

1-1-1998

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Borkhsenius, O., Mason, C., & Moroney, J. (1998). The Intracellular Localization of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase in *Chlamydomonas reinhardtii*. *Plant Physiology*, 116 (4), 1585-1591. <https://doi.org/10.1104/pp.116.4.1585>

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The Intracellular Localization of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase in *Chlamydomonas reinhardtii*¹

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The pyrenoid is a proteinaceous structure found in the chloroplast of most unicellular algae. Various studies indicate that ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is present in the pyrenoid, although the fraction of Rubisco localized there remains controversial. Estimates of the amount of Rubisco in the pyrenoid of *Chlamydomonas reinhardtii* range from 5% to nearly 100%. Using immunolocalization, the amount of Rubisco localized to the pyrenoid or to the chloroplast stroma was estimated for *C. reinhardtii* cells grown under different conditions. It was observed that the amount of Rubisco in the pyrenoid varied with growth condition; about 40% was in the pyrenoid when the cells were grown under elevated CO₂ and about 90% with ambient CO₂. In addition, it is likely that pyrenoidal Rubisco is active in CO₂ fixation because in vitro activity measurements showed that most of the Rubisco must be active to account for CO₂-fixation rates observed in whole cells. These results are consistent with the idea that the pyrenoid is the site of CO₂ fixation in *C. reinhardtii* and other unicellular algae containing CO₂-concentrating mechanisms.

Pyrenoids are electron-dense structures that are found in the plastids of most algae. When pyrenoids are isolated, the most abundant protein present is Rubisco (Kuchitsu et al., 1991; McKay and Gibbs, 1991; Okada, 1992). However, the amount of Rubisco present in the pyrenoid is controversial. Estimates of the amount of Rubisco in the pyrenoid versus the amount in the entire chloroplast range from 60% or higher (Vladimirova et al., 1982; Lacoste-Royal and Gibbs, 1987; Morita et al., 1997) to as low as 5% (Süss et al., 1995).

The function of Rubisco in the pyrenoid is also unclear. Lacoste-Royal and Gibbs (1987) found that a higher percentage of Rubisco was localized to the pyrenoid in stationary cells rather than in actively growing cells of *Chlamydomonas reinhardtii*, an observation that is consistent with the hypothesis that the pyrenoid is a storage body. Süss et al. (1995) interpreted their localization data to indicate that there were two forms of Rubisco in *C. reinhardtii*: form I, which bound to the thylakoid membranes in the stroma, and form II, which was found in the pyrenoid. They further speculated that the pyrenoid-localized form II might function as part of the CO₂-concentrating mechanism.

In photosynthetic organisms that possess a CO₂-concentrating mechanism, Rubisco has a very specific localization. In higher plants with C₄-type photosynthesis,

Rubisco is specifically localized to chloroplasts of bundle-sheath cells (Hatch, 1992). In unicellular cyanobacteria with CO₂-concentrating mechanisms, Rubisco is specifically localized to structures known as carboxysomes (Allen, 1984; Codd and Marsden, 1984). Recent studies of the cyanobacteria's CO₂-concentrating mechanism indicate that HCO₃⁻ accumulated by the cell is dehydrated specifically in the carboxysome, where the resulting CO₂ can be fixed before leaking out of the cell (Badger and Price, 1994). Models of this process have also been proposed (Reinhold et al., 1991).

C. reinhardtii also possesses a CO₂-concentrating mechanism that is inducible (Badger et al., 1980; Aizawa and Miyachi, 1986; Moroney and Mason, 1991). When *C. reinhardtii* is grown under elevated CO₂, the CO₂-concentrating mechanism is not present, but when the alga is grown under low CO₂, the CO₂-concentrating mechanism is operational. With these observations in mind, we investigated the distribution of Rubisco in *C. reinhardtii* under a variety of growth conditions, including high- and low-CO₂ concentrations. Under all growth conditions we found that a significant fraction of Rubisco was localized to the pyrenoid. In particular, under limiting CO₂ more than 90% of the Rubisco was found within the pyrenoid. In addition, we found that the in vivo rates of CO₂ fixation are similar to the total amount of Rubisco activity, as estimated by in vitro Rubisco assays. This means that nearly all of the Rubisco in the cell must be functional to account for the rates of CO₂ fixation observed in cells. This observation implies that the Rubisco localized to the pyrenoid is active in CO₂ fixation.

MATERIALS AND METHODS

Algal Cultures

The wild-type *Chlamydomonas reinhardtii* strain used in this study, 137 mt⁺, was obtained from Dr. R.K. Togasaki (Indiana University, Bloomington). Strain 18-7G (Spreitzer et al., 1985), containing a truncation of the large subunit of Rubisco, was obtained as CC-2653 from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). When grown photoautotrophically, strain 137 was cultured in minimal medium (Sueoka, 1960) in 2.8-L carboys, illuminated with 200 μE m⁻² s⁻¹ at room temperature, and shaken continuously. Cultures were bubbled with 5% CO₂ in air (final C_i concentration = 2 mM) or in ordinary air

¹ This work was supported by the National Science Foundation (grant no. IBN-9632087).

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Abbreviations: C_i, inorganic carbon; high CO₂, air supplemented with CO₂ so that the final CO₂ concentration is 5% (v/v); low CO₂, air containing ambient (350 ppm) CO₂.

(final $C_i = 4 \mu\text{M}$). When grown with acetate as a C source, Tris-acetate-phosphate medium was used (Sueoka, 1960). Strain 18-7G was grown with this medium in the dark.

Electron Microscopy

For transmission electron microscopy, various cell strains were prepared according to Henk et al. (1995). Equal parts of the cell suspension were mixed with equal parts of 4% OsO_4 , 8% formaldehyde, and 2% glutaraldehyde and fixed for 15 min. The sample was then filtered and fixed for an additional 15 min in equal parts of 4% OsO_4 , 8% formaldehyde, 2% glutaraldehyde, and 0.2 M sodium cacodylate buffer (pH 7.2). The final concentration of the fixative components were: 1% OsO_4 , 2% formaldehyde, 0.5% glutaraldehyde, and 50 mM sodium cacodylate buffer. The subsequent steps for obtaining sections and treatment of the grids were the same as described by Henk et al. (1995).

Immunolocalization

The immunocytochemical procedure was similar to the method of Schroeder et al. (1993), with some modifications. Sections were pretreated with 2% sodium-meta-periodate (Sigma) for 15 min to remove any glutaraldehyde, then blocked two times for 30 min each in 2% BSA and 0.1% Tween 20 in PBS. The sections were then incubated for 1 h with diluted primary antibody (1–50 dilutions of anti-Rubisco) or with preimmune serum diluted similarly as a control, washed with 0.5% Tween 20 in PBS four times for a total of 20 min, and blocked again in 2% BSA two times for 15 min each. To detect bound antibodies, we used Protein A conjugated with 15-nm colloidal gold particles (BB International, Cardiff, UK) and diluted 1:50 with 1% BSA for 40 min.

Evaluation of immunogold labeling was made using the computer program Image-1/AT release version 4 (Universal Imaging Co., West Chester, PA). The images from the negatives were frozen and stored in a frame buffer as an 8-bit, 256-gray-scale image. Once this was freshheld, the gold particles associated with the pyrenoid were counted, as were the particles in the same size area in the cytoplasm and thylakoid regions of the individual cells. Because the size of the pyrenoid was different from cell to cell, we calculated the number of particles in a $1\text{-}\mu\text{m}^2$ area as a means for comparing the labeling intensity for the various cell compartments of the different strains in the various experimental conditions.

Enzymes Assays

C. reinhardtii Rubisco was purified according to Spreitzer and Mets (1980). Rubisco activity in vitro was estimated by the method described by Pierce et al. (1982).

Photosynthesis Assays

The photosynthetic rate of algal cells was measured with an O_2 electrode (Rank Brothers, Cambridge, UK). Algae were centrifuged at 5000 rpm for 5 min, and the pellet resuspended at $25 \mu\text{g}$ chlorophyll mL^{-1} in 4 mL of 25 mM

Hepes-KOH (pH 7.3) and transferred to the electrode chamber. Algal cells were assayed at $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ of PAR. They were allowed to consume the C_i of the buffer and the intracellular pool of C_i until no net O_2 exchange was observed, which took between 3 and 10 min. NaHCO_3 at the indicated concentrations was added and the rate of O_2 evolution was measured over the next 30 s to 2 min. Chlorophyll concentrations were determined spectrophotometrically. The $K_{0.5}(\text{CO}_2)$ value is the CO_2 concentration required to give half-maximal rates of O_2 evolution.

SDS-PAGE and Immunoblotting

SDS-PAGE was performed on 12.5% polyacrylamide gels as described by Laemmli (1970). The protocol from Bio-Rad was followed when immunoblotting. The blots were probed with antisera raised against *C. reinhardtii* Rubisco (HTI Bioproducts, Ramona, CA). Immunoblotting was visualized using horseradish peroxidase-conjugated secondary antibodies, as described in the protocol from Bio-Rad.

RESULTS

In this study an antibody raised against Rubisco isolated from *C. reinhardtii* was used. This antibody reacted strongly and specifically with *C. reinhardtii* Rubisco, as judged by immunoblots (Fig. 1). The antibody recognized the *C. reinhardtii* Rubisco large subunit much better than the small subunit (Fig. 1). For this reason, the presence of the small subunit precursor should not complicate the immunolocalization studies.

When the anti-Rubisco antibody was used in immunolocalization studies, immunogold particles were localized preferentially to the chloroplast pyrenoid (Fig. 2). Two

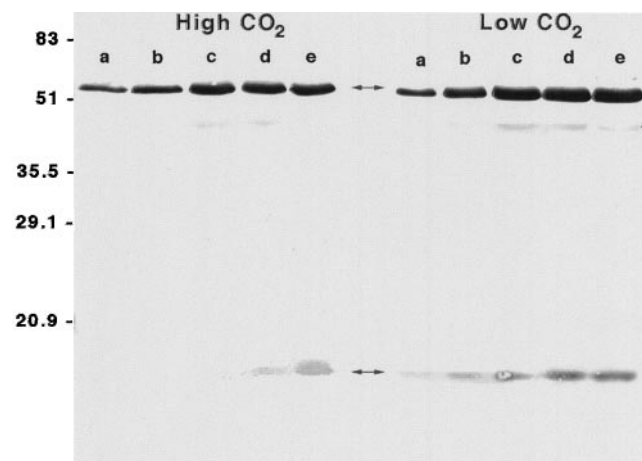


Figure 1. Specificity of the *C. reinhardtii* anti-Rubisco antibody. Different amounts of cell homogenate from high- and low- CO_2 -grown cells were separated by SDS-PAGE, blotted to nitrocellulose, and probed with an anti-Rubisco antibody, as described in "Materials and Methods." Lanes a, 5 μg of total protein; lanes b, 10 μg of protein; lanes c, 20 μg of protein; lanes d, 25 μg of protein; and lanes e, 30 μg of protein. The large and small subunits of Rubisco are indicated with arrows. The weak band at 44 kD (indicated with *) is a breakdown product of the large subunit (Rawat, 1994).

different growth conditions were used for the initial study: growth on elevated CO₂ or growth on air levels of CO₂. In both cases the pyrenoid had a high concentration of immunogold particles. In contrast, the immunogold labeling density in the chloroplast stroma depended on the growth condition. Growth on low CO₂ resulted in a low density of immunogold particles in the stroma (Fig. 2A), with the labeling density being similar to that seen in the cytoplasm.

In contrast, cells grown on elevated CO₂ had higher densities of immunogold particles in the stroma (Fig. 2B). In the case of cells grown on high CO₂ the labeling of the stroma was significantly higher ($P < 0.01$) than the labeling of the cytoplasm. Cells grown under either growth condition showed a very low density of immunogold particles over the entire cell when probed with the preimmune serum (Fig. 2, C and D).

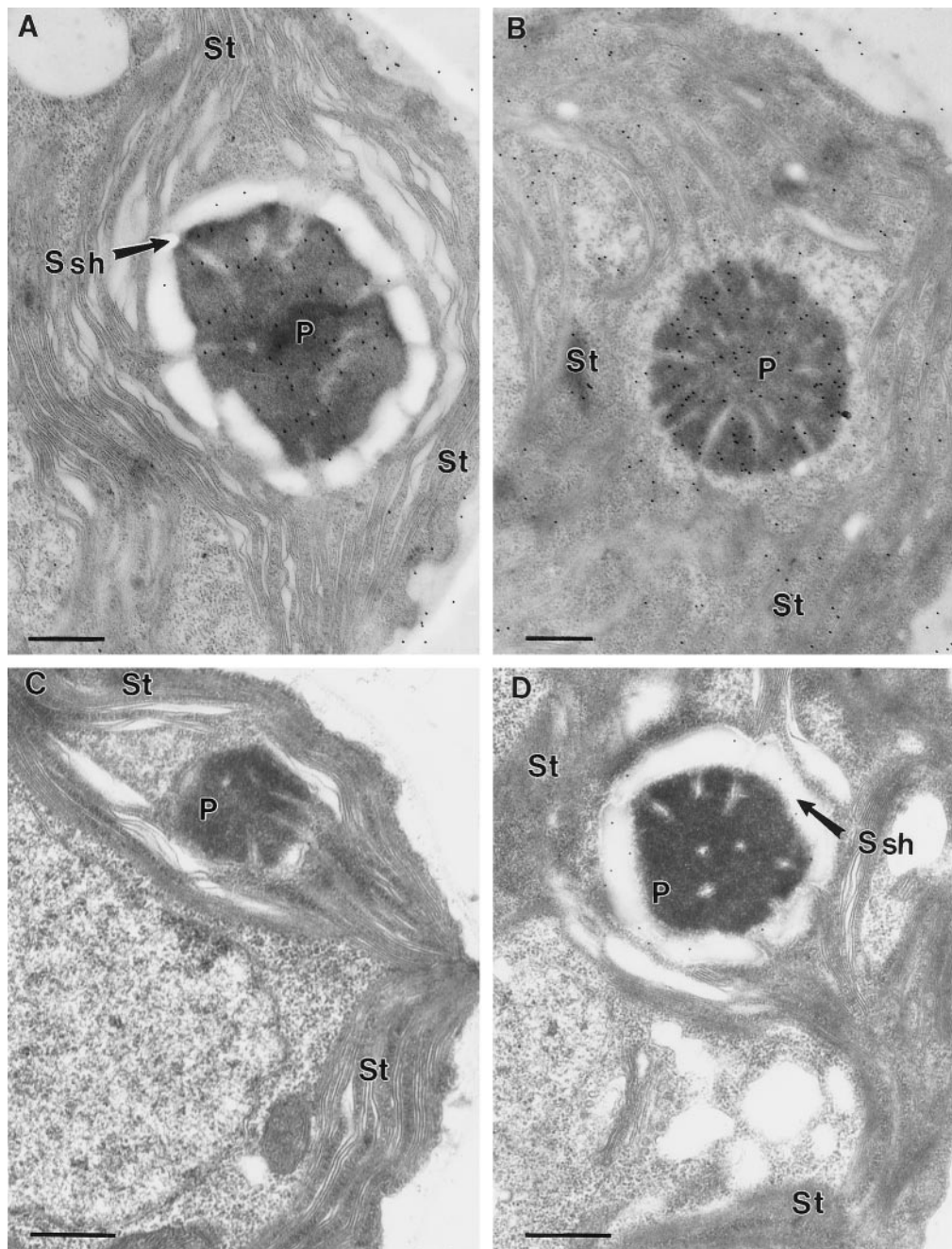


Figure 2. Immunogold labeling of *C. reinhardtii* cells grown on high- or low-CO₂ conditions. A, Low-CO₂-grown wild-type cells grown on minimal medium probed with an antibody raised against *C. reinhardtii* Rubisco. B, High-CO₂-grown cells grown on minimal medium probed with an antibody raised against *C. reinhardtii* Rubisco. C, High-CO₂-grown wild-type cells grown on minimal medium probed with a preimmune serum. D, Low-CO₂-grown cells grown on minimal medium probed with a preimmune serum. Bars indicate 0.5 μm . P, Pyrenoid; Ssh, starch sheath; and St, chloroplast stroma.

Table I. Calculation of Rubisco fraction in both the pyrenoid and stroma of the chloroplast

To obtain the fraction of Rubisco in the pyrenoid, the average density of immunogold particles in the cytoplasm was subtracted from the average density of particles in the stroma or in the pyrenoid. The net particle density of the pyrenoid or stroma was then multiplied by the average volume of the compartment (which is 2.4 mm^3 and 35.6 mm^3 , respectively), giving the total particles for each compartment. To calculate the fraction of Rubisco in the pyrenoid, the total number of particles in the pyrenoid was divided by the combined number of particles in the pyrenoid and in the stroma. The stroma includes the thylakoid area. The data shown are the averages \pm SD of 10 samples and 40 different evaluations. Preimmune sera gave immunogold densities of less than 2 particles mm^{-2} .

Growth Condition	Immunogold Density			Rubisco in Pyrenoid %
	Pyrenoid	Stroma	Cytoplasm	
		<i>particles mm⁻²</i>		
High CO ₂	70 \pm 14	9.0 \pm 2.1	2.0 \pm 0.9	40
Low CO ₂	65 \pm 15	2.5 \pm 1.2	2.0 \pm 0.8	89

When the relative volumes of the pyrenoid and the stroma are taken into account (Lacoste-Royal and Gibbs, 1987), it is clear that almost all of the Rubisco appears to be localized to the pyrenoid in *C. reinhardtii* cells grown under low levels of CO₂ (Table I). Similar calculations using data from the high-CO₂-grown cells show that a significant fraction (approximately 60%) of the Rubisco is localized to the chloroplast stroma in those cells (Table I). These data imply that the localization of Rubisco in *C. reinhardtii* depends in part on the growth conditions of the organism.

We also grew cells under different conditions to determine whether the distribution of Rubisco changes with a different C source. The growth condition tested was mixotrophic growth (Fig. 3). All of the wild-type cultures were grown at $150 \mu\text{mol m}^{-2} \text{ s}^{-1}$ of PAR. In these studies the distribution of immunogold particles in acetate-grown cells indicated that a significant fraction of the Rubisco was in the stroma (Figs. 2B and 3). *C. reinhardtii* strain 18-7G was also tested as a negative control. This strain has a stop codon in the *rbcL* gene and does not have detectable Rubisco (Spreitzer et al., 1985). However, strain 18-7G can grow with acetate as a C source under low light. As shown previously, this strain also does not have a detectable pyrenoid (Rawat et al., 1996). When probed with a Rubisco antibody, strain 18-7G had a very low immunogold density over the chloroplast stroma (Fig. 3A). The low immunogold density seen in this strain is similar to the immunogold density seen in wild-type cells growing on low CO₂, implying that the amount of Rubisco in the stroma of wild-type cells is very low. This result from strain 18-7G also supports the contention that the immunogold particle density in the stroma of wild-type cells grown on high CO₂ or on acetate is higher than background (Table II).

The change in distribution of Rubisco under high- and low-CO₂ growth conditions follows the presence or absence of the CO₂-concentrating mechanism. To further test this correlation, we determined the localization of Rubisco while cells were adapting to low-CO₂ growth conditions. In this experiment cells were first grown on elevated CO₂ and then switched to low CO₂. Using immunogold, the distri-

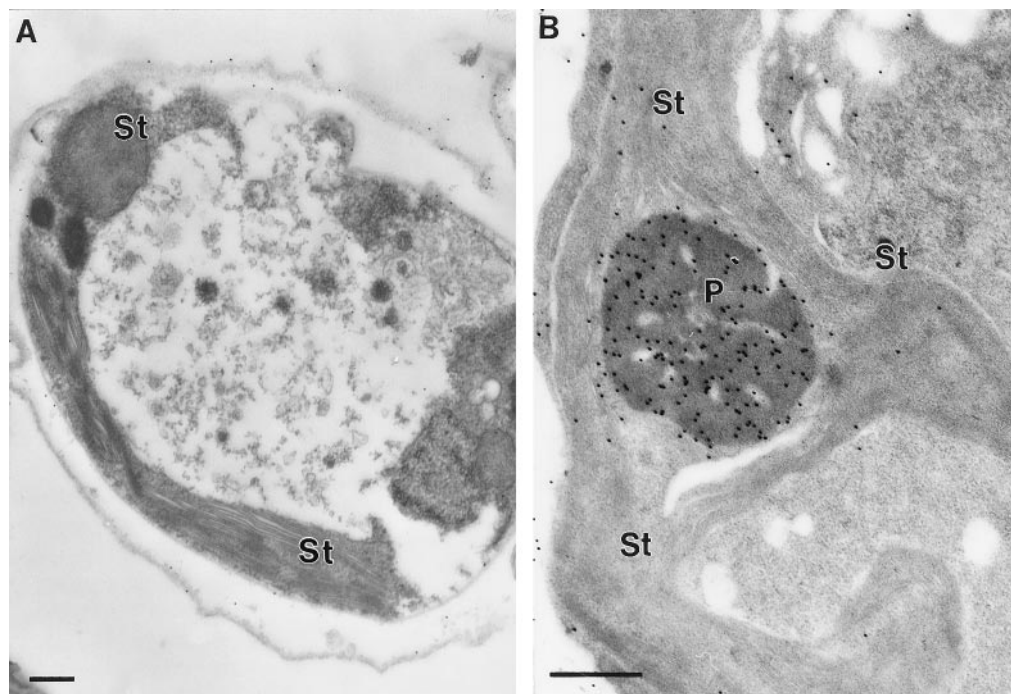


Figure 3. Immunogold labeling of *C. reinhardtii* strain 18-7G or wild-type cells grown on acetate. A, Strain 18-7G. B, Wild-type cells. Sections were probed with an antibody raised against *C. reinhardtii* Rubisco, as described in "Materials and Methods." Bars indicate $0.5 \mu\text{m}$. P, Pyrenoid; and St, chloroplast stroma. No pyrenoids were seen in strain 18-7G, in agreement with previous observations (Rawat et al., 1996).

Table II. Calculation of the Rubisco fraction in both the pyrenoid and the stroma of wild-type cells and strain 18-7G grown on acetate

Both cell lines were grown in moderate light ($150 \mu\text{mol mm}^{-2} \text{s}^{-1}$) on acetate medium. To quantitate the number of immunogold particles in the stroma and in the pyrenoid the method described in the legend to Table I was employed. Because the pyrenoid is absent from 18-7G, the number of particles in the pyrenoid is not applicable (N.A.). Ten samples of each strain were sampled. The data shown are the averages \pm SD.

Strain	Immunogold Density			Rubisco in Pyrenoid
	Pyrenoid	Stroma	Cytoplasm	
	<i>particles mm⁻²</i>			%
Wild type	149 \pm 63	6.2 \pm 1.5	3.8 \pm 1.7	80
18-7G	N.A.	1.3 \pm 0.9	1.6 \pm 0.6	N.A.

bution of Rubisco between the pyrenoid and stroma was estimated for various times after the switch in CO_2 level (Figs. 4 and 5). As seen from the time course (Fig. 4), the distribution of Rubisco changes about 2 h after the change in CO_2 supply. This is also the time when the affinity of the cells for CO_2 , as estimated by the $K_{0.5}(\text{CO}_2)$, is rapidly changing. Representative electron micrographs showing the immunogold distributions are shown in Figure 5. The data in this experiment are consistent with that of Morita et al. (1997) and our earlier experiment (Fig. 2A), indicating that 90% or more of the Rubisco is in the pyrenoid when cells are grown on low CO_2 .

Since 90% of the Rubisco is localized to the pyrenoid, we compared the *in vitro* carboxylation activity of Rubisco isolated from low- CO_2 -grown wild-type cells with the *in vivo* $^{14}\text{CO}_2$ -fixation rate of these same cells. The *in vitro* Rubisco carboxylation rate measured under saturating CO_2 ($186 \pm 50 \mu\text{mol h}^{-1} \text{mg chlorophyll}^{-1}$) closely matched the maximal *in vivo* rate of $^{14}\text{CO}_2$ fixation ($199 \pm 44 \mu\text{mol h}^{-1} \text{mg chlorophyll}^{-1}$). This result supports the suggestion that the majority of Rubisco within the low- CO_2 -grown cell must be active to support the rates of CO_2 fixation observed in whole cells. Since most of the Rubisco is localized to the pyrenoid, it follows that the Rubisco within the pyrenoid is active and that the pyrenoid is the actual location of CO_2 fixation in *C. reinhardtii*.

DISCUSSION

One aim of this report was to resolve the present controversy concerning the localization of Rubisco in *C. reinhardtii*. Lacoste-Royal and Gibbs (1987) reported that between 60 and 70% of Rubisco appeared to be localized to the pyrenoid. Recently, however, Süß et al. (1995) challenged that earlier finding by reporting that only 5% of the Rubisco was localized in the pyrenoid. In addition, Morita et al. (1997) have recently reported that 99% of the Rubisco is localized to the pyrenoid, illustrating the wide range of estimates in the literature. Süß et al. (1995) used cryotechniques to avoid spatial dislocation of soluble enzymes such as Rubisco and other C3 enzymes. They cited this as a possible difference between their results and those of Lacoste-Royal and Gibbs (1987). However, Morita et al.

(1997) used similar techniques in their study. Süß et al. (1995) also argued that Lacoste-Royal and Gibbs (1987) did not take into account the relative volumes of the pyrenoid and stroma in their calculations. The data presented in this report are consistent with the data of Lacoste-Royal and Gibbs (1987) and Morita et al. (1997). The small differences between the reports of Lacoste-Royal and Gibbs (1987), Morita et al. (1997), and this present work are likely due to growth-condition differences. For example, many of the cultures used by Lacoste-Royal and Gibbs (1987) were mixotrophically grown, whereas Morita et al. (1997) grew cells phototrophically on low CO_2 .

Our results are in agreement with all earlier studies that showed a high level of immunogold particles in the pyrenoid when anti-Rubisco antibodies were used. However, we disagree with the conclusions of Süß et al. (1995). The primary reason for our different conclusions is the method of counting the immunogold particles. In this report and in the paper by Lacoste-Royal and Gibbs (1987), the number of immunogold particles is reported as the number of particles per unit area. Süß et al. (1995) counted the total number of particles per section in each subcellular location and did not take into account the area of each organelle in the section sampled.

According to Weibel (1979) a section is a two-dimensional projection of the volume of the organelle or tissue being examined in the electron microscope. As such, the average area of an organelle or cell observed in many sections is proportional to the volume of that object. Therefore, the quantitative method employed by Süß et al. (1995) accounts for the volume difference twice, first in the counting of the particles and a second time when they multiply their number by the relative volumes of the pyrenoid and the stroma. If the number of immunogold particles reported by Süß et al. (1995) is expressed on a particles

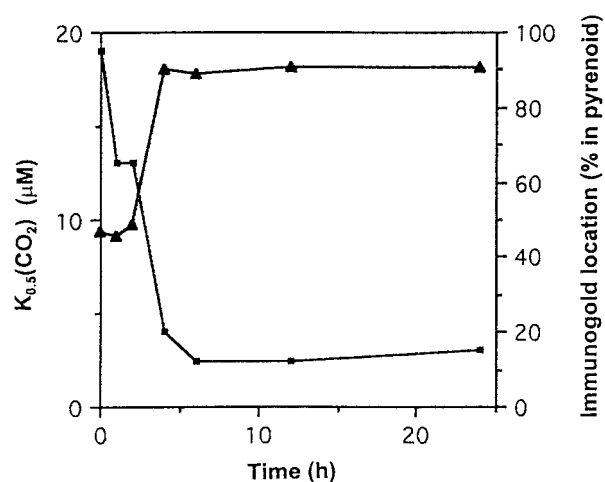


Figure 4. Change in the apparent affinity of cells for C_i and the change in distribution of Rubisco after cells are switched to low CO_2 . At 0 h cells were switched from high- to low- CO_2 conditions. The apparent affinity of the cells was determined by estimating the $K_{0.5}(\text{CO}_2)$ of the cells (■) as described in "Materials and Methods." The percentage of Rubisco in the pyrenoid (▲) is calculated from the distribution of immunogold particles.

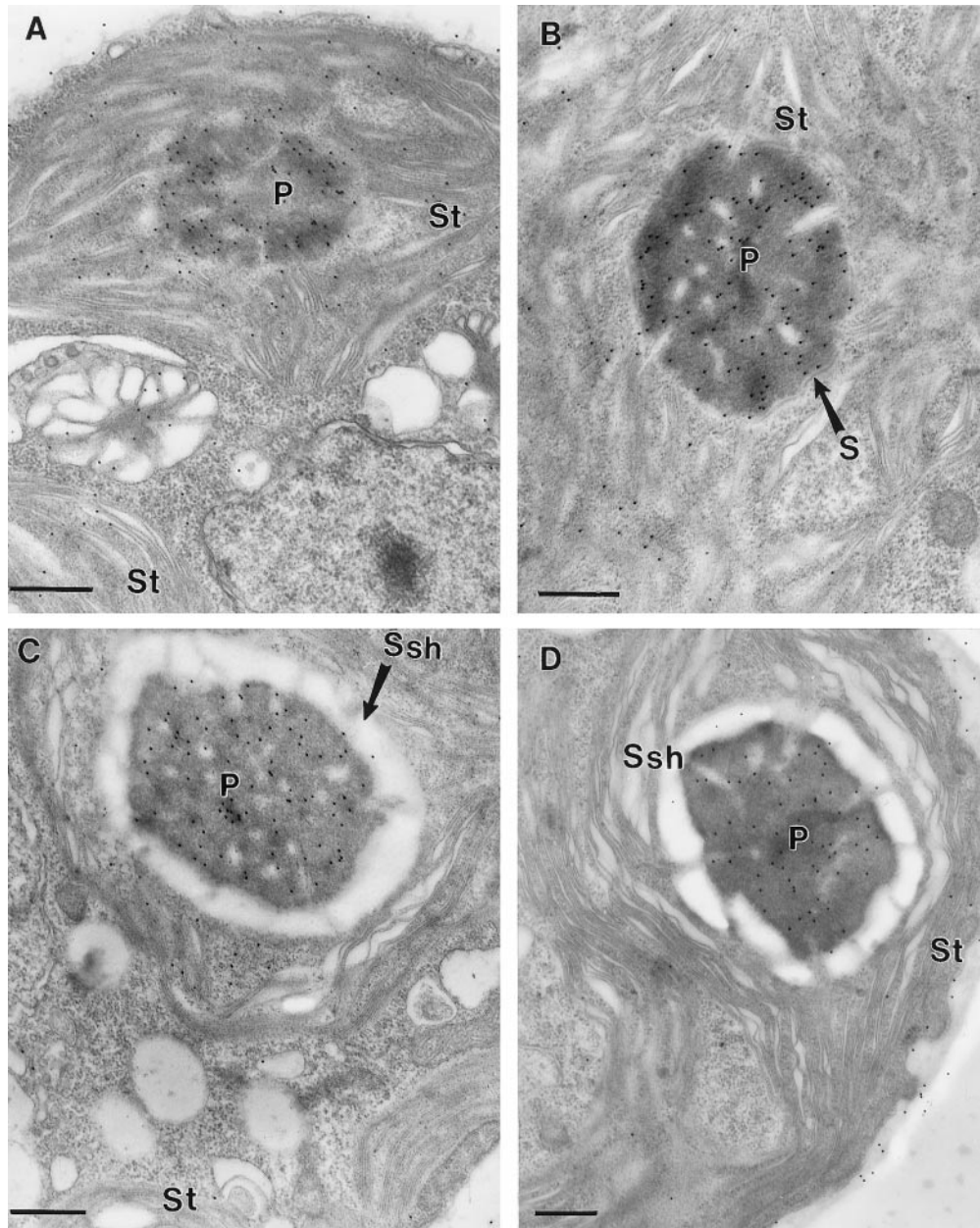


Figure 5. Immunogold labeling of *C. reinhardtii* cells after being switched from high- to low- CO_2 conditions. A, Cells just before transfer to low CO_2 (0 h). B, Cells 2 h after the switch to low CO_2 . C, Cells 6 h after the switch to low CO_2 . D, Cells 12 h after the switch to low CO_2 . Bars indicate $0.5 \mu\text{m}$. P, Pyrenoid; Ssh, starch sheath; and St, chloroplast stroma.

per area basis, their data are much more in line with our results and those of Lacoste-Royal and Gibbs (1987) and Morita et al. (1997). From these studies it appears that at a minimum 60 to 70% of the Rubisco in *C. reinhardtii* is localized to the pyrenoid.

A second aim of this report was to determine whether Rubisco is mobilized or relocated when cells are switched from high to low CO_2 . It appears that growth conditions do affect the intracellular localization of Rubisco. We looked at cells grown on high CO_2 , low CO_2 , or acetate. We consistently found significant levels of Rubisco in the chloroplast stroma if the cells were grown on high CO_2 or acetate. In

the extreme case of strain 18-7G, where no Rubisco is produced, no pyrenoid was observed and the immunogold density in the stroma was at background levels. Our results are consistent with the pyrenoid being the primary location of Rubisco in *C. reinhardtii* under low- CO_2 growth conditions when the components of the CO_2 -concentrating mechanism are being expressed. The shift in Rubisco localization correlates well with the formation of the starch sheath, which also forms shortly after cells are switched to low CO_2 (Ramazanov et al., 1994). Süß et al. (1995) also reported that Rubisco was associated with thylakoid membranes in the chloroplast stroma and the pyrenoid. For the

pyrenoid we found no particular association of Rubisco with the tubules that penetrate the pyrenoid matrix. The concentration of Rubisco is such that it is likely to be found throughout the matrix of the pyrenoid. Our immunolabeling of the stroma was not dense enough to determine whether there is a particular association of Rubisco with the stromal lamellae.

The finding of Rubisco in the pyrenoid has raised the question of whether pyrenoidal Rubisco is active in CO₂ fixation. Clearly, if 100% of the Rubisco is localized to the pyrenoid, then that Rubisco must be active. However, even if a lower estimate of the amount of Rubisco in the pyrenoid is used, our activity measurements are consistent with the idea that pyrenoidal Rubisco is active in vivo. Rubisco activity measurements indicate that there is not an excess of Rubisco activity in the cell, and that most of the Rubisco in the cell must be active to account for the levels of CO₂ fixation observed in whole cells. Because 90% of the Rubisco is localized to the pyrenoid when cells are grown on low CO₂ (Figs. 2A and 4D; Table I) (Morita et al., 1997), these results are consistent with the idea that the primary location of CO₂ fixation in *C. reinhardtii* is the pyrenoid.

A specific localization of Rubisco to the pyrenoid is compatible with the view that organisms with CO₂-concentrating mechanisms package Rubisco in very specific fashions. The most analogous situation is found in cyanobacteria, in which Rubisco is found in carboxysomes (Codd and Marsden, 1984; Reinhold et al., 1991). In cyanobacteria it appears that the CO₂ level is elevated within the carboxysome, thus favoring the carboxylation activity of Rubisco over the oxygenase activity. The pyrenoid may serve the same function in *C. reinhardtii* and other eukaryotic algae.

ACKNOWLEDGMENTS

We thank Ronald Bouchard for his help with the Image-1/AT, and Dr. William Henk and M.C. Henk for their valuable suggestions.

Received October 6, 1997; accepted January 20, 1998.

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