanes F and G). This may be ascribed to contamination of the thylakoid membranes in the cytoplasmic membrane preparations. The amount of the 28-kD protein (marked with an asterisk in Fig. 4) was not much different in M-ndhC and M-ndhK (Fig. 4, lanes B and C) from that in the WT (Figs. 1 and 4, lanes A) and was lower in M55 (Fig. 1, lane D). Thus, the increase of the 28-kD protein is specific to the mutants that have mutations in the \textit{ictA/ndhL} gene.

There are several proteins with the apparent molecular masses of 18, 20, and 26 kD that were increased in the mutants. These protein bands are indicated by the arrows on the right side of Figure 4. The increase of these proteins was clearly observed in M-ndhC and M-ndhK. Although the resolution of the gel in Figure 1 is not high enough, increase of some of these proteins is observed also in RKb, M9, and M-55. The nature of these proteins is being studied.

**DISCUSSION**

The product of the \textit{ictA/ndhL} gene (IctA) was identified in this study using the antibody raised against the protein expressed in \textit{E. coli}. The absence of the immunologically stained band in the RKb and M9 mutants clearly indicated that the band at 6.7 kD is IctA. The CO\textsubscript{2}-uptake activity was abolished in M-ndhK and was 40% of the activity of WT in M-ndhC. Thus, inactivation of any of the \textit{ndh} genes examined (\textit{ndhB, ndhC, and ndhK}) had significant effect on the C\textsubscript{3}-transport activity of the cells. The result implies that a cyanobacterial NADH dehydrogenase is involved in C\textsubscript{3} transport and that the enzyme has a subunit structure similar to mitochondrial NADH dehydrogenase.

The following results indicated that IctA is a subunit of NADH dehydrogenase. (a) IctA was absent in the membranes of M55 (Fig. 1, lane d), (b) The IctA band was very poorly immunostained in the membranes of the M-ndhC and M-ndhK mutants (Fig. 4, lanes b and c). (c) Like other mutants in which \textit{ndh} genes were inactivated, RKb grew very slowly.

![Figure 4](image-url)

**Figure 4.** Electrophoretic profiles showing Coomassie brilliant blue staining patterns (lanes A–C) of polypeptides and immunoblots of IctA (lanes a–c) in the total membranes of WT (lanes A and a), M-ndhC (lanes B and b), and M-ndhK (lanes C and c). The conditions for electrophoresis and the antibody used for immunoblotting are as in Figure 1.

![Figure 5](image-url)

**Figure 5.** Growth curves of WT (left column), M-ndhC and M-ndhK (middle columns), and RKb (right column) of \textit{Synechocystis} PCC6803 under 3% CO\textsubscript{2} (○), air (0.04% CO\textsubscript{2}, ▲), and under air in the presence of 5 mm glucose and 10 μm atrazine (▼).
under phototrophic conditions (Fig. 5). Thus, IctA is assembled with the products of other \( ndh \) genes and is required for phototrophic growth of the cells. The amino acid sequence deduced from the nucleotide sequence of \( ic\tau A/ndhL \) was subjected to homology search by the computer program developed by Pearson and Lipman (17), in the database of Protein Identification Resource (National Biomedical Foundation, Washington, DC). No homologous gene was found in the database. However, a corresponding gene, as yet undiscovered, may be located in nuclear DNA in higher plants.

In the cytoplasmic membrane preparation of WT \textit{Synechocystis}, the IctA band was poorly immunostained (Fig. 1, lane e). This was ascribed to the contamination of the thylakoid membrane. Using the antibodies raised against the products of the \( ndhK \) and \( ndhJ \) genes of \textit{Synechocystis} PCC6803 expressed in \textit{E. coli}, Berger et al. (2) have identified the products (NdhK and NdhJ) of these two genes and concluded that the products are present in both the thylakoid and cytoplasmic membranes. Their conclusion apparently contradicts the results presented in this paper. One possible explanation for this contradiction is that NADH dehydrogenase in the cytoplasmic membrane has a subunit structure different from that in the thylakoid membrane and does not contain IctA. In my experience, however, it is not possible to avoid some contamination of thylakoid membrane in the preparation of cytoplasmic membrane. Whether NADH dehydrogenase is present in the cytoplasmic membrane should be determined after strict assessment of the contamination of thylakoid membrane in the preparation of cytoplasmic membrane, especially when the cross-reactivity with antibody of a band in the cytoplasmic membrane preparation is weaker than that in the thylakoid membrane. Berger et al. (2) showed that the cross-reactivity of their cytoplasmic membrane preparations with the antibodies raised against the products of \( ndhK \) and \( ndhJ \) was much weaker than that with the thylakoid membranes.

The absence of IctA in the cytoplasmic membrane indicates that IctA is not a component of a \( "C" \) transporter but is essential to energization of the \( "C" \)-transporting system. The \( C \) transport is driven only by PSI (12). Thus, IctA (and hence NADH dehydrogenase) appears to be one of the components involved in PSI cyclic electron transport. ATP produced by coupling to the cyclic electron flow may be the direct energy source of the \( C \) transport. Because RkK and M9 mutants grew well under high CO\(_2\) conditions, transport of other ions (NO\(_3^\), SO\(_4^{2-}\), etc.) energized by ATP should be functioning normally. It is conceivable that \( C \) transport requires higher levels of ATP than the transport of other ions.

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**LITERATURE CITED**

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