Plant Physiol. (1990) 93, 305–311
0032-0889/90/93/0305/07/$01.00/0

Received for publication May 31, 1989
and in revised form January 3, 1990

Sequencing and Modification of the Gene Encoding the 42-Kilodalton Protein in the Cytoplasmic Membrane of *Synechococcus* PCC 7942

Tatsuo Omata, Thomas J. Carlson, Teruo Ogawa, and John Pierce*

Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351-01, Japan (T.O., T.O.); *and Central Research & Development Department* (T.J.C.) *and Agricultural Products Department* (J.P.), *E.I. Du Pont de Nemours & Company, Wilmington, Delaware 19880-0402*

ABSTRACT

A 42-kilodalton cytoplasmic membrane protein is synthesized when high CO_2-grown cells of *Synechococcus* PCC 7942 (*Anacystis nidulans* R2) are exposed to low CO_2. The structural gene for this protein (cmpA) has been cloned and sequenced and shown to encode a 450 amino acid polypeptide with a molecular mass of 49 kilodalton. A deletion mutant lacking the 42-kilodalton protein was obtained by transformation of *Synechococcus* PCC 7942 following *in vitro* mutagenesis of the cloned gene. There were no significant differences between the mutant and wild-type cells in their growth rates under either low or high CO_2 conditions. The activity of inorganic carbon (C_i) transport in the mutant was as high as that in the wild-type strain. In both types of cells, CO_2 was the main species of C_i transported and the activities of CO_2 and HCO_3^- transport increased when high CO_2-grown cells were exposed to low CO_2. We conclude that the 42-kilodalton protein is not directly involved in the C_i-accumulating mechanism of *Synechococcus* PCC 7942.

Exposure of H-cells[1] of cyanobacteria to low CO_2 conditions increases their C_i-transporting capability and photosynthetic affinity for extracellular C_i (1, 4, 9, 15, 26). A 42-kD protein is synthesized in the cytoplasmic membrane of *Synechococcus* PCC 7942 and PCC 6301 during adaptation to low CO_2 (15, 16). A number of biochemical studies have shown a close relationship between the amount of the 42-kD protein and the C_i transporting activity of the cells (16, 18). In addition, a high CO_2 requiring mutant of this organism failed to accumulate the 42-kD protein when exposed to low CO_2 concentrations (11). While these studies suggested that the protein may play a role in the C_i-transporting mechanism, Schwarz *et al.* (24) have presented results which argue against the possible involvement of the protein in C_i transport. These authors described a mutant strain (O_{221}) of *Synechococcus* PCC 7942 which increases its C_i-transporting activity upon exposure to low CO_2 conditions yet does not accumulate the 42-kD protein during this adaptation (24). Clearly, increased amounts of the 42-kD protein are not required for increased C_i transport activity. However, since the mutation in O_{221} may not be in the structural gene for the 42-kD protein and since possible functioning of trace amounts of the protein in the mutant could not be excluded, further roles for the 42-kD protein in C_i transport can be envisioned. The 42-kD protein might also have a role in adaptation of the cells to low CO_2 conditions, since mutants which do not accumulate the 42-kD protein, RK1, and O_{221} (11, 24), cannot grow under low CO_2 conditions.

To see whether the 42-kD protein is necessary for C_i-transport and growth under low CO_2 conditions, we have cloned the gene (cmpA) for this protein and constructed a defined mutant of *Synechococcus* PCC 7942 by insertional mutagenesis. The mutant was biochemically and physiologically characterized to evaluate the possible involvement of the 42-kD protein in C_i transport and growth under carbon-limited conditions.

[1] Abbreviations; H-cells, cells grown under high (3%) CO_2 conditions; C_i, inorganic carbon; L-cells, cells grown under high CO_2 and then exposed to low (0.03%) CO_2 conditions for 20h; WT, wild-type strain; Km, Kanamycin; cmpA, cytoplasmic membrane protein A.

MATERIALS AND METHODS

Synechococcus PCC 7942 (provided by S.S. Golden) was grown at 30°C in BG-11 medium (28) supplemented with 50 mM Tes-KOH buffer (pH 7.5). Solid medium was BG-11 supplemented with 1.5% agar, 0.3% sodium thiosulfate, and 50 mM Tes-KOH buffer (pH 8.0). Continuous illumination was provided at 120 μmol PAR$\cdot m^{-2}\cdot s^{-1}$ by incandescent lamps for liquid culture or by fluorescent lamps for cultures on solid medium.

Cloning and Transformation

Chromosomal DNA was extracted and purified from cells of *Synechococcus* PCC 7942 and used for the construction of an expression library in λ-gt11 phage (33). The expression library, containing an average insert size of 5 kb, was screened according to Snyder and Davis (27) using rabbit IgG against the 42-kD protein (17) as a probe. Goat anti-rabbit IgG/alkaline phosphatase conjugate (Bio-Rad) was used to detect the antibody probe.

Synechococcus PCC 7942 was transformed as described by Williams and Szalay (31). A suspension of recipient cells (10^9 cells$\cdot mL^{-1}$) was mixed with 1/50 volume of a solution containing 10 mM Tris-HCl buffer (pH 7.5), 0.1 mM EDTA, and 1 mg$\cdot mL^{-1}$ of plasmid DNA. The constructs that we used contained the gene for aminoglycoside-3′-phosphotransferase

(which confers kanamycin resistance) from the bacterial transposon Tn903 (13) to allow for positive selection of transformants. The cell/DNA mixture was incubated in a glass tube under growth conditions for 6 h. Aliquots (0.1 mL) were plated out on sterile membrane filters (Nucleopore) on solid medium in Petri dishes. After 20 h of incubation under nonselective conditions, the filters were transferred onto solid medium containing 5 μg kanamycin $\cdot$ mL^{-1}. Colonies of transformed cells were visible in 7 d.

The plasmid pUC119 was used for cloning, sequencing, and other DNA manipulations. This plasmid is a pUC19 derivative (32) that contains the intergenic region of M13 phage to allow for production of single stranded DNA. Unless otherwise noted, standard techniques were used for DNA manipulation (8).

Protein Purification and Sequence Analysis

The 42-kD protein was purified by two cycles of SDS-PAGE (6). Gel pieces containing the protein were cut out of the gel, homogenized, and lyophilized. After electroelution of the protein from the gel matrix, the protein was subjected to ion-pair extraction (5) to remove SDS and dye contaminants and sequenced using standard methodologies (3).

Other Techniques

Cytoplasmic (plasma) membranes were prepared from cells of *Synechococcus* as described previously (14). For immunoblotting after electrophoresis, polypeptides were electrotransferred to nitrocellulose and reacted with IgG against the 42-kD protein. Goat anti-rabbit IgG/alkaline phosphatase conjugate was used as the second antibody to detect the reacting polypeptides. Protein amounts were determined according to Lowry *et al.* (7).

Uptake of $^{14}CO_2$ and $H^{14}CO_3^-$ by *Synechococcus* was measured according to Volokita *et al.* (29) using the filtering centrifugation technique. Cells were suspended in 20 mM Hepes-NaOH buffer (pH 8.0) containing 15 mM NaCl and used for the measurement.

DNA was sequenced using the dideoxy chain-termination method (23). Both strands were sequenced twice with no ambiguities.

RESULTS

Isolation and Sequence Analysis of cmpA

A genomic library of *Synechococcus* PCC 7942 constructed in λ-gt11 phage was screened using antibody raised against the 42-kD protein. A recombinant phage that produced peptides recognized by the antibody was cloned and found to contain a 0.94 kb insert within the *Eco*RI site of the phage. The orientation of the insert was determined by restriction analysis. After heat shock and induction with isopropyl-thio-β-galactoside, lysogenic clones from this phage produced a β-galactosidase fusion protein that was slightly larger than authentic β-galactosidase. This fusion protein was predominantly located in the membrane fraction of the *Escherichia coli* lysogens and it cross-reacted well with the anti-42-kD antibody. The 0.94 kb *Eco*RI fragment was ligated into the *Eco*RI site of pUC119 plasmid, the recombinant plasmid (Fig. 1A) was cloned, and single stranded DNA prepared from the clone was sequenced. An open reading frame of 549 nucleotides (183 amino acids), which had been fused in frame to the lacZ sequence in the λ-gt11 recombinant, was found. This indicated that the 0.94 kb fragment contained about half of the structural gene for the 42-kD protein.

The remainder of the gene was then cloned by marking it with an antibiotic resistance gene as follows. Restriction analysis revealed two *Bgl*II sites in the 0.94 kb DNA fragment (Fig. 1A). Digestion of the plasmid with *Bgl*II was followed by ligation of a 1.3 kb *Bam*HI fragment containing the gene for aminoglycoside-3′-phosphotransferase into the newly created *Bgl*II sites. The resulting plasmid contained flanking sequences from cmpA which were interrupted by the antibiotic resistance gene (Fig. 1B). When *Synechococcus* PCC 7942 was incubated with this plasmid and then allowed to grow on Km containing media under an atmosphere of 1% CO_2 in air, numerous Km^r resistant transformants were obtained. To provide adequate time for the newly introduced DNA to

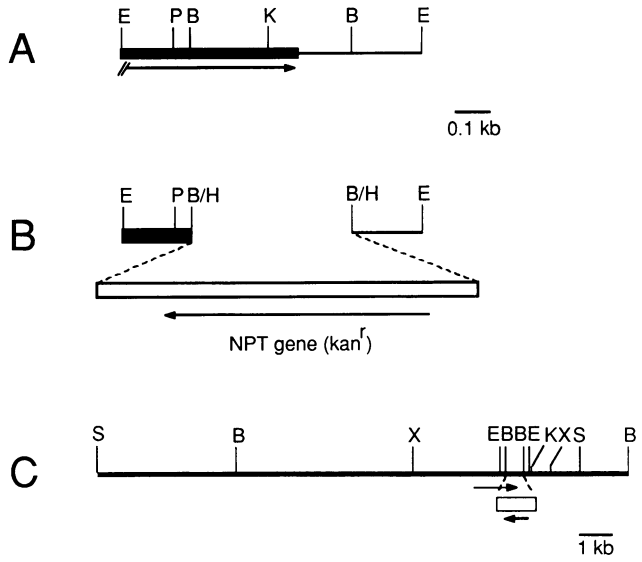

Figure 1. A, Restriction map of a 0.94 kb *Eco*RI fragment isolated from λ-gt11 phage and cloned into pUC119. This DNA constitutes a portion of the gene for the 42-kD protein. The coding region of the DNA is indicated by the thick bar, with an arrow showing the direction and extent of the coding region. (Note: The flanking *Eco*RI sites in this construct derive from cyanobacterial DNA and were not simply introduced during construction of the λ-gt11 library). B, Structure of the modified DNA constructed by replacing the 0.5 kb *Bgl*II fragment with the gene for aminoglycoside-3′-phosphotransferase, conferring Km resistance. The Km-resistance cartridge is indicated by the thick open bar, and the arrow indicates the direction and extent of the protein coding region. C, Restriction map of chromosomal DNA of *Synechococcus* PCC 7942 obtained using the 0.94 kb *Eco*RI fragment as a probe. The rightward facing arrow indicates the direction and extent of the 42-kD protein coding region, and the open bar indicates the position at which the gene was disrupted by insertion of the Km-resistance cartridge. Abbreviations used for restriction enzyme sites: B, *Bgl*II; E, *Eco*RI; H, *Bam*HI; K, *Kpn*I; P, *Pst*I; S, *Sph*I; X, *Xba*I.

segregate to all copies of the cyanobacterial chromosome, approximately 20 individual Km^r transformants were separately and serially restreaked three times onto Km containing media and allowed to grow in air supplemented with 1% CO_2. Southern blot analysis of chromosomal DNA from selected transformants (using the 0.94 kb fragment as a probe) indicated that wild-type DNA had been replaced in all chromosomes by the modified gene via homologous recombination of the flanking cyanobacterial DNA in the construct with chromosomal DNA (not shown, but see Fig. 1C). This type of recombination is expected in *Synechococcus* PCC 7942 for a selectable gene (Km^r) flanked by chromosome-homologous sequences (31), and the resulting gene replacement produced a deletion mutation in cmpA which was marked by the presence of the Km^r gene. A digest of chromosomal DNA from a selected transformant (using either *Xba*I/*Kpn*I or *Bgl*II/*Sph*I) was ligated into pUC119 and the resulting plasmids were used to transform *E. coli* to ampicillin and Km resistance. In this manner, Km resistant clones containing either a 3.7 kb *Xba*I/*Kpn*I fragment or a 10.8 kb *Bgl*II/*Sph*I fragment were recovered and shown to contain both the Km-resistance cartridge as well as flanking DNA from the original 0.94 kb fragment. Single stranded DNA prepared from these plasmids was used for sequencing the remainder of cmpA. An open reading frame of 1350 nucleotides was found (Fig. 2) which encodes a 450 amino acid polypeptide with a calculated molecular mass of 49.1 kD. (The ATG codon at nucleotide position 1 was identified as the translation initiation site by virtue of the presence of a putative ribosome binding site (AGGGGAGA) commencing 11 bases upstream of the ATG codon and the presence of an in-frame stop codon (TAG) 12 bases upstream.) The deduced molecular mass is significantly larger than the apparent molecular mass of the 42-kD protein estimated from its electrophoretic mobility (15, 16).

Protein sequence analysis of the purified 42-kD protein revealed a sequence of X-X-X-Pro-Gln-Ala-Tyr-Leu-Gln-X-X-X (where X denotes an ambiguous amino acid) which was not found in a recent data base of published sequences. This sequence was found in the derived sequence in Figure 2, but surprisingly was not at the amino terminus as expected but rather was near the carboxyl end of the deduced protein sequence (amino acids 438–443). Although we have not investigated the matter further, we surmise that the protein may be blocked at the amino terminus and was inadvertantly cleaved at the Asn^{434}–Pro^{435} bond during its manipulation after purification. Asp-Pro and Asn-Pro bonds are well known to be sensitive to acid hydrolysis (20), and such a cleavage could explain our results. In any event, the correspondence of this sequence with the sequence derived from the DNA analysis (together with the immunological evidence cited above) indicates that the cloned gene encodes the 42-kD protein isolated from cytoplasmic membranes of *Synechococcus* PCC 7942.

Characterization of a cmpA Deletion Mutant

The Km-resistant transformant obtained above is a deletion mutant lacking 330 nucleotides of the structural portion of cmpA. As a result, the modified gene in this transformant encodes a protein lacking 110 amino acids of the carboxyl terminus of the native protein.

Cytoplasmic membranes purified from the Km-resistant transformant were analyzed to see whether the product of the modified cmpA was present. The polypeptide composition of the cytoplasmic membranes from H-cells of the transformant was essentially the same as that of WT cells (Fig. 3A, lanes a and c). Exposure of WT H-cells to low CO_2 conditions led to a large increase in the amount of the 42-kD protein (Fig. 3A, lane b) as has been observed previously (15, 16). In contrast, no apparent change in polypeptide composition was observed in the transformant even after 20 h of exposure to low CO_2 (Fig. 3A, lane d). The absence of 42-kD protein derivatives in the transformant was further confirmed by immunoblotting using the antibody against the 42-kD protein as the probe (Fig. 3B). The 42-kD protein was densely stained when cytoplasmic membranes of WT L-cells (Fig. 3B, lane b) were analyzed, whereas only a very faint 42-kD band was observed in L-cells of the Km-resistant transformant (lane d). Since the first faint 42-kD band was also observed in H-cells of both the WT and the transformant (lanes a and c), it would appear that this band represents a minor, noninducible protein which cross-reacts with the antibody. No other immunoreactive proteins were found in the cytoplasmic membrane from L-cells of the transformant (land d). Further, prolonged incubation of the transformant for up to 60 h under low CO_2 conditions did not induce any protein reactive with the antibody (not shown). Thus, the product of the modified gene was absent in the cytoplasmic membrane of the transformant.

When the total membrane fraction (cytoplasmic membrane, thylakoid membrane plus cell wall) from the transformant was analyzed by immunoblotting, faint bands at 50, 45, 40, and 35 kD were observed (not shown). However, these bands were neither specific to the transformant nor to L-cells, indicating that they were due to nonspecific binding of the antibody. These results indicated that the product of the modified gene was absent in any of the membranes in the transformant. Thus, the transformant is a simple mutant in which the 42-kD protein is totally missing. This mutant will be denoted as M42 hereafter.

Properties of the M42 Mutant

M42 could grow under both low and high CO_2 conditions (Fig. 4). The growth rate of M42 under low CO_2 was comparable to that under high CO_2, and there were no significant differences in the growth rates of M42 and WT under low or high CO_2 conditions. Clearly, the 42-kD protein is not necessary for low CO_2 growth.

We further characterized the M42 mutant's ability to pump and accumulate C_i in order to determine whether active accumulation of C_i requires the 42-kD protein. In WT H-cells, light-dependent CO_2 uptake was much faster than HCO_3^- uptake (Fig. 5a, curves C and D). At low (10 μM) HCO_3^- concentrations, uptake rates were severely depressed (not shown). Thus, CO_2 is the species preferentially taken up by *Synechococcus* cells under these conditions. H-cells of M42 also took up CO_2 and HCO_3^- in the light (curves C and D in Fig. 5b), although the rates of CO_2 and HCO_3^- uptake were lower than those in H-cells of WT. Exposure of H-cells of

```
                                                                                                    GGTTATCAGCCTTATCGGTCT  -120

GGAATAACCAGTTGGCCTAAAGTCATGCAGACAGAGCGTTTCTGCGCCTCTCGTGAAGCAATTCGCACAACTTGTCCATCTTTAGAGGCATCTCCTGTTGTGGGATGTAGGGGAGACGT   -1

ATG AAC GAA TTT CAA CCA GTC AAT CGT CGT CAG TTT CTG TTC ACG CTC GGA GCA ACC GCT GCT AGC GCT ATT TTG CTG AAG GGT TGC GGT    90
Met Asn Glu Phe Gln Pro Val Asn Arg Arg Gln Phe Leu Phe Thr Leu Gly Ala Thr Ala Ala Ser Ala Ile Leu Leu Lys Gly Cys Gly
1

AAT CCT CCT TCC AGT AGC GGC GGC GGG ACT TCT AGT ACA ACT CAG CCA ACT GCT GCA GGG GCG AGT GAT CTG GAA GTC AAG ACA ATC AAA   180
Asn Pro Pro Ser Ser Ser Gly Gly Gly Thr Ser Ser Thr Thr Gln Pro Thr Ala Ala Gly Ala Ser Asp Leu Glu Val Lys Thr Ile Lys
31

TTG GGC TAC ATC CCC ATC TTT GAA GCG GCT CCA CTG ATC ATT GGC CGC GAA AAA GGC TTT TTT GCC AAA TAT GGC TTG GAT GTT GAA GTC   270
Leu Gly Tyr Ile Pro Ile Phe Glu Ala Ala Pro Leu Ile Ile Gly Arg Glu Lys Gly Phe Phe Ala Lys Tyr Gly Leu Asp Val Glu Val
61

TCG AAA CAA GCC AGC TGG GCA GCT GCT CGC GAT AAC GTC ATT CTC GGT TCT GCT GGT GGC GGC ATC GAT GGC GGT CAG TGG CAA ATG CCG   360
Ser Lys Gln Ala Ser Trp Ala Ala Ala Arg Asp Asn Val Ile Leu Gly Ser Ala Gly Gly Gly Ile Asp Gly Gly Gln Trp Gln Met Pro
91

ATG CCT GCC TTG CTA ACG GAA GGT GCG ATC AGC AAC GGT CAA AAA GTT CCC ATG TAT GTC TTG GCT TGC TTG AGC ACC CAA GGC AAT GGC   450
Met Pro Ala Leu Leu Thr Glu Gly Ala Ile Ser Asn Gly Gln Lys Val Pro Met Tyr Val Leu Ala Cys Leu Ser Thr Gln Gly Asn Gly
121

ATC GCT GTT TCC AAT CAG CTC AAG GCC CAA AAT CTG GGC TTG AAG CTA GCG CCC AAC CGC GAC TTT ATC CTC AAC TAC CCG CAA ACT AGC   540
Ile Ala Val Ser Asn Gln Leu Lys Ala Gln Asn Leu Gly Leu Lys Leu Ala Pro Asn Arg Asp Phe Ile Leu Asn Tyr Pro Gln Thr Ser
151

GGC CGG AAG TTC AAA GCA TCC TAC ACC TTC CCG AAC GCC AAC CAA GAC TTC TGG ATT CGC TAT TGG TTT GCA GCT GGC GGT ATC GAT CCT   630
Gly Arg Lys Phe Lys Ala Ser Tyr Thr Phe Pro Asn Ala Asn Gln Asp Phe Trp Ile Arg Tyr Trp Phe Ala Ala Gly Gly Ile Asp Pro
181

GAT AAA GAC ATT GAA CTC TTG ACC GTT CCC AGC GCA GAA ACT CTA CAA AAT ATG CGC AAT GGC ACG ATC GAT TGC TTC AGT ACC GGC GAT   720
Asp Lys Asp Ile Glu Leu Leu Thr Val Pro Ser Ala Glu Thr Leu Gln Asn Met Arg Asn Gly Thr Ile Asp Cys Phe Ser Thr Gly Asp
211

CCC TGG CCG TCG CGG ATT GCC AAA GAT GAC ATC GGC TAT CAA GCT GCG CTG ACA GGT CAA ATG TGG CCT TAC CAC CCC GAG GAA TTC TTG   810
Pro Trp Pro Ser Arg Ile Ala Lys Asp Asp Ile Gly Tyr Gln Ala Ala Leu Thr Gly Gln Met Trp Pro Tyr His Pro Glu Glu Phe Leu
241

GCG CTG CGA GCA GAC TGG GTA GAC AAA CAT CCG AAA GCT ACG CTC GCC TTG CTG ATG GGC TTG ATG GAA GCG CAG CAA TGG TGC GAT CAG   900
Ala Leu Arg Ala Asp Trp Val Asp Lys His Pro Lys Ala Thr Leu Ala Leu Leu Met Gly Leu Met Glu Ala Gln Gln Trp Cys Asp Gln
271

AAA GCA AAT CGG GCA GAG ATG GCC AAG ATC CTC TCC GGT CGC AAC TTC TTT AAC GTG CCG GTT TCG ATC CTG CAG CCG ATT CTG GAA GGT   990
Lys Ala Asn Arg Ala Glu Met Ala Lys Ile Leu Ser Gly Arg Asn Phe Phe Asn Val Pro Val Ser Ile Leu Gln Pro Ile Leu Glu Gly
301

CAA ATC AAA GTT GGA GCA GAC GGA AAA GAT CTC AAC AAC TTT GAT GCC GGC CCG CTC TTC TGG AAG AGT CCG CGC GGC AGT GTC TCC TAT  1080
Gln Ile Lys Val Gly Ala Asp Gly Lys Asp Leu Asn Asn Phe Asp Ala Gly Pro Leu Phe Trp Lys Ser Pro Arg Gly Ser Val Ser Tyr
331

CCC TAC AAA GGG CTC ACC CTC TGG TTC TTG GTG GAG TCG ATC CGC TGG GGC TTC AAC AAG CAA GTG CTA CCT GAC ATT GCA GCC GCC CAG  1170
Pro Tyr Lys Gly Leu Thr Leu Trp Phe Leu Val Glu Ser Ile Arg Trp Gly Phe Asn Lys Gln Val Leu Pro Asp Ile Ala Ala Ala Gln
361

AAA CTC AAC GAT CGC GTG ACT CGT GAA GAC CTC TGG CAA GAG GCA GCC AAG AAA TTA GGG GTG CCC GCT GCG GAT ATC CCA ACC GGA TCG  1260
Lys Leu Asn Asp Arg Val Thr Arg Glu Asp Leu Trp Gln Glu Ala Ala Lys Lys Leu Gly Val Pro Ala Ala Asp Ile Pro Thr Gly Ser
391

ACT CGC GGT ACC GAG ACC TTC TTT GAT GGC ATC ACC TAC AAC CCA GAC AGT CCG CAA GCT TAT CTC CAA AGC TTG AAG ATT AAA CGG GCA  1350
Thr Arg Gly Thr Glu Thr Phe Phe Asp Gly Ile Thr Tyr Asn Pro Asp Ser Pro Gln Ala Tyr Leu Gln Ser Leu Lys Ile Lys Arg Ala
421

TAA GTAGGGGCTTCAATCATCAACCTTAGTTCAGTCACTATCAGGAGATAGACAGACCATGGTTACTGCACGGGAAACAAGACGAAACGGAAGTCGTCCTTCTGGCTTAAAAAAATGG  1468
 *

CGTCAGAAACTCGATGGCATCTTGCTACCGCTAGCAGGAATTTTGGGTTTCCTCATCATTTGGCAGATCTTTTCTAGCACGGGCAACCCGCTTGCCCGGCCCTGCTCAGTCTCTTCACA  1587

GAAGAGAGAACACGCGAGTTGCTGCCCTATCCCTTCTTGGATCGCGGCGGGCTTGATAAAGGTCTGTTCTGGCAGACGTATCGCTTAGTTCTGACGCGGGTGGCCCAGGGCTTTTCGAT  1706

CCGCAGCCATCATCGGCATCGGCATTTCCGTTGGAATTC  1745
```

Figure 2. Nucleotide sequence of a 1885-bp fragment from *Synechococcus* PCC 7942 DNA, containing the gene for the 42-kD protein (cmpA). The deduced amino acid sequence from the open reading frame between nucleotides 1 and 1350 is included. The underlined amino acid sequence (residues 438–443) was found when isolated 42-kD protein was subjected to amino acid sequence analysis. This sequence has been submitted to Genbank under accession number M32999.

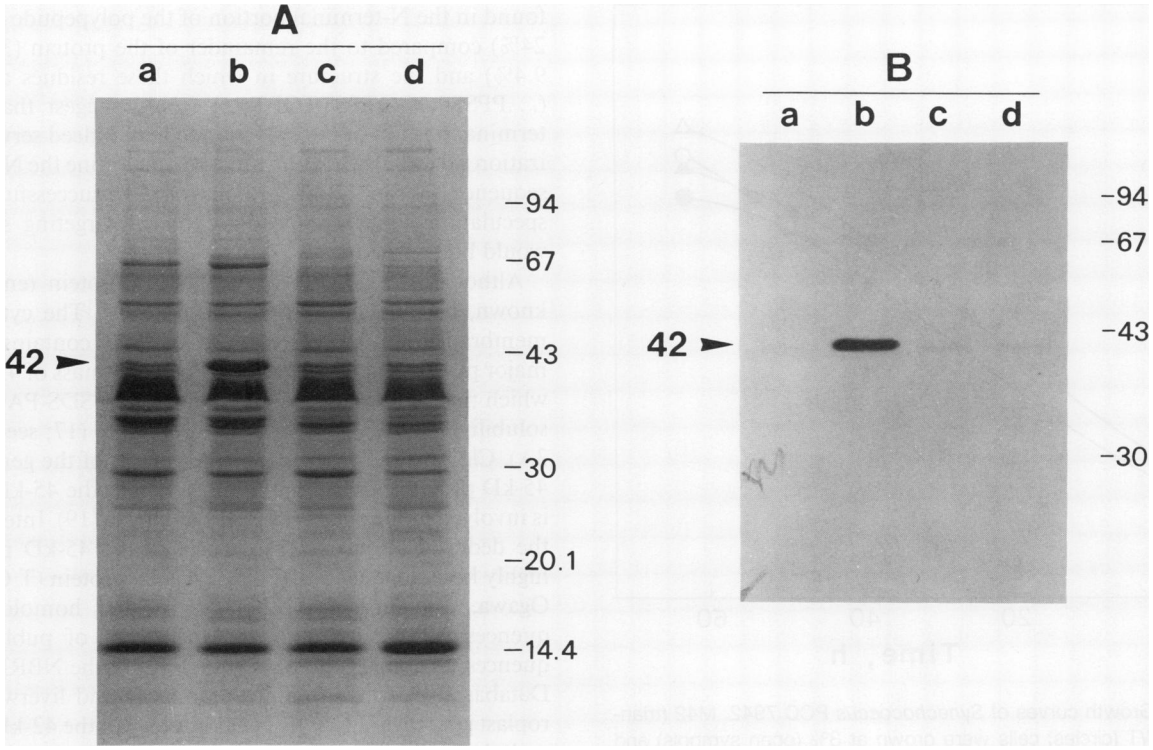

Figure 3. A, Electrophoretic profiles showing Coomassie brilliant blue-staining patterns of polypeptides in the cytoplasmic membranes from H-cells (lanes a and c) and L-cells (b and d) of WT (a and b) and the Km-resistant transformant (c and d). Samples (25 μg protein each) were solubilized at room temperature for 30 min and proteins were separated in an SDS-polyacrylamide gel (8–15% acrylamide gradient). B, Immunoblotting profiles of the polypeptides in the cytoplasmic membranes from H-cells (lanes a and c) and L-cells (b and d) of WT (a and b) and the Km-resistant transformant (c and d). IgG against the 42-kD protein was used as the probe. Samples (2.5 μg protein each) were solubilized at room temperature and run on a 10% SDS-polyacrylamide gel. The separated polypeptides were then electrotransferred to nitrocellulose and probed with the antibody. The numbers to the right of the figures represent the position of the marker proteins.

M42 to low CO_2 resulted in a large increase in both CO_2 and HCO_3^- uptake rates (curves A and B in Fig. 5b). In WT, L-cells also transport HCO_3^- faster than H-cells (curve A in Fig. 5a), but there was not much difference between L-cells and H-cells in their CO_2 uptake activity (curve B). Since M42 has the ability to take up CO_2 and HCO_3^- into the intracellular C_i pool at substantial rates, we conclude that the 42-kD protein does not participate directly in C_i uptake. The observation that the activity of C_i uptake in M42 increased after exposure to low CO_2 confirmed the results obtained by Schwarz *et al.* (24) using the O_{221} mutant.

DISCUSSION

We have prepared a mutant (M42) of *Synechococcus* PCC 7942 which completely lacks the 42-kD cytoplasmic membrane protein. This mutant could grow under low CO_2 conditions, and the CO_2 and HCO_3^- transport activities in the mutant were as high as those in WT. Clearly, the 42-kD protein is not required either for growth under low CO_2 conditions or for transport of CO_2 or HCO_3^-. Furthermore, the 42-kD protein is not required for the process of adaptation to low CO_2 as exposure of H-cells of M42 to low CO_2 conditions increased their C_i-transporting capability. Since the adaptation of cyanobacteria does not occur in the presence of inhibitors of protein synthesis (9, 16), some other protein(s) must be synthesized during this process. Although the 42-kD protein is actively synthesized during the adaptation of *Synechococcus* PCC 7942 to low CO_2 conditions (16), the present results suggest that another protein (or proteins) which is essential for increasing the C_i-transporting activity must be synthesized under low CO_2 conditions. The failure to detect such a protein in the earlier studies would suggest that the protein is a minor component.

The mutant strains of *Synechococcus* PCC 7942 which do not accumulate the 42-kD protein require high CO_2 concentrations for growth (11, 24). These mutants (RK1 and O_{221}) are considered to be defective in their ability to utilize the intracellular C_i pool efficiently. Although the cellular component(s) responsible for this process of C_i utilization has not been identified, it is possible that the essential component(s) for C_i utilization as well as the 42-kD protein are synthesized under the same regulatory scheme. Analyses of the mRNA derived from cmpA are being undertaken to test this possibility.

The cyanobacteria are structurally the simplest organisms which have differentiated intracellular membranes (thylakoid membranes) with little or no physical continuity with the cytoplasmic membrane. Since the polypeptide compositions

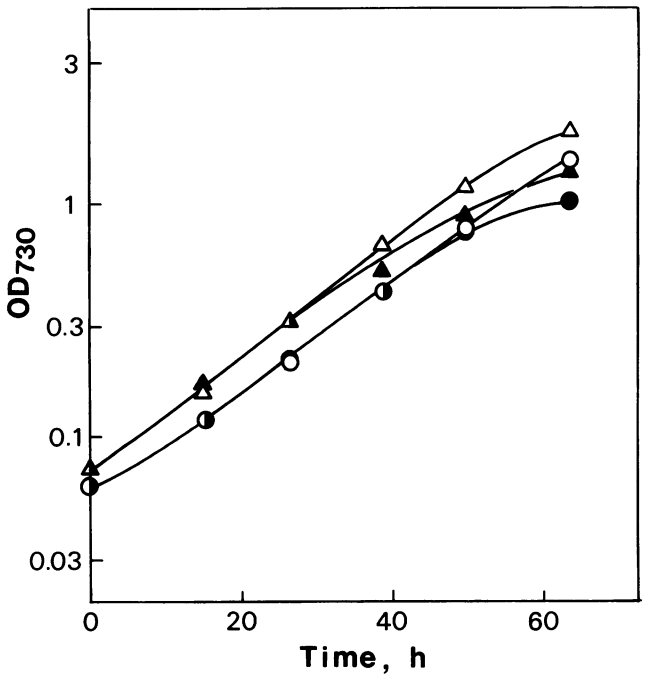

Figure 4. Growth curves of *Synechococcus* PCC 7942. M42 (triangles) and WT (circles) cells were grown at 3% (open symbols) and 0.04% (closed symbols) CO_2 in air.

of the cytoplasmic and thylakoid membranes are distinct, there must exist a mechanism to target newly synthesized proteins to the appropriate membrane. cmpA encodes a polypeptide of 49 kD. Although apparent molecular masses of proteins determined by SDS-PAGE are often aberrant, it is also possible that the N-terminal region of the polypeptide serves as a sequence for localization of the 42-kD polypeptide in the cytoplasmic membrane. For example, transit sequences used for targeting proteins into chloroplasts are often found to contain an abundance of hydroxylated amino acids (for a review see ref. 22). The high number of Thr and Ser residues found in the N-terminal portion of the polypeptide (14/58 or 24%) compared to the remainder of the protein (37/392 or 9.4%) and the structure in which these residues are found (. . .PPSSSGGGTSSTTQPT. . .) would suggest that the N-terminal portion of the polypeptide may indeed serve a localization function. Since our efforts to determine the N-terminal sequence of the 42-kD protein were unsuccessful, further speculation about the possible role of targeting sequences would be premature.

Although the function of the 42-kD protein remains unknown, its sequence may be informative. The cytoplasmic membrane of *Synechococcus* PCC 7942 contains another major protein with an apparent molecular mass of 45 kD (17) which migrates as a 37-kD protein band in SDS-PAGE when solubilized with SDS at room temperature (17; see also Fig. 3A). Cloning and insertional mutagenesis of the gene for the 45-kD protein has been used to show that the 45-kD protein is involved in the active transport of nitrate (19). Interestingly, the deduced amino acid sequence of the 45-kD protein is highly homologous to that of the 42-kD protein (T Omata, T Ogawa, unpublished results). No further homologous sequences were found in recent databases of published sequences (Wilbur-Lipman search [2, 30] of the NBRF Protein Database-Release 20.0, and the tobacco and liverwort chloroplast genomes [12, 25]). The homology of the 42-kD protein and the 45-kD protein may suggest a role for the 42-kD protein in transport of some ion(s) or nutrients, or may represent a common structure for proteins localized in the cytoplasmic membrane of the cyanobacterium. Further characterization of the M42 and M45 mutants are underway to test these possibilities.

Since the completion of these studies, it has come to our attention that another group of workers have also isolated the gene for the 42-kD membrane protein (21). These workers cloned the gene by utilizing antibodies directed against a 42-kD protein that they find in *Synechococcus* PCC 7942 upon exposure of cells to high light conditions (10). Since the nucleotide sequence that they report is identical to that for cmpA, it is clear that the gene obtained by using the antibodies

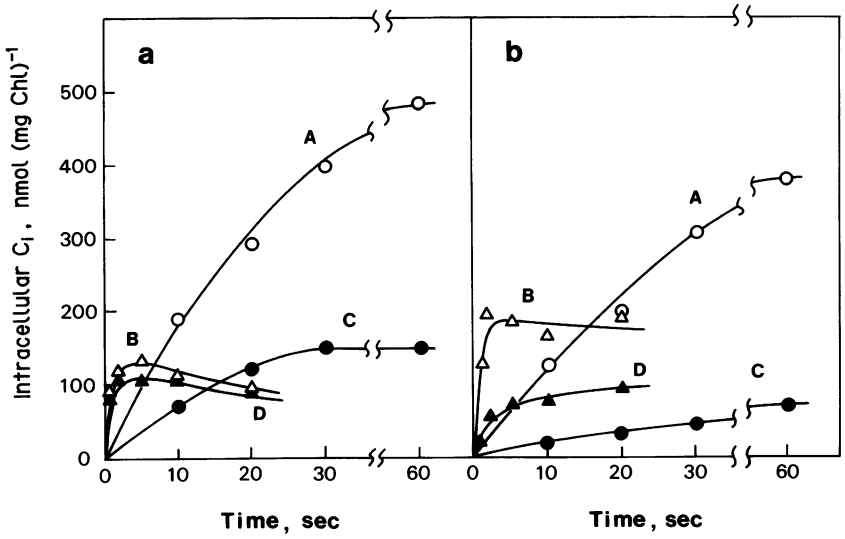

Figure 5. Accumulation of C_i by *Synechococcus* PCC 7942. WT (a) and M42 (b) cells were incubated for various periods of time in the light, and intracellular C_i was determined as the amount of internal, acid-labile ^{14}C. The responses of both L-cells (open symbols, curves A and B) and H-cells (closed symbols, curves C and D) were determined. Uptake was initiated with either 10 μM $^{14}CO_2$ (triangles, curves B and D) or 300 μM $H^{14}CO_3^-$ (circles, curves A and C).

directed against the 'light-induced' 42-kD protein is the same as the one that we have obtained using antibodies directed against the 'low CO_2-induced' 42-kD protein. Their original localization of this protein in the thylakoid membranes is now questionable as these workers show clear immunocytochemical localization of the protein in the cytoplasmic membrane. They also showed that the transcript for cmpA is induced by light. However, it should be noted that the high light conditions used by these other workers (1.9 $mW \cdot cm^{-2}$ [10]) are similar to those we have used in studying the M42 mutant (120 μmol $PAR \cdot m^{-2} \cdot s^{-1}$ = 2.0 $mW \cdot cm^{-2}$). Since M42 grew quite well under these conditions, it is unlikely that the 42-kD protein is required for adaptation to growth under these light regimes. Taken together, these studies suggest that the 42-kD cytoplasmic membrane protein is synthesized during growth under high light and low CO_2, but that its presence is not absolutely required for autotrophic growth under these conditions.

ACKNOWLEDGMENT

We thank R. Kutny for performing the sequence analysis of the 42-kD protein.

LITERATURE CITED

1. **Badger MR, Andrews TJ** (1982) Photosynthesis and inorganic carbon usage by the marine cyanobacterium, *Synechococcus* sp. Plant Physiol **70:** 517–523
2. **Devereux J, Haeberli P, Smithies O** (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res **12:** 387–395
3. **Hunkapiller MW, Hewick RM, Hood LE, Dreyer WJ** (1983) High sensitivity sequencing with a gas phase sequenator. Methods Enzymol **91:** 399–413
4. **Kaplan A, Badger MR, Berry JA** (1980) Photosynthesis and the intracellular inorganic carbon pool in the blue-green alga *Anabaena variabilis*: response to external CO_2 concentration. Planta **149:** 219–226
5. **Konigsberg WH, Henderson L** (1983) Removal of sodium dodecyl sulfate from proteins by ion-pair extraction. Methods Enzymol **91:** 254–259
6. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
7. **Lowry OH, Roseborough NJ, Farr AL, Randall RJ** (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275
8. **Maniatis T, Fritsch EF, Sambrook J** (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, ISBN 0-87979-136-0
9. **Marcus Y, Zenvirth D, Harel E, Kaplan A** (1982) Induction of HCO_3^- transporting capability and high photosynthetic affinity to inorganic carbon by low concentration of CO_2 in *Anabaena variabilis*. Plant Physiol **69:** 1008–1012
10. **Masamoto K, Riethman HC, Sherman LA** (1987) Isolation and characterization of a carotenoid-associated thylakoid protein from the cyanobacterium *Anacystis nidulans* R2. Plant Physiol **84:** 633–639
11. **Ogawa T, Kaneda T, Omata T** (1987) A mutant of *Synechococcus* PCC 7942 incapable of adapting to low CO_2 concentration. Plant Physiol **84:** 711–715
12. **Ohyama K, Fukuzawa H, Kohchi T, Shirai H, Sano T, Sano S, Umesono K, Shiki Y, Takeuchi M** et al (1986) Chloroplast gene organization deduced from complete sequence of liverwort. Nature **322:** 572–574
13. **Oka A, Sugisaki H, Takanami M** (1981) Nucleotide sequence of the kanamycin resistance transposon Tn 903. J Mol Biol **147:** 217–226
14. **Omata T, Murata N** (1983) Isolation and characterization of the cytoplasmic membranes from the blue-green alga (cyanobacterium) *Anacystis nidulans*. Plant Cell Physiol **24:** 1101–1112
15. **Omata T, Ogawa T** (1985) Changes in the polypeptide composition of the cytoplasmic membrane in the cyanobacterium *Anacystis nidulans* during adaptation to low CO_2 conditions. Plant Cell Physiol **26:** 1075–1081
16. **Omata T, Ogawa T** (1986) Biosynthesis of a 42-kD polypeptide in the cytoplasmic membrane of the cyanobacterium *Anacystis nidulans* strain R2 during adaptation to low CO_2 concentration. Plant Physiol **80:** 525–530
17. **Omata T, Ogawa T** (1987) Immunochemical studies on the major proteins in cytoplasmic membrane of cyanobacteria. *In* J Biggins, ed, Progress in Photosynthesis Research, Vol IV. Martinus Nijhoff Publishers, Dordrecht, pp 309–312
18. **Omata T, Ogawa T, Marcus Y, Friedberg D, Kaplan A** (1987) Adaptation to low CO_2 level in a mutant of *Anacystis nidulans* R2 which requires high CO_2 for growth. Plant Physiol **83:** 892–894
19. **Omata T, Ohmori M, Arai N, Ogawa T** (1989) Genetically engineered mutant of the cyanobacterium *Synechococcus* PCC 7942 defective in nitrate transport. Proc Natl Acad Sci USA **86:** 6612–6616
20. **Piskiewicz D, Landon M, Smith EL** (1970) Anomalous cleavage of aspartyl proline peptide bonds during amino-acid sequence determinations. Biochem Biophys Res Commun **40:** 1173–1178
21. **Reddy KJ, Masamoto K, Sherman DM, Sherman LA** (1989) DNA sequence and regulation of the gene (*cpb*A) encoding the 42-kilodalton cytoplasmic membrane carotenoprotein of the cyanobacterium *Synechococcus* sp. strain PCC 7942. J Bacteriol **171:** 3486–3493
22. **Robinson A, Austen B** (1987) The role of topogenic sequences in the movement of proteins through membranes. Biochem J **246:** 249–261
23. **Sanger F, Nicklen S, Coulson AR** (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467
24. **Schwarz R, Friedberg D, Kaplan A** (1988) Is there a role for the 42 kilodalton polypeptide in inorganic carbon uptake by cyanobacteria? Plant Physiol **88:** 284–288
25. **Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayashida N, Matsubayashi T, Zaita N, Chunwongse J, Obokata J** *et al.* (1986) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. EMBO J **5:** 2043–2049
26. **Shiraiwa Y, Miyachi S** (1985) Role of carbonic anhydrase in photosynthesis of blue-green alga (cyanobacterium) *Anabaena variabilis* ATCC 29413. Plant Cell Physiol **26:** 109–116
27. **Snyder M, Davis RW** (1985) Screening gt11 expression libraries with antibody probes. In T Springer, ed, Hybridoma Technology in Biosciences and Medicine. Plenum, New York, pp 397–406
28. **Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G** (1971) Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol Rev **35:** 171–205
29. **Volokita M, Zenvirth D, Kaplan A, Reinhold L** (1984) Nature of the inorganic carbon species actively taken up by the cyanobacterium *Anabaena variabilis*. Plant Physiol **76:** 599–602
30. **Wilbur WJ, Lipman DJ** (1983) Rapid similarity searches of nucleic acid and protein data banks. Proc Natl Acad Sci USA **80:** 726–730
31. **Williams JGK, Szalay AA** (1983) Stable integration of foreign DNA into the chromosome of the cyanobacterium *Synechococcus* R2. Gene **24:** 37–5
32. **Yanisch-Perron C, Vieira J, Messing J** (1985) Improved M-13 phage cloning vectors and host strains: nucleotide sequence of the M13-MP18 and pUC-19 vectors. Gene **33:** 103–119
33. **Young RA, Davis RW** (1983) Efficient isolation of genes by using antibody probes. Proc Natl Acad Sci USA **80:** 1194–1198