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A gene homologous to the subunit-2 gene of NADH dehydrogenase is essential to inorganic carbon transport of *Synechocystis* PCC6803

(*ndhB*/*ndh*/high CO_2-requiring mutant/insertional inactivation)

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ABSTRACT **A clone that transforms the RKa mutant of *Synechocystis* PCC6803 defective in inorganic carbon (C_i) transport to the wild-type phenotype was isolated from a cyanobacterial genomic library. The clone contained an 11.8-kilobase-pair DNA insert. Sequencing of the insert DNA in the region of the mutation in RKa revealed an open reading frame (designated as *ndhB*), which showed extensive amino acid sequence homology to the subunit-2 genes of NADH dehydrogenase (EC 1.6.99.3) (*ndhB*) of chloroplasts and mitochondria. The homology was much stronger with the chloroplast genes. Sequence analysis of the *ndhB* gene of RKa mutant revealed a G → A substitution that results in a Gly → Asp substitution in the deduced amino acid. A defined mutant (M55), constructed by inactivating the *ndhB* gene in wild-type *Synechocystis*, required high CO_2 conditions for growth and was unable to transport CO_2 and HCO_3^- into the intracellular C_i pool. The results indicate that the *ndhB* gene is required for C_i transport. Dark respiration was also depressed by the inactivation of the *ndhB* gene. A possible role of the *ndhB* gene product in the energization of C_i transport is discussed.**

Cyanobacteria possess a mechanism for concentrating inorganic carbon (C_i) internally to high levels, which enables them to cope with the low affinity for CO_2 of their ribulose 1,5-bisphosphate carboxylase/oxygenase and to grow with air levels of CO_2 (1, 2). This mechanism involves an active C_i transport system that utilizes CO_2 and HCO_3^- as substrates (3, 4) and that is activated and energized by light (5, 6). The activation requires photosystems 1 and 2, whereas the energization requires only photosystem 1. To study the molecular mechanism of C_i transport, attempts have been made to isolate mutants defective in C_i transport, which are presumed to require high CO_2 for growth. Most of the high CO_2-requiring mutants isolated from *Synechococcus* PCC7942 (7–10) and *Synechocystis* PCC6803 (11), however, had high activity for C_i transport and appeared to be mutants that were unable to utilize the intracellular C_i pool for photosynthesis. Ogawa (11) isolated two mutants of *Synechocystis* PCC6803, RKa and RKb, that were defective in C_i transport. The clone, which complements one of these mutants, RKb, has been isolated and analyzed (12). It contained an open reading frame (ORF), designated as *ictA*, which codes for a protein of 80 amino acids. The function of this putative protein is not known.

In this study, I cloned and analyzed a DNA fragment of wild-type (WT) *Synechocystis* PCC6803 that, upon transformation, restored WT capabilities to the RKa mutant.* Sequencing of the DNA fragment in the region of the mutation in RKa revealed an ORF (designated as *ndhB*), which showed extensive amino acid sequence homology to the *ndhB* genes of chloroplasts [presumed to encode the subunit 2 of NADH dehydrogenase (EC 1.6.99.3), refs. 13–15] and of mitochondria (16–19). To confirm that the *ndhB* gene is essential for C_i transport, I constructed a defined mutant (M55) of *Synechocystis* by inactivating this gene. Physiological characterizations of the RKa and M55 mutants suggested that the *ndhB* gene encodes a protein involved in energization of the C_i-transporting system.

MATERIALS AND METHODS

Growth Conditions. WT and mutant cells of *Synechocystis* PCC6803 were grown in liquid culture at 30°C in BG11 medium (20) during aeration with 3% CO_2 in air. For growth on solid medium, BG11 was supplemented with 1.5% agar and 5 mM sodium thiosulfite. Where appropriate, BG11 was supplemented with kanamycin (20 μg/ml). All media were buffered with 20 mM *N*-tris(hydroxymethyl)-2-aminomethanesulfonic acid·KOH (pH 8.0). Continuous illumination was provided at 120 μmol of photosynthetically active radiation per m^2·s by incandescent lamps for liquid cultures and by fluorescent lamps for cultures on solid medium.

Cloning and Transformation. A genomic library of WT *Synechocystis* PCC6803 constructed in pUC18, which had been fractionated by electrophoresis into 96 fractions, was a gift from J. G. K. Williams (DuPont). Complementation tests were performed by the method of transformation reported by Dzelzkalns and Bogorad (21). A fraction of the genomic library that had the highest complementation ability was used to transform *Escherichia coli* (DH5α). One thousand clones from the enriched library were tested for their ability to complement RKa. Four clones capable of complementing the mutant were obtained. One of these clones was used for further analysis.

Insertional Inactivation. The cloned DNA fragment was interrupted by an aminoglycoside 3′-phosphotransferase gene (which confers kanamycin resistance, the Km^r cartridge) that originated from the bacterial transposon Tn*903* (22). The WT cells of *Synechocystis* were transformed with the plasmid containing the modified DNA fragment according to the method reported by Williams and Szalay (23). The mixture of cells (100 μl, 4×10^8 cells per ml) and the plasmid (5 μl, 1 mg of DNA per ml) was incubated in a plastic tube under growth conditions for 6 hr and aliquots (0.1 ml) were plated on a sterile membrane filter on solid medium. After 20 hr of incubation under nonselective conditions (3% CO_2), the filters were transferred onto solid medium containing 5 μg of kanamycin per ml. Colonies of transformed cells, which were visible in 7 days, were streaked onto solid medium containing 15 μg of kanamycin per ml and the plates were placed in a box

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Abbreviations: WT, wild-type; C_i, inorganic carbon; ORF, open reading frame; Chl, chlorophyll; Km^r, kanamycin resistance(t); PCR, polymerase chain reaction; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DTT, dithiothreitol.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. D90288).

aerated with 3% CO_2 in the light. This step was repeated several times, and a total of 100 colonies from the plates of the final step was screened on duplicate plates containing 15 μg of kanamycin per ml under air and 3% CO_2 conditions.

Analysis of Mutant DNA. A fragment of the *ndhB* gene of RKa [698-base-pair (bp) nucleotides] was amplified by the method of polymerase chain reaction (PCR, ref. 24) using the genomic DNA of the mutant as the template and cloned into pUC119 for sequencing. The oligonucleotides used as the primers were synthesized according the sequence shown in Fig. 2 (sequence from bases 347 to 365 and complementary sequence from bases 1149 to 1132). The same primers were used when the DNA fragments of WT and M55 were amplified by the PCR method for analysis of segregation of the modified *ndhB* gene in M55.

Other DNA Methods. Unless otherwise stated, standard techniques were used for DNA manipulation (25). Nucleotides were deleted from the cloned DNA fragment using a deletion kit (Takara, Tokyo) that contains exonuclease III and mung bean nuclease. The number of nucleotides deleted was determined by sequencing the DNA fragment after the deletion. The nucleotide sequences were determined using a DuPont DNA analysis system (Genesis 2000). Both strands were sequenced with no ambiguities.

Silicone Oil-Filtering Centrifugation. The uptake of $^{14}CO_2$/$H^{14}CO_3^-$ was measured in 3% CO_2-grown cells before and after 17 hr of aeration with air in the light, using the silicone oil-filtering method (3). Cells were suspended in BG11 buffered with 20 mM Hepes·KOH (pH 8.0) at a chlorophyll (Chl) concentration of 5 μg/ml. C_i uptake was initiated by the addition of $^{14}CO_2$ or $H^{14}CO_3^-$ in the light and terminated by centrifugation.

Gas Exchange Measurements. The mutant and WT cells of *Synechocystis* grown with 3% CO_2 were aerated with air for 17 hr in the light without 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and dithiothreitol (DTT) and then suspended in 20 mM Hepes·NaOH buffer (pH 7.0) containing 15 mM NaCl at a Chl level of 5.5 μg/ml. CO_2 exchange of the cell suspension was measured at 30°C in the presence of 10 μM DCMU with and without 10 mM reduced DTT using an open gas-analysis system described previously (5, 8).

Other Measurements. Growth curves were determined from the rise in the OD at 730 nm using a Shimadzu (Kyoto) recording spectrophotometer (UV200). Oxygen uptake by dark respiration was measured in 3% CO_2-grown cells suspended in the growth medium, using a Clark-type O_2 electrode (Rank Brothers, Cambridge, U.K.). Pigments in the cells were extracted by methanol, and Chl concentration in the extract was determined (26).

RESULTS

Sequence Analysis of the WT Locus Required for C_i Transport. A clone that complemented RKa was isolated from a genomic library of WT *Synechocystis* PCC6803. The clone contained an 11.8-kilobase-pair (kbp) DNA insert (the restriction map is shown in Fig. 1). Deletion analysis of this insert indicated that the mutation in RKa is located between 662- and 742-bp nucleotides from the *Bam*HI site (between 1036- and 1116-bp nucleotides from the *Eco*RI site upstream of the *Bam*HI site as numbered in Fig. 2); a subclone constructed after deletion of 661-bp nucleotides from the *Bam*HI site had no effect on its complementing activity, whereas a subclone with a 743-bp deletion failed to complement the mutant. Sequence analysis of 1816-bp nucleotides between the *Eco*RI and *Bgl* II sites revealed an ORF that covers the above region of mutation in RKa and encodes a highly hydrophobic protein consisting of 521 amino acids (341 hydrophobic, 103 hydrophilic, and 77 neutral residues, Fig. 2). The calculated molecular weight of this protein is 55,453.

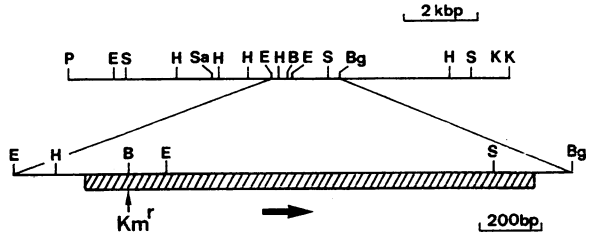

FIG. 1. Restriction map of an 11.8-kbp-insert DNA in the clone that complements the RKa mutant. The 1563-bp-nucleotide ORF (*ndhB* gene) is indicated by the hatched bar, with an arrow showing the direction of the ORF. When M55 was constructed, the Km^r cartridge was inserted in the site as indicated. B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn* I; P, *Pst* I; S, *Sph* I; Sa, *Sac* I.

The ORF was designated as *ndhB* based on the homology with chloroplast and mitochondrial genes (see Fig. 3). A Shine–Dalgarno (SD, ref. 27) sequence (GAG) was found upstream of the postulated initiation codon of the *ndhB* gene. A possible promoter sequence can be found upstream of the initiation codon at bases 67–72 (TTGCCA, −35 box) and 90–95 (AATACT, −10 box). An inverted repeat downstream of the *ndhB* gene (bases 1759–1779) may be involved in transcription termination.

Homologous Genes. The amino acids encoded in the *ndhB* gene showed significant sequence homology to those of the *ndhB*(*ndh2*) genes of chloroplasts and mitochondria. The mitochondrial *ndhB* gene encodes the subunit 2 of the respiratory-chain NADH dehydrogenase (16–19, 28), and the chloroplast genes homologous to the mitochondrial gene have been designated as *ndhB* or *ndh2* (13–15). Fig. 3 shows the deduced amino acid sequence homology of the *ndhB* gene with the *ndhB* genes of *Marchantia polymorpha* (liverwort) chloroplasts (13) and human mitochondria (URF2, ref. 16). Significant homology exists among these three amino acid sequences over their entire length and very strong homology exists in specific regions. The *ndhB* gene of *Synechocystis* had stronger homology with the chloroplast genes than with the mitochondrial genes; the sequence homologies of amino acids encoded in the *ndhB* gene of *Synechocystis* with those of the *ndhB* genes of liverwort (13), tobacco (14), and rice (15) chloroplasts were 50.3% (homology score = 1451), 50.3% (1055), and 51.0% (1399), respectively, whereas the homologies with those of the *ndhB* genes of human (16), bovine (17), sugarbeet (18), and *Chlamydomonas* (19) mitochondria were 24.8% (347), 24.5% (280), 28.8% (515), and 22.2% (385), respectively.

Mutation in RKa. Sequence analysis of a fragment of respective *ndhB* gene of RKa in the region of mutation revealed a G → A substitution at position 1061 (Fig. 2). This change resulted in a Gly → Asp substitution in the deduced amino acid at residue 295 (Fig. 3). The glycine is one of the conserved amino acids among protein products of the *ndhB* genes of chloroplast and mitochondrial origin and, therefore, could be an important amino acid for the function of the proteins.

Construction of a Defined Mutant. The significance of the *ndhB* gene for C_i transport was further demonstrated by the generation of a high CO_2-requiring mutant (M55) following inactivation of the WT gene by inserting the Km^r cartridge in the *Bam*HI site (see Fig. 1). Electrophoretic analysis of the modified and unmodified *ndhB* gene fragments in M55 and WT amplified by the PCR method showed that the 0.7-kbp *ndhB* fragment in WT was replaced by a 2.0-kbp DNA fragment in M55 (data not shown). The 0.7-kbp *ndhB* gene fragment was absent in the PCR product of M55, indicating that no WT *ndhB* gene remained in M55 and that segregation of the modified gene was complete. The size of the modified gene fragment agreed with the expected size (0.7-kbp nucle-

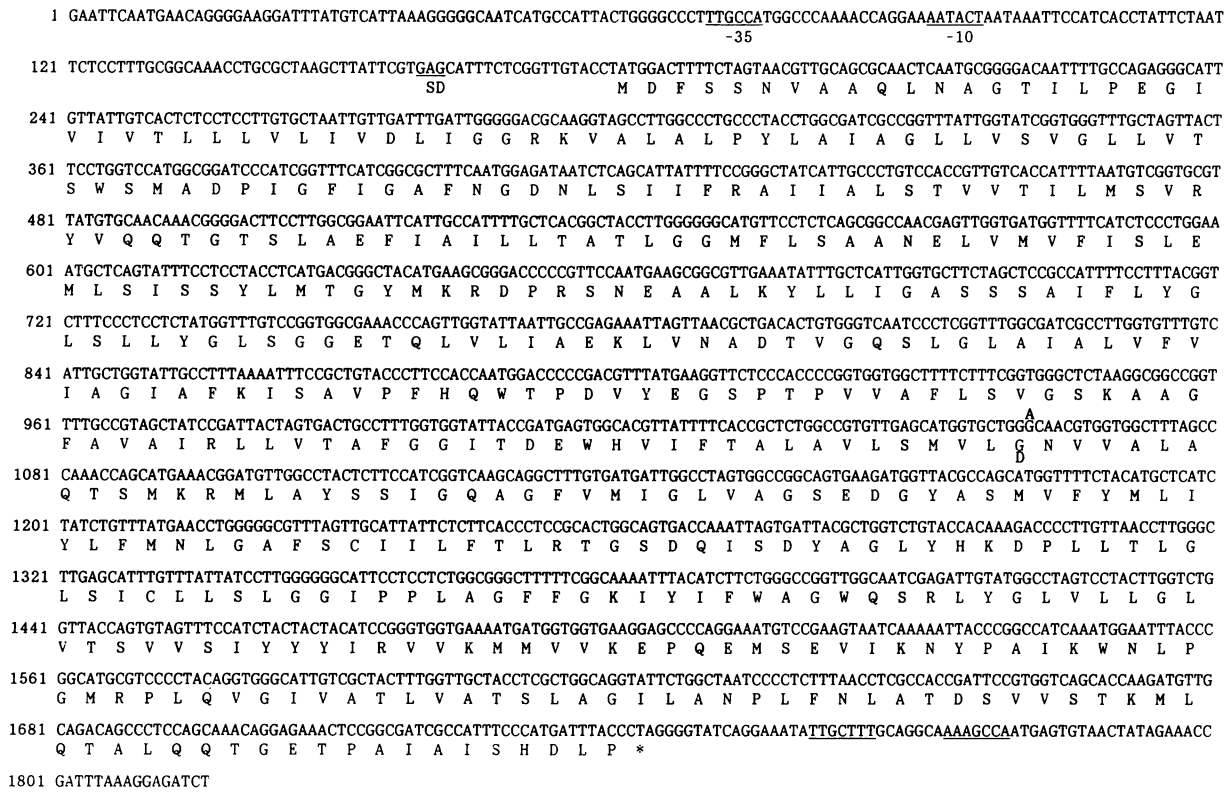

FIG. 2. Nucleotide sequence of the insert DNA between the *Eco*RI and *Bgl* II sites and deduced amino acid sequence (standard one-letter symbols) of the protein encoded in the *ndhB* gene. The A residue at position 1061 is the base substitution in the RKa mutant (G → A). This results in a Gly (G) → Asp (D) substitution in the deduced amino acid. The −10 and −35 sequences are underlined and a possible ribosome binding site is labeled SD and underlined. An inverted repeat downstream of the *ndhB* gene, possibly involved in transcription termination, is also underlined.

otides for the *ndhB* gene fragment *plus* 1.3-kbp nucleotides for the Km^r cartridge). Transformants obtained after insertion of the Km^r cartridge at the *Bgl* II site downstream of the *ndhB* gene were able to grow under low CO_2 as well as under high CO_2 conditions. The result indicated that the generation of the high CO_2-requiring mutant is a result of inactivation of the *ndh* gene and is not due to polar effects on the downstream sequences.

Physiological Characteristics of M55 and RKa. The WT cells of *Synechocystis* grow with air levels of CO_2 as fast as with 3% CO_2 (Fig. 4 *Left*). In contrast, M55 and RKa were unable to grow with air (Fig. 4 *Right and Center*), presumably due to the defect in C_i transport. Growth rates of WT and RKa were similar under 3% CO_2, indicating that high CO_2 conditions overcame the defect in RKa. The growth rate of M55 was lower than that of WT even under 3% CO_2. Thus, the complete inactivation of the *ndhB* gene in M55 appears not only to inactivate C_i transport but also to affect other aspects of metabolism.

Direct evidence for a defect of C_i transport in RKa and M55 was obtained by measuring CO_2 and HCO_3^- uptake into the intracellular C_i pool. C_i uptake was low in high CO_2-grown

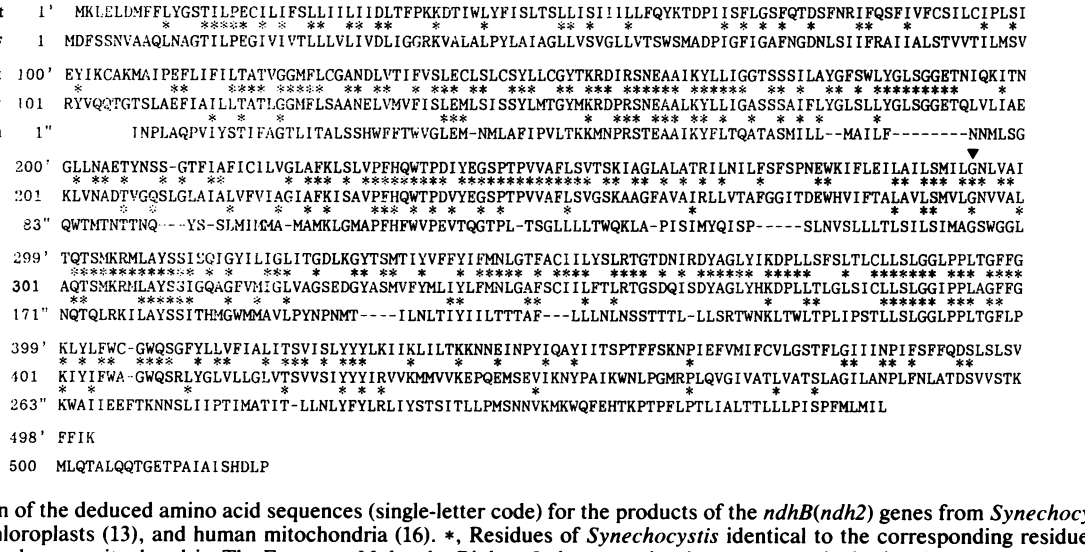

FIG. 3. Comparison of the deduced amino acid sequences (single-letter code) for the products of the *ndhB*(*ndh2*) genes from *Synechocystis* PCC6803, liverwort chloroplasts (13), and human mitochondria (16). *, Residues of *Synechocystis* identical to the corresponding residues in liverwort chloroplasts or human mitochondria. The European Molecular Biology Laboratory data base was searched using the computer program developed by Pearson and Lipman (29). ▼, Gly that was substituted by Asp in the RKa mutant.

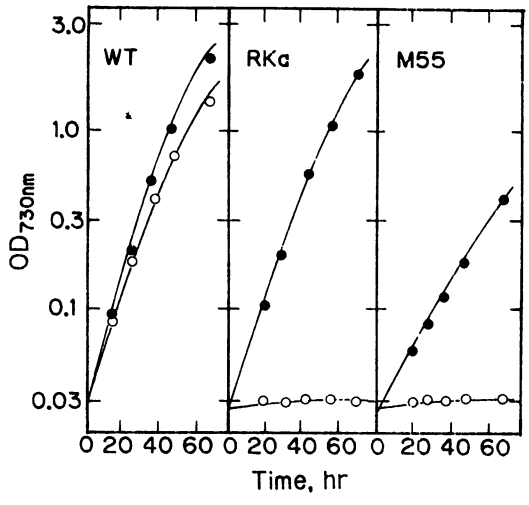

FIG. 4. Growth curves of WT, RKa, and M55 under 3% CO_2 (●) and air (0.04% CO_2, ○).

cells of WT *Synechocystis* but increased after aeration with air. CO_2 was the C_i species preferentially transported in high CO_2-grown and low CO_2-adapted WT cells (Fig. 5 *Left*). These characteristics of C_i transport in WT *Synechocystis* are similar to those in other cyanobacterial strains (3, 4, 8). In contrast to WT cells, RKa and M55 cells, before and after aeration with air, showed very little activity of CO_2 uptake (Fig. 5 *Right*). In addition, HCO_3^- uptake was negligibly low in high CO_2-grown cells of RKa and M55 and was only 10% the WT activity in air-adapted cells of RKa. In M55, the HCO_3^- uptake was not detectable even in air-adapted cells. These characteristics of the mutants indicate that the *ndhB* gene is essential to C_i transport.

The rates of O_2 uptake by dark respiration in WT, RKa, and M55 cells were 21.7, 6.7, and 1.6 μmol/mg of Chl per hr, respectively, which were completely abolished by 1 mM KCN. Thus, dark respiration was depressed by the inactivation of the *ndhB* gene. These results indicate that the *ndhB* gene encodes a protein involved in dark respiration and justify the presumption that the gene encodes a subunit of NADH dehydrogenase.

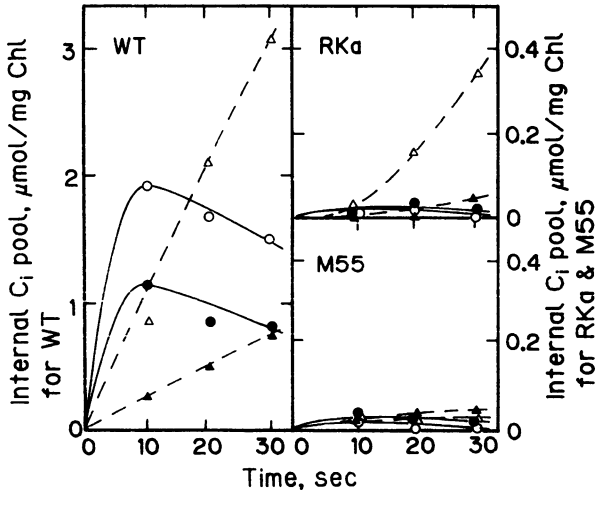

FIG. 5. Time courses of uptake of CO_2 and HCO_3^- into the intracellular C_i pool of WT, RKa, and M55 cells. Circles, CO_2 was supplied; triangles, HCO_3^- was supplied; closed symbols, 3% CO_2-grown cells; open symbols, cells aerated with air for 17 hr in the light. The concentrations of CO_2 and HCO_3^- were 9.3 and 255 μM, respectively.

C_i transport in cyanobacteria is driven by light energy from only photosystem 1. Light is also required for activation of the transport system. In contrast to the energization, the activation requires a very low level of photosystem-2 activity, which can be substituted by reduced DTT. The depression of dark respiration by the inactivation of the *ndhB* gene suggests that a protein encoded in this gene is not a C_i transporter but is involved in energization and/or activation of C_i transport. To test the possibility whether the defect in C_i transport in RKa and M55 is due to a defect in the activation process, CO_2 uptake by air-adapted WT and mutant cells was measured in the presence and absence of reduced DTT (Fig. 6). When the photosystem-2 activity was inhibited by DCMU, WT cells had only low activity of CO_2 uptake, which was increased by addition of reduced DTT (upper curves). Thus, reduced DTT can be used for activation of the C_i-transporting system of *Synechocystis*, as has been observed with *Synechococcus* (6). If the activation process was impaired in RKa and M55, reduced DTT will restore CO_2 uptake in these mutants. However, the mutants did not show CO_2 uptake even in the presence of reduced DTT (middle and lower curves). It appears most probable that a protein product of the *ndhB* gene is involved in energization of the C_i-transporting system.

DISCUSSION

A clone that transforms RKa to a WT phenotype was isolated from a genomic library of WT *Synechocystis*. Analysis of the nucleotide sequence in the region of the mutation revealed an ORF, designated as *ndhB* (Figs. 1 and 2), which showed extensive sequence homology with the *ndhB* (or *ndh2*) genes of chloroplasts and mitochondria (Fig. 3). Inactivation of the *ndhB* gene in WT *Synechocystis* led to the significant reduction of the ability of the cells to transport CO_2 and HCO_3^- into the intracellular C_i pool (Fig. 5). These results clearly demonstrated that the *ndhB* gene is essential to C_i transport.

The following possibilities can be considered for the role of the *ndhB* gene product in C_i transport: it is a component of the C_i transporter that has a dehydrogenase-like structure or it is an enzyme involved in the activation or energization of the C_i transport system. Dark respiration was depressed by the inactivation of the *ndhB* gene, which suggests that the *ndhB* gene product is not a component of the C_i transporter but is involved in energization and/or activation of the C_i-transporting system. The possibility that the *ndhB* gene functions only in the activation of the system may be ruled

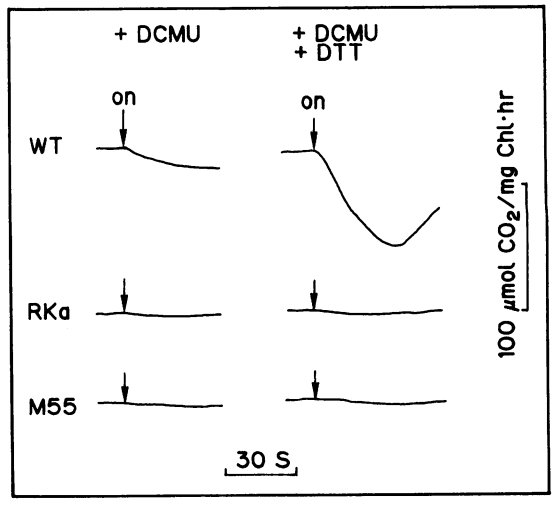

FIG. 6. The CO_2 exchange of WT, RKa, and M55 cells in the presence of DCMU as affected by light and reduced DTT.

out since RKa and M55 did not show C_i transport activity even in the presence of reduced DTT (which activates the C_i transport system in WT, Fig. 6). The growth rate of M55 was slower than that of the WT even under high CO_2 conditions (Fig. 4). Thus, the inactivation of the *ndhB* gene affects not only C_i transport but also has pleotropic effects, probably due to the depression of dark respiration. C_i transport in cyanobacteria proceeds under anaerobic conditions, indicating that respiration is not essential for transport (5). Thus, the depression of C_i transport in RKa and M55 mutants is not a result of the depression of dark respiration. In cyanobacteria, the respiratory and photosynthetic electron transport chains share components within the thylakoid membrane (30). NADH dehydrogenase is one of these components. Thus, it appears most probable that the *ndhB* gene encodes a subunit of NADH dehydrogenase that plays a role in photosynthetic electron transport to energize the C_i-transporting system.

In human mitochondria, six of the ORFs (URFs 1–5 and 4L) have been identified as genes for components of the NADH dehydrogenase (16, 28). Several chloroplast genes were designated as *ndh* based on their homology to the human mitochondrial genes (13–15). Genes designated as *ndhC*, *ndhD*, and *ndhE*, respectively, have recently been cloned from *Synechocystis* PCC6803 (31, 32). The *psbG* gene, which was once regarded as being involved in photosystem-2, and *ORF157* are also considered to be members of the group of the *ndh* genes (31, 33). To date, however, no evidence has been shown on the function of the *ndh* genes in photosynthetic organisms. The present study clearly demonstrated that the *ndhB* gene of *Synechocystis* is essential for C_i transport and dark respiration. If other *ndh* genes of *Synechocystis* encode proteins that make a complex with the *ndhB* gene product, they may also be essential for C_i transport. This could be clarified by studying the effect of inactivation of each of the *ndh* genes on C_i transport. Recently, another gene (*ictA*) essential to C_i transport has been cloned (12). Dark respiration was depressed by the inactivation of the *ictA* gene (unpublished). It appears plausible that the *ictA* gene is also a member of the *ndh* gene group, although no homologous genes were present in chloroplasts or mitochondria.

Because of the hydrophobic nature of the protein encoded in the *ndhB* gene, it may be located in the thylakoid membrane and/or cytoplasmic membrane. C_i transport is energized only by photosystem-1 light energy (5). If the protein is confined to the thylakoid membrane, there may be a photosystem-1 cyclic electron transport that involves this protein and produces energy required for C_i transport. If the protein is located in the cytoplasmic membrane, there is a possibility that it is involved in producing a H^+ gradient across the membrane that is coupled to the transport of C_i. Further studies are necessary to identify the protein product of the *ndhB* gene and to locate it in the cell.

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