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

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ABSTRACT

A 42-kilodalton cytoplasmic membrane protein is synthesized when high CO2-grown cells of Synechococcus PCC 7942 (Anacystis nidulans R2) are exposed to low CO2. 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 CO2 conditions. The activity of inorganic carbon (C1) transport in the mutant was as high as that in the wild-type strain. In both types of cells, CO2 was the main species of C transported and the activities of CO2 and HCO3- transport increased when high CO2-grown cells were exposed to low CO2. We conclude that the 42-kilodalton protein is not directly involved in the C1-accumulating mechanism of Synechococcus PCC 7942.

Exposure of H-cells† of cyanobacteria to low CO2 conditions increases their C1-transporting capability and photosynthetic affinity for extracellular C1 (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 CO2 (15, 16). A number of biochemical studies have shown a close relationship between the amount of the 42-kD protein and the C1 transporting activity of the cells (16, 18). In addition, a high CO2 requiring mutant of this organism failed to accumulate the 42-kD protein when exposed to low CO2 concentrations (11). While these studies suggested that the protein may play a role in the C1-transporting mechanism, Schwarz et al. (24) have presented results which argue against the possible involvement of the protein in C1 transport. These authors described a mutant strain (O22I) of Synechococcus PCC 7942 which increases its C1-transporting activity upon exposure to low CO2 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

C1 transport activity. However, since the mutation in O22I 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 C1 transport can be envisioned. The 42-kD protein might also have a role in adaptation of the cells to low CO2 conditions, since mutants which do not accumulate the 42-kD protein, RK1, and O22I (11, 24), cannot grow under low CO2 conditions.

To see whether the 42-kD protein is necessary for C1 transport and growth under low CO2 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 C1 transport and growth under carbon-limited conditions.

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·m⁻²·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 λgt11 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 (10⁶ cells·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·mL⁻¹ of plasmid DNA. The constructs that we used contained the gene for aminoglycoside-3' -phosphotransferase

† Abbreviations: H-cells, cells grown under high (3%) CO2 conditions; C1, inorganic carbon; L-cells, cells grown under high CO2 and then exposed to low (0.03%) CO2 conditions for 20h; WT, wild-type strain; Km, Kanamycin; cmpA, cytoplasmic membrane protein A.

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(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·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^CO₂ and H^3^CO₃⁻ 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 EcoRI 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 *Escherichia coli* lysogens and it cross-reacted well with the anti-42-kD antibody. The 0.94 kb EcoRI fragment was ligated into the EcoRI 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 BglII sites in the 0.94 kb DNA fragment (Fig. 1A). Digestion of the plasmid with BglII was followed by ligation of a 1.3 kb BamHI fragment containing the gene for aminoglycoside-3′-phosphotransferase into the newly created BglII 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% CO₂ in air, numerous Km' resistant transformants were obtained. To provide adequate time for the newly introduced DNA to

![Figure 1](https://example.com/figure1.png)
segregate to all copies of the cyanobacterial chromosome, approximately 20 individual Km' transformants were separately and serially restreaked three times onto Km containing media and allowed to grow in air supplemented with 1% CO₂. 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 chromosmes 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') 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' gene. A digest of chromosomal DNA from a selected transformant (using either XbaI/KpnI or BglII/SphI) 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 XbaI/KpnI fragment or a 10.8 kb BglII/SphI fragment were recovered and shown to contain both the Km-resistant 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 (AGGGAGA) 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 inadvertently cleaved at the Asp-Pro-Pro-Pro 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 CO₂ 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 CO₂ (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, nondetectable 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 CO₂ 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 CO₂ conditions (Fig. 4). The growth rate of M42 under low CO₂ was comparable to that under high CO₂, and there were no significant differences in the growth rates of M42 and WT under low or high CO₂ conditions. Clearly, the 42-kD protein is not necessary for low CO₂ growth.

We further characterized the M42 mutant's ability to pump and accumulate CO₂ in order to determine whether active accumulation of CO₂ requires the 42-kD protein. In WT H-cells, light-dependent CO₂ uptake was much faster than HCO₃⁻ uptake (Fig. 5a, curves C and D). At low (10 μM) HCO₃⁻ concentrations, uptake rates were severely depressed (not shown). Thus, CO₂ is the species preferentially taken up by Synechococcus cells under these conditions. H-cells of M42 also took up CO₂ and HCO₃⁻ in the light (curves C and D in Fig. 5b), although the rates of CO₂ and HCO₃⁻ uptake were lower than those in H-cells of WT. Exposure of H-cells of
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.
M42 to low CO₂ resulted in a large increase in both CO₂ and HCO₃⁻ uptake rates (curves A and B in Fig. 5b). In WT, L-cells also transport HCO₃⁻ faster than H-cells (curve A in Fig. 5a), but there was not much difference between L-cells and H-cells in their CO₂ uptake activity (curve B). Since M42 has the ability to take up CO₂ and HCO₃⁻ into the intracellular Cᵢ pool at substantial rates, we conclude that the 42-kD protein does not participate directly in Cᵢ uptake. The observation that the activity of Cᵢ uptake in M42 increased after exposure to low CO₂ confirmed the results obtained by Schwarz et al. (24) using the O₂₂₁ 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 CO₂ conditions, and the CO₂ and HCO₃⁻ 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 CO₂ conditions or for transport of CO₂ or HCO₃⁻. Furthermore, the 42-kD protein is not required for the process of adaptation to low CO₂ as exposure of H-cells of M42 to low CO₂ conditions increased their Cᵢ-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 CO₂ conditions (16), the present results suggest that another protein (or proteins) which is essential for increasing the Cᵢ-transporting activity must be synthesized under low CO₂ 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 CO₂ concentrations for growth (11, 24). These mutants (RK1 and O₂₂₁) are considered to be defective in their ability to utilize the intracellular Cᵢ pool efficiently. Although the cellular component(s) responsible for this process of Cᵢ utilization has not been identified, it is possible that the essential component(s) for Cᵢ 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...
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 (...PPSSGGGTSSSTTQPT...) 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 4](image1.png)

**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) CO₂ in air.

![Figure 5](image2.png)

**Figure 5.** Accumulation of C₄ by *Synechococcus* PCC 7942. WT (a) and M42 (b) cells were incubated for various periods of time in the light, and intracellular C₄ was determined as the amount of internal, acid-labile eCO₂. 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 eCO₂ (triangles, curves B and D) or 300 μM H⁺CO₂⁻ (circles, curves A and C).
directed against the 'light-induced' 42-kDa protein is the same as the one that we have obtained using antibodies directed against the 'low CO₂-induced' 42-kDa 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 cm⁻² [10]) are similar to those we have used in studying the M42 mutant (120 μmol PAR m⁻² s⁻¹ = 2.0 mW cm⁻²). Since M42 grew quite well under these conditions, it is unlikely that the 42-kDa protein is required for adaptation to growth under these light regimes. Taken together, these studies suggest that the 42-kDa cytoplasmic membrane protein is synthesized during growth under high light and low CO₂, but that its presence is not absolutely required for autotrophic growth under these conditions.

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LITERATURE CITED