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Low Activation State of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase in Carboxysome-Defective *Synechococcus* Mutants[1]

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The high-CO_2-requiring mutant of *Synechococcus* sp. PCC 7942, EK6, was obtained after extension of the C terminus of the small subunit of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco). The carboxysomes in EK6 were much larger than in the wild type, but the cellular distribution of the large and small subunits of Rubisco was not affected. The kinetic parameters of in vitro-activated Rubisco were similar in EK6 and in the wild type. On the other hand, Rubisco appeared to be in a low state of activation in situ in EK6 cells pretreated with an air level of CO_2. This was deduced from the appearance of a lag phase when carboxylation was followed with time in cells permeabilized by detergent and subsequently supplied with saturating CO_2 and RuBP. Pretreatment of the cells with high CO_2 virtually abolished the lag. After low-CO_2 treatment, the internal RuBP pool was much higher in mutant cells than in the wild-type cells; pretreatment with high CO_2 reduced the pool in mutant cells. We suggest that the high-CO_2-requiring phenotype in mutants that possess aberrant carboxysomes arises from the inactivated state of Rubisco when the cells are exposed to low CO_2.

Cyanobacteria possess polyhedral bodies that contain most of the cellular Rubisco (see Codd, 1988; McKay et al., 1992) and CA activities (Price et al., 1992). Quantitative models (Reinhold et al., 1989, 1991) assigned a critical role to the structural organization of the carboxysomes in the operation of the CCM. They postulate that CO_2 is generated within the carboxysomes at CA sites in close proximity to Rubisco. The CCM enable the cells to overcome the 20-fold difference between the concentration of dissolved CO_2 at equilibrium with air and the $K_m(CO_2)$ of their Rubisco (see Aizawa and Miyachi, 1986; Badger, 1987; Pierce and Omata, 1988; Kaplan et al., 1990, 1991, 1994; Miller et al., 1990; Coleman, 1991; Badger and Price, 1992, for recent reviews and literature citations).

Aberrant carboxysomes were observed in several *Synechococcus* sp. PCC 7942 mutants, which demand high CO_2 for growth. These mutants were isolated after modifications of ORF78, *ccmL*, *ccmM*, *ccmN*, and *ccmO*, which were located upstream of *rbc* (Friedberg et al., 1989; Price and Badger, 1991; Orus et al., 1992; Price et al., 1993; Marco et al., 1994; M. Ronen-Tarazi, J. Lieman-Hurwitz, C. Gabay, M. Orus, A. Kaplan, unpublished data). The mutants exhibit normal ability to accumulate C_i within the cells but, nevertheless, show an apparent photosynthetic affinity for C_i approximately 100-fold lower than that of the wild type. They are defective in their ability to use the internal C_i pool in photosynthesis (Marcus et al., 1986; Schwarz et al., 1988; Lieman-Hurwitz et al., 1991).

The high-CO_2-requiring mutant of *Synechococcus* sp. PCC 7942, EK6, resembles the carboxysome-defective mutants discussed above by possessing a very low apparent affinity for external C_i in spite of its ability to accumulate an internal C_i concentration as large as that in the wild type. This mutant was obtained by extending the 3′ end of *rbcS* (encoding the small subunit of Rubisco) by 84 nucleotides. The small subunit in EK6 was 17 kD rather than 14 kD, as in the wild type (Lieman-Hurwitz et al., 1991). In the present study we investigated the physiological and structural bases for the high-CO_2-requiring phenotype of this mutant. Evidence is presented that this mutant contains grossly modified carboxysomes, and that the phenotype results from a low state of activity of Rubisco, in situ, when the mutant has been exposed to low CO_2.

MATERIALS AND METHODS

Mutant EK6 of *Synechococcus* sp. strain PCC 7942 was obtained as described by Lieman-Hurwitz et al. (1991). The wild type and the mutant were grown in the presence of 5% CO_2 in air as previously described (Schwarz et al., 1988). The rates of C_i-dependent O_2 evolution were measured by an O_2 electrode as described earlier (Kaplan et al., 1988).

Cultures of the wild type and EK6 were harvested in their exponential phase of growth and resuspended in 1 mL of 50 mM Tricine buffer, pH 7.8, and supplemented with 1 mM of each of the protease inhibitors PMSF, caproic acid, and benzamidine. The cells were broken by a Brown homogenizer (15 s, repeated three times) in the presence of an equal volume of glass beads (Sigma, <106 μm). To compare the results obtained with intact cells and cell-free extracts, it was essential to quantify the fraction of cells

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Abbreviations: CA, carbonic anhydrase; CCM, inorganic carbon-concentrating mechanism; C_i, inorganic carbon; RuBP, ribulose-1,5-bisphosphate.

broken. The amount of Chl (estimated after extraction with 80% acetone) in the intact cells and in the cell-free extract was used to assess the fraction of broken cells. After the treatment in the Brown homogenizer, the suspension was layered over a 1:1 mixture of dibutyl phthalate and bis(2-ethylhexyl) phthalate (Fluka). Centrifugation (1 min, 12,000 rpm in an Eppendorf centrifuge) resulted in precipitation of intact cells. The amount of Chl in the upper layer was used to assess the fraction of broken cells in each experiment.

In Vitro Carboxylation Assay

The cell-free fraction was centrifuged (12,000 rpm for 10 min in an Eppendorf centrifuge) and the pellet (carboxysome-enriched fraction, see Price et al. [1992]) was resuspended in 0.5 mL of 50 mM Tricine, pH 7.8. Rubisco was activated for 30 min on ice by the addition of 20 mM HCO_3^-, 20 mM $MgCl_2$, and 1 mM DTT. Fixation of $^{14}CO_2$ was then allowed to take place at 30°C in the presence of 100 mM Tricine, pH 7.8, 20 mM $MgCl_2$, 5 mM DTT, CA (approximately 10 enzyme units), and a range of RuBP and $^{14}C_i$ concentrations as indicated in the text. The specific activity of the $^{14}C_i$ was approximately 15 Bq/nmol, and it varied slightly with different C_i concentrations. Carboxylation was terminated after 2 min by the addition of acetic acid, and the acid-stable ^{14}C (representing fixed CO_2) was determined.

Determination of the Amount of Rubisco

This determination was performed essentially as described by Cannon et al. (1991) for *Thiobacillus neapolitanus*. Briefly, Rubisco (in 100 μL of the cell-free fraction used in the carboxylation experiments) was activated as described above, but in the presence of 20 mM $^{14}C_i$ (30 Bq/nmol), followed by the addition of carboxyarabinitol bisphosphate (about 10 nmol). The sample was loaded on a Sephadex G-75 (fine) column and eluted with 50 mM Tricine, pH 8.0, supplemented with 1 mM DTT. Fractions of 0.5 mL were collected and their radioactivity was measured. The amount of Rubisco (as nmol of binding sites) was calculated from the amount of ^{14}C in the peak of the high mol wt material eluted from the column and the specific activity.

In Situ Carboxylation Assay and Determination of Internal RuBP Pool

To measure the activity of Rubisco under conditions close to the in vivo situation, the cells were harvested, resuspended in 70 mM Hepes-NaOH, pH 7.8, at 30°C, and aerated with air or 5% CO_2 in air for 5 to 10 min in the light. Samples (200 μL) were rapidly transferred to small (300 μL) vials containing (final concentration) 80 mM Tricine, pH 7.6, 10 mM $MgCl_2$, 0.4 mM RuBP, and 0.05% detergent (mixed alkyltrimethylammonium bromide, Sigma). The carboxylation reaction was initiated, after 20 s of pretreatment in the presence of the detergent, by the addition of 15 mM $^{14}C_i$ (15 Bq/nmol), and terminated after various intervals by addition of acetic acid. This procedure enabled us to follow the time course of carboxylation as affected by the pretreatment conditions. In another set of experiments, the amount of CO_2 fixed by cells pretreated as above but without the addition of RuBP was determined. In this case carboxylation was terminated after 45 min to allow complete utilization of RuBP carried into the reaction mixture with the cells. The amount of RuBP present in these cells at the end of the light and CO_2 pretreatment was thus assessed.

The effect of the C_i concentration on the internal concentration of RuBP at the steady-state rate of photosynthesis was examined as described by Mayo et al. (1989). Briefly, the cells (equivalent to 25 μg Chl/mL) were placed in the oxygen electrode, at 30°C, illuminated with two projector lamps, and supplemented with various concentrations of C_i. Samples (1 mL) were drawn into a syringe containing perchloric acid (final concentration, 10%) when steady-state rates of oxygen evolution were reached. The amount of RuBP in these samples was determined (after neutralization with KOH and precipitation of the potassium perchlorate) by carboxylation reaction.

Immunoelectron Microscopy

High-CO_2-grown wild-type and EK6 cells were fixed in 1% glutaraldehyde in 0.1 M cacodylate, pH 7.5, for 2 h at room temperature. The cells were then washed three times with the same buffer, dehydrated through a graded ethanol series, and embedded in a 1:1 mixture of 70% ethanol and LR White medium-grade acrylic resin at 4°C overnight. Blocking was performed in gelatin capsules at 50°C for 24 h, and cut sections were placed on grids coated with 4% collodium solution. For the immunoreaction, the sections were incubated in 10 mM Tris, pH 8.2, supplemented with 0.5% BSA, 0.9% NaCl, and 0.1% Tween 20. They were then blocked with normal anti-goat antibodies for 10 min, washed in the same buffer, and incubated with antibodies at room temperature for 2 h. The antibodies were raised either against the native holoenzyme of Rubisco from spinach (*Spinacia oleracea*) or against the small subunit of Rubisco from *Synechococcus*. Western analysis indicated that the former antibody does not react with the small subunit of Rubisco from *Synechococcus*. The sections were then conjugated with 18-nm colloidal gold anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) and washed with the same buffer. The immunolabeled sections were stained with uranyl acetate and lead citrate prior to viewing in a Philips (Mahwah, NJ) 300 electron microscope.

RESULTS

Since the high-CO_2-requiring mutant EK6 was obtained following the extension of the C terminus of the small subunit of Rubisco, it was possible that the kinetic parameters of the enzyme had been modified, which would explain the observed, very low, apparent photosynthetic affinity for extracellular C_i (Lieman-Hurwitz et al., 1991). Figure 1 presents the carboxylation rate of in vitro-activated Rubisco as a function of CO_2 concentration. The data are expressed as nmol CO_2 fixed per nmol active sites of Rubisco as determined on the same cell-free pellet fraction

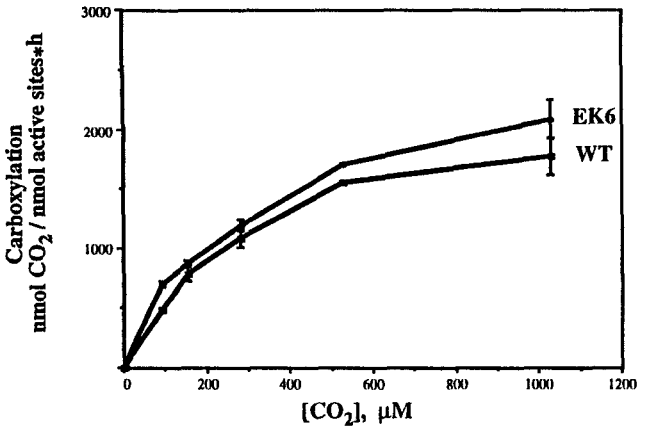

Figure 1. The rate of carboxylation as a function of CO_2 concentration by in vitro-activated Rubisco from *Synechococcus* sp. strain PCC 7942 and from mutant EK6.

(carboxysome enriched; see Price et al., 1992) as was used in the carboxylation experiments. This fraction contained 0.43 and 0.25 nmol binding sites per μg Chl in the wild type and EK6, respectively. Figure 1 indicates similar kinetic parameters for Rubisco in the wild type and in EK6. The deduced $K_m(CO_2)$ and V_{max} were 230 to 240 μM CO_2 and 1950 to 2070 nmol CO_2 fixed per nmol active site per h, respectively. In another set of experiments the affinity of the in vitro-activated enzyme for RuBP was determined in the presence of a saturating CO_2 concentration. The K_m(RuBP) was 25 to 30 μM for both the wild type and the mutant. These values are in accord with the kinetic parameters of *Synechococcus* 6301 Rubisco expressed in *Escherichia coli* (see Paul et al., 1991). They indicate that the extension of *rbcS* did not affect the intrinsic kinetic properties of the enzyme.

An important prediction of our theoretical model is that the correct organization of Rubisco in carboxysomes is critical for the formation of the local, elevated CO_2 concentration near carboxylation sites (Reinhold et al., 1991). Therefore, we examined whether the modification of the small subunit resulted in aberrant carboxysomes or altered the distribution of Rubisco between the carboxysomes and the cytoplasm.

Figure 2 presents typical electron micrographs prepared after immunogold labeling of high-CO_2-grown wild-type and EK6 cells harvested during the log phase of growth. Several prominent differences may be noted between the wild type and EK6. Cells of the latter are larger and their carboxysomes are much larger than in the wild type. In most cases they occupy the entire width of the cells, unlike the wild type, where they are about one-third of the cell's diameter. Approximately 10% of the cells of EK6 contained a displaced carboxysome located between the thylakoids and the cytoplasmic membrane. In most cases this was observed at the tip of the cells, but carboxysomes located next to the cytoplasmic membrane at the equator of the cell were also noted (Fig. 2A).

Using the antibodies against the large and small subunits of Rubisco (see "Materials and Methods") we verified that the very large bodies observed in EK6 were indeed carboxysomes, since Rubisco subunits were located mainly in these bodies. The density of the gold particles within the carboxysomes was much lower in the mutant than in the wild type. The number of cells where gold particles were observed in the cytoplasm was also lower in the mutant. We did not detect noticeable differences between the wild type and the mutant with respect to the distribution of the large and small subunits of Rubisco between the carboxysomes and the cytoplasm.

The modification of the small subunit of Rubisco in EK6 did not alter the activity of the enzyme when it was activated in vitro (Fig. 1). However, in view of the abnormal carboxysomes in the mutant (Fig. 2), it was possible that the organization of Rubisco within the carboxysomes and the ability to raise the local concentration of CO_2 had been affected. Since the activation state of Rubisco is strongly affected by the concentration of CO_2 (Andrews and Lorimer, 1987), this activation state in vivo might be lower in EK6 than in the wild type. To test this possibility, it was desirable to investigate the in situ activity of Rubisco after pretreatment of the cells with either high or low CO_2. The experimental approach used here was as close to the in vivo situation as we could create. The detergent treatment enabled determinations of the enzyme activity within 20 s after the pretreatment. Figure 3 presents the time courses of carboxylation in EK6 and wild-type cells aerated either with air or with 5% CO_2 and subsequently permeabilized by a detergent treatment. Exposure of EK6 to low CO_2 resulted in an extended lag of 15 to 20 s, during which carboxylation activity was not detected at all (Fig. 3A). Examination of another high-CO_2-requiring mutant, E1, which also contains defective carboxysomes (Friedberg et al., 1989), yielded a very similar curve (not shown). In contrast, in the case of the wild-type cells, considerable carboxylation activity was already apparent after 5 s (Fig. 3B).

The extended lag phase seen in Figure 3A might thus be interpreted as indicating that the exposure of EK6 to low CO_2 resulted in a nonactive state of Rubisco in the mutant, in situ, due to inefficient operation of the CCM. Subsequent reactivation by the high concentration of CO_2 present in the reaction mixture raised the carboxylation rate to a level comparable to that in the wild type. To examine this possibility, the wild-type and EK6 cells were aerated with 5% CO_2 in air (instead of air) during the pretreatment. Under these conditions the lag phase was eliminated (Fig. 3A).

In another set of experiments, cells were exposed to the detergent for 40 rather than for 20 s to test whether the observed lag resulted from less-efficient permeabilization of the cellular membranes in the mutant. This treatment did not eliminate the lag period, disposing of this possibility (not shown). Furthermore, the absence of a lag period in mutant cells exposed to high CO_2 (Fig. 3A) did not lend support to this possibility.

If the activation state of Rubisco in the mutant under low CO_2 is indeed lower than that in the wild type, this should be reflected in the curve relating the level of the endogenous RuBP pool to the concentration of C_i (Woodrow and

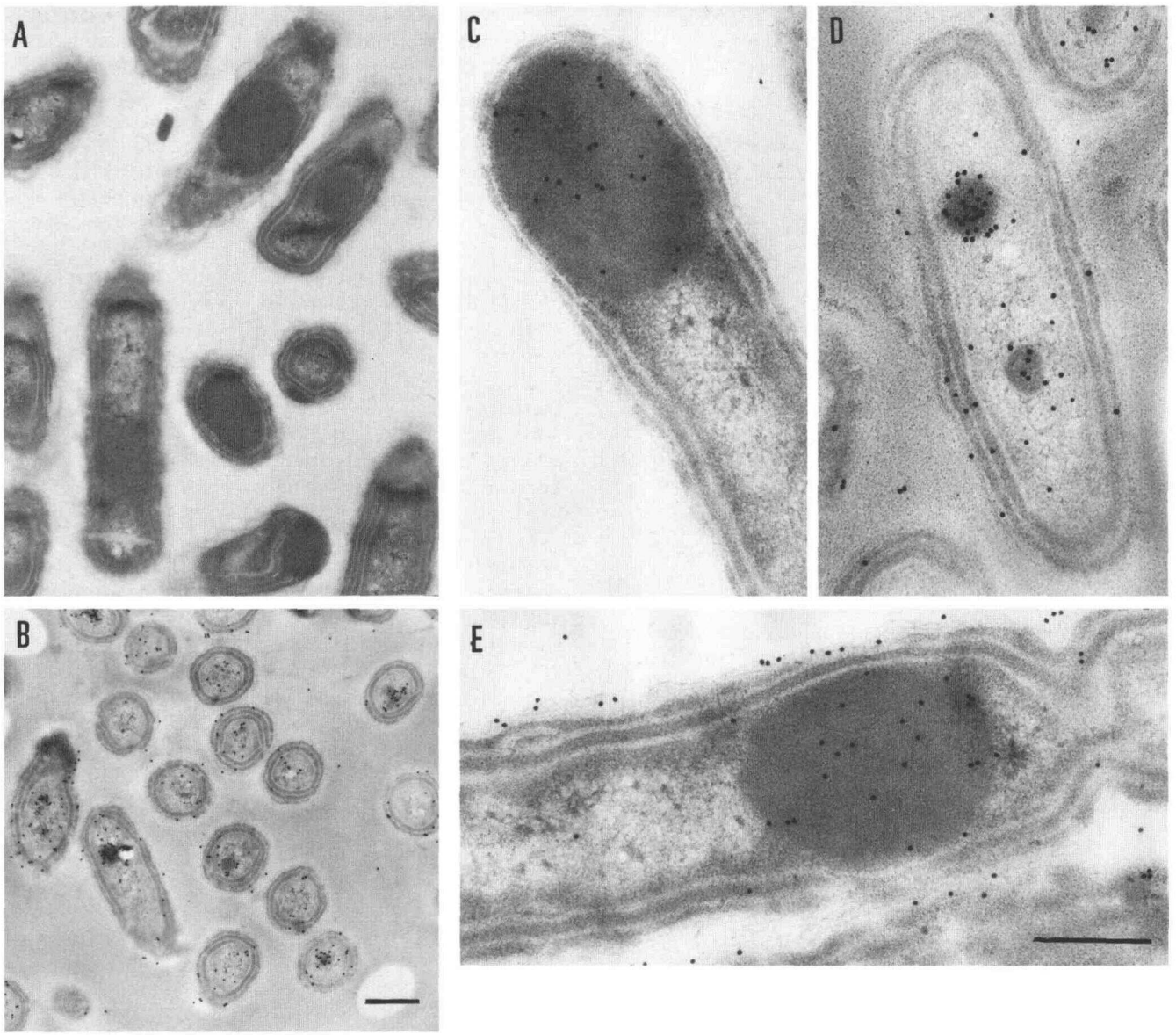

Figure 2. Electron micrographs of immunogold-labeled *Synechococcus* sp. strain PCC 7942 (B and D) and mutant EK6 (A, C, and E). The antibodies used reacted with the large subunit (A–D) or the small subunit (E) of Rubisco. The bars indicate 0.5 μm (note the different magnifications in A and B, compared with C, D, and E).

Berry, 1988; Mayo et al., 1989). Figure 4 presents the results of experiments where the steady-state rates of photosynthetic oxygen evolution and the size of the internal RuBP pools were determined as a function of the extracellular C_i concentration. The apparent photosynthetic affinity for extracellular C_i was much lower in the mutant ($K_{1/2}[C_i]$ = 8 mM) than in the wild type ($K_{1/2}[C_i]$ = 0.1 mM), in agreement with our earlier report (see Lieman-Hurwitz et al., 1991). The maximal rate of photosynthesis at saturating C_i was higher in the wild type, probably reflecting the higher level of Rubisco per unit Chl in these cells. In both types of cells, the highest level of RuBP was observed near the C_i concentration at which the half-maximium rate of photosynthesis was reached. The highest level of RuBP detected in wild-type cells that were exposed to 0.1 mM C_i (0.8 nmol/μg Chl) was similar to that determined in high-C_i-grown *Synechococcus leopoliensis* (Mayo et al., 1989). The highest level of RuBP in EK6, in the presence of 8 mM C_i, was about 50% lower than the highest level in the wild type. Again, this might be attributed to the lower amount of Rubisco (per unit Chl) in EK6. In both the wild type and the mutant, the highest level of RuBP was approximately twice the concentration of Rubisco binding sites.

Raising the external C_i concentration resulted in decreasing the internal RuBP level, in agreement with the results of Mayo et al. (1989). At C_i concentrations where the photosynthetic rate was maximal, the internal RuBP pool fell below the concentration of Rubisco binding sites. This may indicate that the maximal photosynthetic rate was limited by the formation of RuBP.

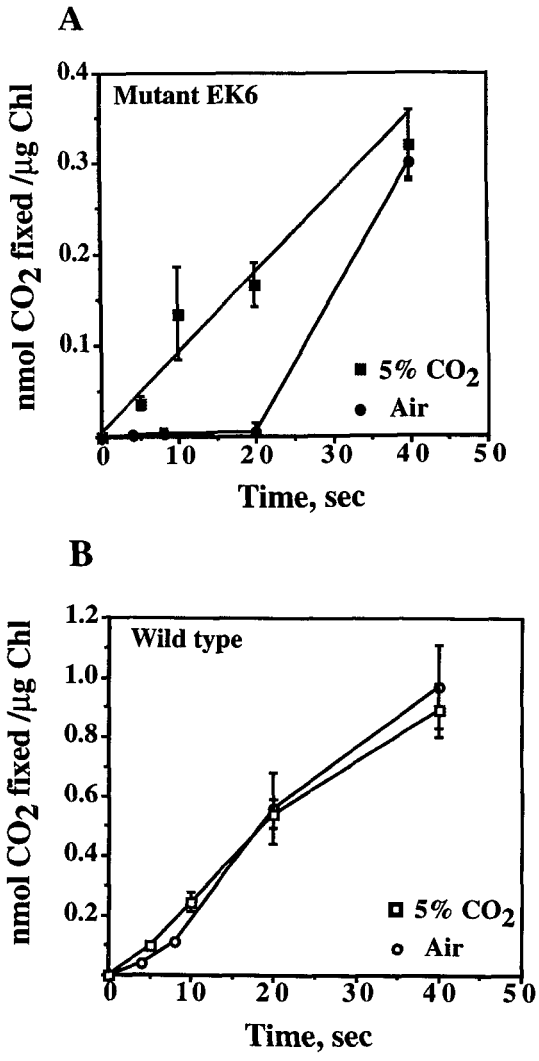

Figure 3. Time courses of carboxylation by mutant EK6 (A) and the wild type (B) *Synechococcus* sp. strain PCC 7942. The cells were grown under high CO_2 and aerated with air or 5% CO_2 in air for 5 min followed by a 20-s treatment with the detergent. The cells were then (at time zero) supplied with saturating $^{14}C_i$ (15 mM) and RuBP (0.4 mM). Carboxylation was terminated by acetic acid after various intervals.

As opposed to the experiments presented in Figure 4, some of the results in Figure 3 were obtained with mutant cells aerated with air during the pretreatment period, conditions under which they are unable to perform net photosynthesis. To determine the RuBP pool under the conditions of Figure 3, the amount of CO_2 fixed was measured under similar conditions, except that exogenous RuBP was not provided. The amount of CO_2 fixed thus gave an indication of the size of the endogenous RuBP pool (Fig. 5).

These experiments were also conducted with two other high-CO_2-requiring mutants of *Synechococcus* sp. PCC 7942 to test whether the results obtained with EK6 are general for mutants that possess aberrant carboxysomes. Those chosen were mutant E1, which contains visibly defective carboxysomes (Friedberg et al., 1989), and mutant No. 68, in which carboxysomal CA activity is 30-fold lower (kindly provided by Dr. D. Price and Dr. M. Badger). Pretreatment of the mutants with 5% CO_2 in air resulted in a RuBP level comparable to that of the wild type (within the range of 0.05–0.17 nmol CO_2 fixed/μg Chl). However, after pretreatment with low CO_2, the endogenous RuBP pool in the mutants was considerably higher than that in the wild type (0.7–0.95 nmol CO_2 fixed/μg Chl, as compared with 0.06 nmol CO_2 fixed/μg Chl by the wild type). The higher RuBP level in the mutants after aeration with air supported the suggestion that under these conditions their Rubisco is in a low state of activation.

DISCUSSION

The role of the small subunit of Rubisco is not fully understood (see Paul et al., 1991). Analysis of the three-dimensional structure of the holoenzyme indicated that the N terminus of the small subunit is buried within the holoenzyme close to the active site, whereas its C terminus (modified in mutant EK6) is facing outward (Newman and Gutteridge, 1993). The electron micrographs (Fig. 2) demonstrated that the carboxysomes in EK6, where the small subunit had been extended by 28 amino acids (Lieman-Hurwitz et al., 1991), were much larger than in the wild type and in many cases were displaced in the cells. Immunogold labeling suggested that the distribution of the large subunit of Rubisco between carboxysome and cytoplasm was similar to that of the small subunit in both types of cells. However, the density of the gold grains within the carboxysomes was considerably higher in the wild type, possibly due to the swollen nature of the carboxysomes in the mutant.

Very little information is available on carboxysome biogenesis and the process of organization of the constituents of these bodies (see Kaplan et al., 1994). Substitution of *rbcLS* in *Synechocystis* sp. PCC 6803 with the *rbc* encoding the large subunit of Rubisco from *Rhodospirillum rubrum* resulted in a high-CO_2-requiring mutant that lacked visible carboxysomes (Pierce et al., 1988). This observation, together with the results presented here, suggests a significant role for the native, unaltered Rubisco in the organization of the carboxysomes in cyanobacteria. The extension of the small subunit in EK6 might well have affected the packing of the enzyme within the carboxysomes. It might possibly have interfered with the association of the small subunit with the carboxysomal shell, if in cyanobacteria as in *Thiobacillus* (Holthijzen et al., 1986) such an association exists.

It is well established that Rubisco requires activation by CO_2 and Mg^{2+} for full activity of the enzyme (Andrews and Lorimer, 1987; Gutteridge, 1990). When Rubisco was activated in vitro (Fig. 1) the kinetic parameters of the enzyme were not detectably affected by the modification of the small subunit in EK6. However, the results reported here (Figs. 3 and 5) indicate that Rubisco in situ in the mutants, which contain aberrant carboxysomes, may be in a low state of activation. This inference is based on the extended lag phase observed in the time curve for ^{14}C fixation in cells of EK6 pretreated with low CO_2 and sub-

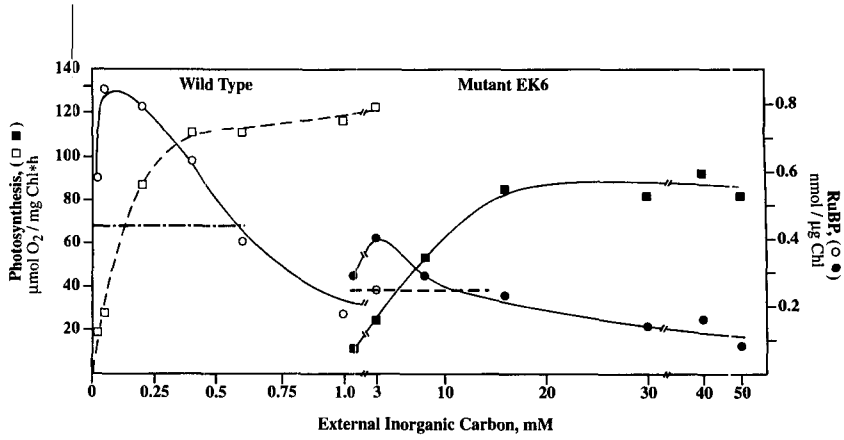

Figure 4. The rate of photosynthesis and the pool size of RuBP in *Synechococcus* sp. strain PCC 7942 and mutant EK6 as a function of the C_i concentration in the medium. The horizontal broken lines indicate the concentration of Rubisco active sites in the wild type and the mutant as determined by carboxyarabinitol bisphosphate binding.

sequently supplied with saturating RuBP and C_i concentrations (Fig. 3).

The rise in slope of the time curve after the lag phase most probably indicates an increasing degree of activation of Rubisco consequent on the high CO_2 concentration present in the reaction medium. An alternative explanation, that the lag represents the delay before $^{14}CO_2$ concentration at the carboxylation site reaches its steady-state level, can probably be rejected. Pretreatment of the mutant cells with a high level of CO_2 virtually abolished the lag phase (Fig. 3), which would not be the case if $^{14}CO_2$ supply to the carboxylation site were delayed. However, this finding does support the activation hypothesis. The latter is supported further by the observation that in vitro activation of the enzyme resulted in kinetic parameters that did not differ detectibly between the wild type and the mutant (Fig. 1). Further, the data in Figure 5 suggest that a large internal RuBP pool was present in the mutant cells exposed to low CO_2 but not in those exposed to high CO_2. This would be expected if the enzyme was in a low state of activation in situ in mutant cells exposed to low CO_2; the very high internal RuBP level would be expected to inactivate Rubisco even further.

The low state of activation of Rubisco in mutants carrying altered carboxysomes could be explained on the basis of our theoretical quantitative model (Reinhold et al., 1991). The latter proposes that the carboxysomes are the site of the elevated internal CO_2 concentration, which results from the activity of the CCM. It is envisaged that the C_i species do not reach equilibrium in the cytoplasm (Reinhold et al., 1989). The accumulated HCO_3^- penetrates into the carboxysomes, where CO_2 is generated at CA sites, and part of it is fixed as it diffuses outward past Rubisco sites. The model thus suggests that a major part of the biological significance of the packing of Rubisco and CA into carboxysomes is that it enables the formation of a high CO_2 concentration at the fixation site and also minimizes leakage of CO_2 from the cell (Reinhold et al., 1991). Defective carboxysomes might well be incapable of forming or maintaining this CO_2 pool, which might result in a CO_2 level too low for adequate activation of Rubisco. Further, the concentration of CO_2 as a substrate would be lower than in the wild type.

It is difficult to determine the concentration of CO_2 at the site of carboxylation directly, particularly since CO_2 and bicarbonate are not at a chemical equilibrium within the cells (Volokita et al., 1984; Schwarz et al., 1988; Price and Badger 1989; Kaplan et al., 1991). Results presented by Omata et al. (1987) were interpreted as indicating that the internal CO_2 concentration is lower in mutant cells, which contain aberrant carboxysomes, than in the wild type.

A second explanation for the low state of activation of Rubisco in carboxysome-defective mutants exposed to low CO_2 should be considered. Modification of the small subunit of Rubisco in EK6 might have resulted in a higher $K_{act}(CO_2)$, i.e. a lower affinity of the activation site for CO_2. However, the data presented in Figure 1 do not lend support to this possibility. These data were obtained after 2 min of exposure of in vitro activated enzyme to different concentrations of CO_2. Time courses (not shown) indicated linear dependence of the accumulation of acid-stable ^{14}C on exposure time. If the mutation in EK6 had resulted in a considerably higher $K_{act}(CO_2)$, high enough to lead to the inactivation state of the Rubisco in cells exposed to low CO_2 (Fig. 3), a decreased activity of the mutant enzyme

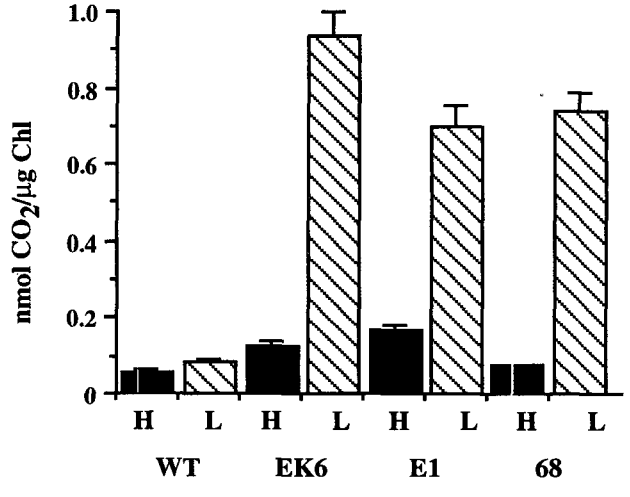

Figure 5. The amount of CO_2 fixed by the wild type and mutants EK6, E1, and No. 68 in carboxylation assays in which exogenous RuBP was not provided. Pretreatment conditions are indicated in the figure. The cells were grown under 5% CO_2 and exposed to high (H) or low (L) CO_2 during the pretreatment period and then treated by the detergent as described in "Materials and Methods" and the legend to Figure 3.

would be expected, particularly over a low-CO_2 concentration range. Moreover, a similar behavior was observed in other carboxysome-defective mutants, E1 and No. 68, which contain unmodified Rubisco. We suggest, therefore, that the altered Rubisco activity in the mutants is related to lower CO_2 concentration rather than an alteration of the CO_2 activation site.

We propose that it is the relatively low carboxysomal CO_2 concentration and the consequently low state of activity of Rubisco that underlie the low, apparent photosynthetic affinity for extracellular C_i observed in carboxysome-defective mutants and their resulting demand for high CO_2 for growth. Exposure of EK6 cells to high CO_2 activated Rubisco (Fig. 3) and enabled them to achieve a photosynthetic rate and, hence, a growth rate comparable to that of the wild type (Lieman-Hurwitz et al., 1991).

The size of the RuBP pool observed in the wild type (Fig. 4) is within the range determined by Mayo et al. (1989) in *S. leopoliensis*. However, in *S. leopoliensis* the pool size of RuBP in high-C_i-grown cells did not, as here, fall below the concentration of Rubisco binding sites (Mayo et al., 1989). On a Chl basis, the amount of Rubisco binding sites determined here was approximately 2-fold higher than that reported for *S. leopoliensis* (Mayo et al., 1989). Species variability or difficulties involved in efficient breakage of the cells and in assessing the percentage of broken cells may have contributed to the different results. Another experimental constraint in these studies is the need to use a rather high cell density in the oxygen electrode chamber. The pool size of RuBP and the changes therein are, otherwise, too small for accurate determination. Consequently, the cells are not uniformly illuminated and at any given time some of them may be light limited, with a resulting decrease in their RuBP pool. Although two projector lamps were used in these experiments, a possible light limitation cannot be ruled out. Accurate determination of the relative concentrations of Rubisco binding sites and of RuBP is essential for the elucidation of the factors that limit the rate of photosynthesis in cyanobacteria.

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