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Biochemistry

A cyanobacterial mutant requiring the expression of ribulose bisphosphate carboxylase from a photosynthetic anaerobe

(carboxysomes/photoheterotrophy/CO_2 utilization)

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ABSTRACT **Ribulose bisphosphate carboxylase is essential for both photoautotrophic and photoheterotrophic growth of the cyanobacterium *Synechocystis* 6803. However, a mutant lacking cyanobacterial carboxylase could be obtained by replacing the natural carboxylase gene with the corresponding gene from *Rhodospirillum rubrum*, a photosynthetic anaerobe. This treatment produced an organism whose growth depended on the activity of the structurally and functionally dissimilar foreign carboxylase. As a further consequence of this mutagenic replacement, the mutant also lacked microscopically observable carboxysomes, the subcellular inclusion bodies in which the wild-type carboxylase naturally resides. The mutant, dependent on a carboxylase with an inferior relative specificity for CO_2 versus O_2 and apparently lacking carboxysomes, is extremely sensitive to the CO_2/O_2 ratio supplied during growth and is unable to grow at all in air. This response to the gas composition should prove useful for selection of various *R. rubrum* carboxylase mutants with altered specificities for CO_2 and O_2.**

The growth of many plants and other photoautotrophic organisms is often limited by the availability of CO_2. A diminished photosynthetic capacity and the oxidative loss of reduced metabolites are major components of this limitation. Both of these effects result from the competition between CO_2 and O_2 for the active site of ribulose 1,5-bisphosphate (RbuP_2) carboxylase. Interestingly, RbuP_2 carboxylases from different natural sources have intrinsically different relative specificities for CO_2 and O_2 (1). That is, at equal concentrations of CO_2 and O_2, the least specific enzymes (e.g., from *Rhodospirillum rubrum*) perform ≈10 carboxylations for every oxygenation whereas the most specific enzymes (e.g., from spinach) produce ≈80 carboxylations for every oxygenation. In solutions equilibrated with normal atmospheric levels of CO_2 and O_2, these values correspond to enzymes that spend 70 and 22% of their time, respectively, functioning as oxygenases.

To understand the structural basis for this discrimination, we have sought a functional genetic approach for direct selection of mutant RbuP_2 carboxylases with altered substrate specificity. This approach requires the expression of an RbuP_2 carboxylase gene in a genetically and experimentally accessible biological environment in which the carboxylase enzyme's relative substrate specificity for CO_2 versus O_2 can be made to be growth-limiting to the organism. For a variety of reasons, we have chosen to study the enzyme from *R. rubrum*: its crystal structure is being refined to a high resolution (2); it is a simple dimer of identical 50.5-kDa subunits encoded by a single gene (3); it has one of the lowest specificities for CO_2 yet found in nature (so there is every reason to expect that further increases in this enzyme's relative substrate specificity are obtainable) (1); it has apparently never been under environmental selection with regard to its relative specificity for CO_2 and O_2 (as *R. rubrum* grows photoautrophically under anaerobic conditions).

In this report, the expression of the *R. rubrum* carboxylase in the cyanobacterium *Synechocystis* 6803, an oxygen-evolving photosynthetic prokaryote, is shown to constitute a biological system that allows for selection of carboxylase function. In addition to describing the physiological consequences of this expression, we provide evidence suggesting that RbuP_2 carboxylase function is essential for photoautotrophic and photoheterotrophic growth in this organism.

MATERIALS AND METHODS

Strains and Plasmids. *Synechocystis* PCC 6803 is an *Aphanocapsa* species from the American Type Culture Collection (ATCC 27184) (4). pUC118 (from J. Vieira, Rutgers University) is a pUC18 derivative (5) that contains the intergenic region of M13 to allow for production of single-stranded DNA. pUC4K contains the neomycin phosphotransferase (NPT) gene (6) flanked by useful restriction enzyme sites. pRR2119 containing the *R. rubrum* RbuP_2 carboxylase gene (7) was provided by C. Somerville (Michigan State University). The gene for the large subunit of *Anabaena* RbuP_2 carboxylase (8) was a gift from R. Haselkorn (University of Chicago).

Growth of *Synechocystis* 6803. This cyanobacterium was grown in the presence or absence of 5 mM glucose at a light intensity of ≈20 $\mu E \cdot m^{-2} \cdot s^{-1}$ in BG-11 medium (9) supplemented with 150 mM of the K^+ salt of *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (pH 8.0). When desired, the medium was further supplemented with 10 μg of kanamycin per ml or 10 μM atrazine. Cultures were maintained at 30°C and aerated with humidified gases of various CO_2, O_2, and N_2 concentrations.

Electron Microscopy. Glutaraldehyde [70% (vol/vol) Polysciences] was added to growing cultures to a final concentration of 2%. After 2 hr at 22°C, cells were twice rinsed by centrifugation in 1.0 mM sodium cacodylate (pH 7.0) and then post-fixed in buffered 1% osmium tetroxide for 1 hr at 22°C. After two water rinses and *en bloc* staining with aqueous 1% uranyl acetate for 12 hr at 4°C, cells were pelleted and embedded in 1% low-melting agar (Sigma; agarose, type XI) that had been cooled to 30°C. Cubes (1 mm^3) of agarose were slowly dehydrated in ethanol [25, 50, 75 and 90% (vol/vol), 15 min for each solution] and then infiltrated with LR white resin (for two 1-hr periods with 100% resin, then with fresh resin for 3.5 days). Blocks were polymerized in Beem capsules at 60°C for 24 hr. Thin sections were stained with uranyl acetate and lead citrate.

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Abbreviations: RbuP_2, ribulose 1,5-bisphosphate; NPT, neomycin phosphotransferase; wt, wild type.

Other Methods. Polyacrylamide gel electrophoresis was performed in the presence of 0.1% lithium dodecyl sulfate on a 6–15% polyacrylamide gradient gel (1.5 W, 14 hr, 4°C). Standard protocols were used for Western (10) and Southern (11) blot analyses and for DNA manipulations (12).

RESULTS

Mutagenesis of the Wild-Type (wt) Carboxylase Gene. The cyanobacterium *Synechocystis* 6803 is a readily transformable photosynthetic organism that is capable of photoautotrophic growth as well as heterotrophic growth using glucose as a carbon source (4, 13). We sought to use these growth properties to prepare an RbuP_2 carboxylase null mutant that could be maintained on glucose and that could be transformed to photoautotrophic growth by foreign carboxylase genes. A plasmid library was constructed with *Synechocystis* 6803 chromosomal DNA. A 1.1-kilobase *Eco*RI–*Hin*cII fragment containing a portion of the cyanobacterial carboxylase structural gene for the large subunit (*rbcL*) was identified in this library by Southern blot using the gene for the large subunit of *Anabaena* RbuP_2 carboxylase as a probe. Partial sequence analysis of this fragment revealed significant homology to known *rbcL* sequences and allowed us to orient the structural gene fragment on the DNA insert (pTC1, Fig. 1*a*). The carboxylase coding region was then modified by the insertion of DNA encoding NPT, which provides resistance

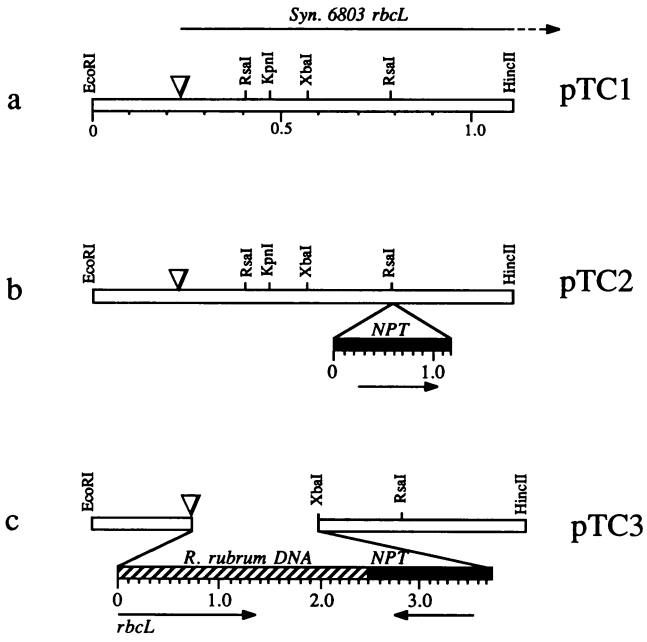

FIG. 1. Plasmids used for transformation of *Synechocystis* 6803. The structures shown are all inserts in the multiple cloning site of plasmid pUC118. Inverted triangles, translation initiation codons; horizontal arrows, translational direction and extent of structural genes. Numbers are in kilobase pairs. (*a*) A 1.1-kilobase fragment cloned from *Synechocystis* 6803 contains 5′ untranslated sequences and the major portion of the large subunit gene for RbuP_2 carboxylase. The ≈400-base sequence in from the *Eco*RI site was determined and used to align the structural gene on the restriction map. (*b*) Insertion of a NPT gene from pUC4K into the RbuP_2 carboxylase structural sequence to allow for subsequent detection of insertionally inactivated RbuP_2 carboxylase by selection on medium containing kanamycin. (*c*) Construct for introduction of the *R. rubrum* carboxylase gene into *Synechocystis* 6803. The construct contains the *R. rubrum* RbuP_2 carboxylase gene from pRR2119 and a 3′-proximal NPT gene flanked by *Synechocystis* 6803 sequences. The base sequence surrounding the carboxylase initiation codon was determined to verify that the *R. rubrum* structural gene had been precisely fused to the cyanobacterial carboxylase promoter region.

to the antibiotic kanamycin. Since stable transformants of *Synechocystis* 6803 arise by homologous recombination and gene replacement mechanisms (4), uptake of this construct (pTC2, Fig. 1*b*) by *Synechocystis* 6803 was expected to cause transformation by recombination with the chromosome through the homologous sequences flanking the kanamycin-resistance gene. This recombination would produce a kanamycin-resistant mutant with an insertionally inactivated carboxylase gene. After allowing gene segregation to take place [there are multiple copies of the chromosome in this cyanobacterium (24)], the mutants would be expected to be devoid of RbuP_2 carboxylase activity.

Incubation of *Synechocystis* 6803 with pTC2 did indeed yield transformants that were capable of growing on minimal agar plates supplemented with glucose and kanamycin. However, the transformants also grew well in the absence of glucose and, therefore, had retained the capacity for photoautotrophic growth. Southern blot analysis indicated that the transformants contained not only the insert of pTC2 in the expected chromosomal location but also unmodified wt carboxylase DNA. Repeated attempts at forcing gene segregation by serial restreaking in the presence of glucose and ever increasing concentrations of kanamycin failed. Since *Synechocystis* 6803 can grow in the presence of photosystem II inhibitors when provided with glucose (4), we also tried to force gene segregation in the presence of atrazine to help ameliorate any problems that might be associated with photoinhibition caused by an excess of reducing equivalents (derived from photosystem II and glucose). These efforts were also unsuccessful, and we were unable to obtain clones lacking the wt carboxylase gene and the associated enzyme activity. We were left with heterogeneous mutants containing wt carboxylase DNA on one or more chromosomes. As we have no evidence from DNA, protein, or enzymatic analyses for multiple forms of RbuP_2 carboxylase in *Synechocystis* 6803, we tentatively concluded (see below for further evidence) that RbuP_2 carboxylase function was required by this organism even in the presence of glucose.

Functional Replacement of the wt Carboxylase Gene. Because carboxylase function may be essential, we embarked on an alternate approach of disabling the native carboxylase gene by replacing it with a foreign gene encoding a carboxylase enzyme that is antigenically, structurally, and functionally distinct from the cyanobacterial protein. We chose for this purpose the carboxylase gene from a photosynthetic anaerobe, *R. rubrum*, which codes for the production of a single polypeptide. The active form of the *R. rubrum* carboxylase is a dimer (L_2) of identical subunits (holoenzyme M_r = 101,000) (3) whereas the carboxylase from cyanobacteria is a M_r 508,000 hexadecameric enzyme composed of equal portions of large and small subunits (L_8S_8) (14). In addition, the two enzymes have different relative substrate specificities for CO_2 and O_2; the cyanobacterial enzyme has a 3- to 4-fold higher relative specificity for CO_2 versus O_2 than does the *R. rubrum* enzyme (1) and hence is less sensitive to inhibition by oxygen.

To introduce the *R. rubrum* carboxylase gene into the cyanobacterium, a portion of the cyanobacterial *rbcL* gene was replaced with structural DNA encoding the *R. rubrum* carboxylase and the selectable marker NPT. The initiation codon for the *R. rubrum* gene was then precisely fused to the cyanobacterial carboxylase promoter region by oligonucleotide-directed deletion mutagenesis. These procedures yielded the plasmid pTC3 (Fig. 1*c*) that contains the structural gene for *R. rubrum* carboxylase fused to the cyanobacterial carboxylase 5′ untranslated elements and a 3′-proximal selectable marker for kanamycin resistance. The flanking cyanobacterial DNA in this construction (5′ upstream region and 3′ carboxylase gene fragment) provides the sequences necessary for gene replacement by homologous recombina-

tion with the cyanobacterial chromosome. Successful integration of this construct into the chromosome would place the *R. rubrum* carboxylase gene under the control of the cyanobacterial carboxylase promoter while simultaneously inactivating the native *rbcL* gene.

Incubation of wt *Synechocystis* 6803 with pTC3 yielded kanamycin-resistant transformants on glucose-containing plates under an atmosphere of 99% air/1% CO_2. Transformants were restreaked as before to verify the resistant phenotype and to allow for gene segregation. These transformants could also be grown in the absence of glucose if they were provided with an atmosphere of 5% CO_2/95% N_2 but could not grow in an unsupplemented air environment in the presence or absence of glucose. Subsequently, isolates were grown photoautotrophically in liquid culture perfused with 5% CO_2/95% N_2. Chromosomal DNA was prepared and analyzed by Southern blot analysis using pTC1 (wt carboxylase structural gene) and pRR2119 (*R. rubrum* carboxylase structural gene) as probes. The appropriate mutant chromosomal fragments bearing the *R. rubrum* carboxylase and NPT structural genes were found, and no intact wt carboxylase genes were evident (data not shown).

Soluble protein extracts prepared from mutant and wt cells were analyzed after electrophoresis using antibodies prepared against either purified *R. rubrum* carboxylase or purified spinach carboxylase (which cross reacts well with the cyanobacterial carboxylase). The results (Fig. 2) verified the presence of *R. rubrum* carboxylase and the absence of cyanobacterial carboxylase in the mutant extract. When RbuP_2 carboxylase from the mutant was purified by size-exclusion and anion-exchange chromatographies, no carboxylase activity (<0.1% wt levels) was found in the regions of the chromatograms in which cyanobacterial carboxylase is normally found. We conclude that the "cyanorubrum" mutants are devoid of wt RbuP_2 carboxylase genes as well as wt carboxylase protein and that they are able to grow photoautotrophically because of the presence of *R. rubrum* carboxylase.

Essentiality of RbuP_2 Carboxylase. The above results suggest that insertional inactivation of the wt carboxylase gene was successful only if a functional foreign carboxylase gene was simultaneously provided. Further evidence that expression of the foreign carboxylase gene was indeed required was obtained by transformation studies with a construction similar to pTC3 but lacking 23 bases adjacent to the translation initiation codon that span a putative ribosomal binding site. (See pTC3Δ and comparative sequences below, wherein coding sequences are bold type and *R. rubrum* sequences are also underlined.)

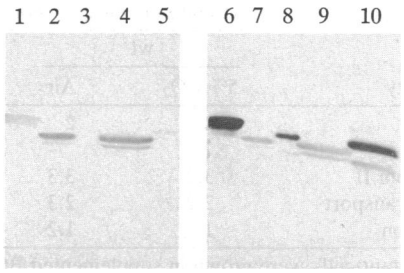

FIG. 2. Analysis of RbuP_2 carboxylase in wt and mutant forms of *Synechocystis* 6803. wt and mutant cells of *Synechocystis* 6803 were grown photosynthetically at 30°C to near stationary phase under aeration in a humidified atmosphere of 5% CO_2/95% N_2. The medium for growing the mutant was further supplemented with 10 μg of kanamycin per ml. Cells were collected by centrifugation and resuspended in 100 mM Tris·HCl (pH 8.0) containing 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM ε-aminocaproic acid, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, DNase at 1 mg/ml, and lysozyme at 1 mg/ml. The cells were lysed by three successive passages through a French pressure cell (84 kg/m^2) and soluble proteins that precipitated between 35 and 65% saturated ammonium sulfate were resuspended in 1 ml of 25 mM Tris·HCl. This solution was subsequently dialyzed for 6 hr at 4°C against 25 mM Tris·HCl (pH 8.0) containing 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride (final volume, 2 ml). Portions (2 or 37 μl) of the extracts (the wt extract contained ≈25 μg of RbuP_2 carboxylase per ml) were solubilized with lithium dodecyl sulfate and subjected to PAGE in the presence of lithium dodecyl sulfate on a 6–15% gradient gel (1.5 W, 14 hr, 4°C). Western blot analysis was performed with rabbit antibodies prepared against purified spinach (lanes 1–5) or *R. rubrum* (lanes 6–10) RbuP_2 carboxylase, and colorimetric visualization was afforded by horseradish peroxidase linked to goat anti-rabbit IgG antibodies. Recombinant *R. rubrum* carboxylase (1.2 μg) purified from *Escherichia coli* containing pRR2119 was included as a marker (lanes 1 and 6). [This is a fusion protein containing a 25-amino acid N-terminal extension and is, therefore, larger (M_r, 53,000) than the native *R. rubrum* enzyme (M_r, 50,500).] Lanes: 1 and 6, *R. rubrum* carboxylase; 2 and 7, wt (2 μl); 3 and 8, mutant (2 μl); 4 and 9, wt (37 μl); 5 and 10, mutant (37 μl). Note that although the anti-spinach carboxylase antibody is very poorly cross-reactive with *R. rubrum* carboxylase (lanes 1, 3, and 5), the anti-*R. rubrum* carboxylase antibody cross-reacts fairly well with cyanobacterial carboxylase (lanes 7 and 9).

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...AAGCTAGGCTATTCAATGTTTATGGAGGACTGACCTAG ATG GTA CAA...wt
...AAGCTAGGCTATTCAATGTTTATGGAGGACTGACCTAG ATG GAC CAG...pTC3
...AAGCTAGGCTATTCA.....................ATG GAC CAG...pTC3Δ
                                        Translational start
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When pTC3Δ was incubated with wt *Synechocystis* 6803, kanamycin-resistant transformants were obtained on plates supplemented with glucose. However, as found with the insertional mutants (from pTC2), these transformants could also grow photoautotrophically and Southern analysis indicated that the transformants maintained wt carboxylase sequences on one or more chromosomes (data not shown). Again, we were unable to force segregation of the mutant gene under photoheterotrophic growth conditions in the presence or absence of atrazine. Although these mutants contained the complete coding sequence of the *R. rubrum* carboxylase gene, they lacked detectable *R. rubrum* carboxylase protein (<1% of that found in mutants transformed with pTC3), presumably due to translational problems. It would appear that insertional inactivation of the wt carboxylase gene of *Synechocystis* 6803 precludes both photoautotrophic and photoheterotrophic growth unless the organism is provided with an alternate carboxylase.

Finally, the requirement of the cyanorubrum mutant for high CO_2 concentrations was related to the presence of *R. rubrum* RbuP_2 carboxylase by transforming the cyanorubrum mutant with *Synechocystis* 6803 *rbcL* DNA (pTC1). We found that photoautotrophic growth in air was restored by introduction of the wt gene encoding the more efficient cyanobacterial enzyme. This result suggests that a single genetic lesion (the replacement of the wt carboxylase gene with the *R. rubrum* carboxylase gene) is responsible for the altered growth characteristics of the mutant.

Metabolism and Physiology of the Cyanorubrum Mutant. Biochemical analysis of the cyanorubrum mutant revealed important distinctions from wt *Synechocystis* 6803 (Table 1). Rates of photosynthetic electron transport were slightly elevated in the mutant, but the maximum rate of CO_2 fixation was ≈2 times lower. This lowered capacity correlated well with an ≈3 times lower overall carboxylase activity in the mutant. Interestingly, the *R. rubrum* carboxylase enzyme naturally has an ≈3 times lower maximum catalytic rate than the cyanobacterial enzyme. This result suggests that the

Table 1. Photosynthesis in wt and mutant *Synechocystis* 6803

Activity	wt		Mutant
	5% CO_2	Air	5% CO_2
Carboxylase	2	2	0.6
Photosynthesis			
Photosystem II	3.2	3.3	4.3
Electron transport	2.2	2.3	3.3
CO_2 fixation	1.3	1.2	0.65

wt and mutant cells were grown in supplemented BG-11 medium to late logarithmic phase. Concentrations of CO_2 in air used during growth of the cells are given. Cells were harvested by centrifugation, washed twice with 50 mM Mes (Na^+ salt, pH 6.1), and resuspended in this buffer at a density of 110 μg of chlorophyll per ml. Chlorophyll content was determined spectrophotometrically. RbuP_2-dependent rates of CO_2 fixation (carboxylase activity) were determined spectrophotometrically at 25°C and reported as μmol of CO_2 per min per mg of chlorophyll. Photosynthetic activities were determined at 25°C in an oxygen electrode at cell concentrations of <10 μg of chlorophyll per ml and are reported as μmol of O_2 per min per mg of chlorophyll. Photosystem II activity was estimated as the light-saturated rate of O_2 evolution in the presence of 1 mM *p*-benzoquinone. Photosynthetic electron transport activity was estimated as the light-saturated rate of O_2 consumption (corrected for a small amount of dark respiration) in the presence of 0.1 mM methylviologen. *In vivo* rates of CO_2 fixation were estimated as the light-saturated rate of O_2 evolution in the presence of saturating concentrations of CO_2 at an initial dissolved O_2 concentration of <25 μM.

amount of *R. rubrum* carboxylase protein produced in the cyanorubrum mutant is very similar to the amount of cyanobacterial carboxylase protein produced in the wt cells, and that the precise fusion of the *R. rubrum* structural gene to the wt carboxylase promoter DNA produced a functionally equivalent transcriptional unit in the appropriate chromosomal context. Our further finding that the maximum growth rate of the cyanorubrum mutant is ≈2 times slower than that of the wt cyanobacteria (generation times of ≈24 hr versus ≈13 hr) provides circumstantial evidence that the growth of these organisms is limited by RbuP_2 carboxylation rates.

To further test the effects of the foreign carboxylase protein, we grew both wt and cyanorubrum cells under atmospheres containing various proportions of CO_2 and O_2. The growth of wt cells was hardly affected by the range of gas concentrations that we used. In contrast, the growth of the cyanorubrum mutant was progressively retarded as CO_2/O_2 ratios decreased (Table 2) and was so sensitive to O_2 that natural levels of O_2 (i.e., ≈20%) were inhibitory even in the presence of 5% CO_2. The mutant did not grow in air (0.035% CO_2/21% O_2), an atmosphere that provides nearly maximal

Table 2. Effect of O_2/CO_2 ratios on growth of wt and mutant cells

O_2, %	CO_2, %	O_2/CO_2 ratio	Doubling time, hr	
			Mutant	wt
0	5	0	24	15
10	5	2	26	17
20	5	4	32	15
21	5	4.2	38	—
21	3	7	46	16
40	5	8	50	17
21	1	21	68	—
21	0.5	42	180	—
21	0.1	210	NG	16

Cells were grown at 30°C in 50-ml cultures in supplemented BG-11 medium under various gas regimes. Exponentially growing cells were inoculated into fresh medium to an optical density (730 nm) of <0.1. The optical densities of the cultures were taken during the subsequent exponential growth phase, and growth rates were determined (±20%). Values not shown were not determined. NG, no growth.

growth rates with wt cells. It is important to note that it is the CO_2/O_2 ratio and not just the absolute levels of the two gases that is the growth-controlling factor (Table 2). This latter observation clearly draws our attention to the kinetic properties of the different RbuP_2 carboxylases as being determinative.

Lastly, electron microscopic analysis of the cyanorubrum mutant revealed the complete absence of the electron-dense polyhedral carboxysome structures (15) that are readily observed in wt cells (Fig. 3). Possible physiological consequences of this pleiotropy will be discussed below.

DISCUSSION

Photoheterotrophy in *Synechocystis* 6803. *Synechocystis* 6803 can grow slowly in the dark on glucose [generation time, ≈84 hr (4)] and must, therefore, be able to provide itself with reducing equivalents and ATP from this sugar. If true heterotrophy is characterized by such slow growth, obligate heterotrophs would be unable to compete with photosynthesizing cells (which grow 6- to 7-fold faster), and this may help to explain our inability to obtain carboxylase null mutants in cultures grown in the light. Earlier evidence for an incomplete glycolytic pathway in the cyanobacteria (16) supports the hypothesis that the main pathway for glucose utilization by this organism in the light may be the pentose monophosphate pathway in which glucose and ATP are converted to ribulose 5-phosphate and CO_2 with the concomitant production of two molecules of reduced pyridine nucleotide. If efficient metabolism to the level of triose sugars required phosphorylation of the monophosphate to RbuP_2 and subsequent reaction with RbuP_2 carboxylase, this series of metabolic events could explain our findings that RbuP_2 carboxylase is required even in the presence of glucose.

The ability of *Synechocystis* 6803 to grow in the absence of a functional photosystem II when provided with glucose has been used to great advantage by researchers studying the effects of mutation in genes encoding photosystem II polypeptides (4, 17). However, it would appear from the present study that this photoheterotrophy is of a very limited sort in that RbuP_2 carboxylase function and presumably CO_2 fixation are still required for growth on glucose. In this view, glucose serves predominantly as a source of reducing equivalents that are absolutely necessary in the absence of photosystem II function, and ATP would be generated by way of cyclic photophosphorylation. Under these circumstances,

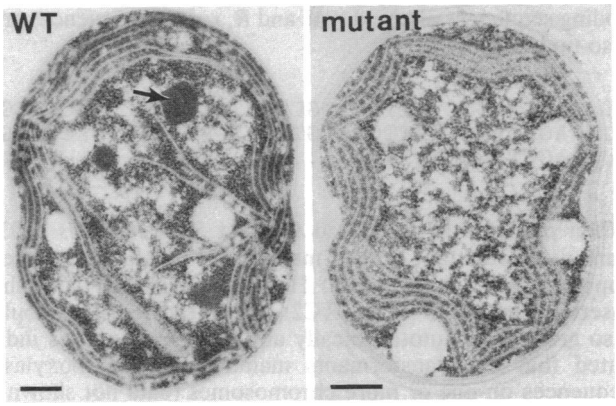

FIG. 3. Electron microscopy of wt *Synechocystis* 6803 (*Left*) and the cyanorubrum mutant (*Right*). Random sections were made through embedded cells that had been grown under 5% CO_2/95% air. None of the several hundred mutant cells that were examined contained the polyhedral, electron-dense bodies (carboxysomes, arrow) which were readily observed in wt cells. Carboxysomes were present in similar amounts in wt cells grown under low or high CO_2 concentrations. (Bars = 250 nm.)

photoheterotrophic growth on glucose would be permissive of inactivating mutations in only a small number of photosynthetic genes. Accordingly, the use of *Synechocystis* 6803 in mutational studies of other photosynthetic functions may be very limited, and it would be of interest to determine whether other photoheterotrophic cyanobacteria [e.g., *Agmanellum quadruplicatum*, which grows photoheterotrophically on glycerol (18)] are more generally heterotrophic and amenable to these types of studies.

CO_2 Utilization by *Synechocystis* 6803. Since maximum rates of carboxylation, photosynthesis, and growth of the cyanorubrum mutant were all 2–3 times slower than in wt cells, it would appear that photoautotrophic growth of the mutant is quantitatively limited by the amount of RbuP_2 carboxylase activity. In addition, the extreme sensitivity of the mutant to changes in the CO_2/O_2 ratio supplied during growth suggests that the growth of the mutant is also determined by the qualitative differences in substrate specificity between the cyanobacterial and *R. rubrum* enyzmes. It would seem, at first, that an increased sensitivity to O_2 is to be expected of an organism that contains a carboxylase enzyme that is less specific for CO_2 and more reactive toward O_2. However, the relative specificities of the *R. rubrum* and cynobacterial carboxylases differ by a factor of 3–4, and this small factor cannot account for the extreme O_2 sensitivity that was observed.

Other, incidental effects of our mutation could explain the anomalous oxygen sensitivity of the mutant. These include repression of the CO_2 transport and accumulation processes normally present in wt cyanobacteria (19) and failure to package the *R. rubrum* carboxylase into carboxysomes, the subcellular inclusion bodies in which the wt carboxylase naturally resides (15, 20). The active accumulation of CO_2 and bicarbonate is a process that is normally induced in wt *Synechocystis* 6803 grown under stressfully low CO_2 concentrations. However, the mechanism for concentrating inorganic carbon may be repressed in the mutant cells at the high CO_2 levels required for their growth. Although we have not determined whether the cyanorubrum mutant actively accumulates inorganic carbon when grown at high CO_2 levels, the available evidence (19, 21, 22) suggests that lack of this capacity could contribute a factor of $\approx$3 to the mutant's sensitivity to decreases in the CO_2/O_2 ratio supplied to the cells. Perhaps our finding that the cyanorubrum mutants are devoid of microscopically observable carboxysomes* may support the proposal that carboxysomes are also involved in efficient CO_2 utilization by cyanobacteria (15, 23). The basic tenets of this model (23) are that the carboxysome membrane is rather impermeable to CO_2 and that carbonic anhydrase and RbuP_2 carboxylase are located in the carboxysome. The HCO_3^- that builds up to high levels in the cytosol as a result of active transport is then free to diffuse into the carboxysome where it is dehydrated to CO_2 and ultimately fixed by the action of RbuP_2 carboxylase. This subcellular localization of RbuP_2 carboxylase would thereby provide a useful barrier to diffusion of CO_2 away from the carboxylase. If carboxysomes do indeed serve to sequester RbuP_2 carboxylase in an environment of high CO_2 concentration, their functional absence in the cyanorubrum mutant could contribute to the mutant's enhanced sensitivity to O_2.

A Selectable System for RbuP_2 Carboxylase Function. These studies provide us with a unique organism in which to study the effects of mutation on the *in vivo* function of RbuP_2 carboxylases. Introduction of genes encoding foreign carboxylases with intrinsically higher relative specificity for CO_2 would be expected to produce transformants whose growth is less sensitive to O_2, and the cyanorubrum mutant could be used to screen libraries of carboxylase mutants for genes that encode active or altered carboxylases. Similarly, various mutants of *R. rubrum* carboxylase could be screened directly for changes in substrate specificity.

*It is quite possible that carboxysomes are present in the mutant cells but are structurally indistinct in the absence of wt carboxylase. Since the only genetic lesion in the mutants appears to be the functional replacement of the wt carboxylase gene, one would expect that the proteinaceous carboxysome membrane is still synthesized. However, it is highly likely that the dimeric structure of the *R. rubrum* carboxylase is different enough from the hexadecameric cyanobacterial enzyme to preclude its sequestration into the carboxysome. Although it is possible that ultrastructurally indistinct (empty) carboxysomes are present in the mutants, they would not be functional.

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