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Ultrastructural and biochemical adaptation of algal cells to limiting CO_2 concentrations*

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SUMMARY: The pyrenoid is a prominent proteinaceous structure found in the stroma of the chloroplast in unicellular eukaryotic algae, most multicellular algae, and some hornworts. The pyrenoid contains the enzyme ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco) and is sometimes surrounded by a carbohydrate sheath. In *Chlamydomonas reinhardtii* the pyrenoid starch sheath is formed rapidly in response to a decrease in the CO_2 concentration in the environment. In this report we show that the unicellular green algae *Chlamydomonas acidophila* and *Chlorella spK* also form a starch sheath when adapted to low CO_2 conditions. We also report that in some high CO_2-requiring *C. reinhardtii* strains, pyrenoid starch sheath formation is partly inhibited or absent. The inhibition of the pyrenoid starch sheath is also seen when the carbonic anhydrase inhibitor ethoxyzolamide is added to cells placed in an environment low in CO_2. These observations support the idea that the ultrastructural reorganization of the pyrenoid starch sheath under low CO_2 conditions plays a role in the CO_2 concentrating mechanism in *C. reinhardtii* as well as in other eukaryotic algae.

Key words: CO_2, photsynthesis, pyrenoid, starch, *Chlamydomonas*, *Chlorella*.

INTRODUCTION

Since the discovery of the reductive photosynthetic carbon cycle by Calvin and his colleagues over forty years ago a number of variations on this basic metabolic pathway have been identified. In terrestrial plants, C_4 photosynthesis has evolved in response to decreasing atmospheric CO_2 levels while Crassulacean Acid Metabolism (CAM) is found in many plants growing in arid conditions. Like C_4 plants, aquatic organisms must be able to efficiently acquire C_i from the environment since the diffusion of CO_2 and HCO_3^- is thousands of times slower in aqueous solutions than in air. Most aquatic photosynthetic organisms have adapted to the aqueous environment by having some form of a CO_2 concentrating mechanism (CCM), a means of concentrating CO_2 at the site of Rubisco (Badger *et al.*, 1980). Cells with a CCM have the ability to accumulate inorganic carbon (CO_2 and HCO_3^-) to levels higher than can be obtained by simple diffusion. Algae with the CCM can grow on very low CO_2 concentrations, lower than that tolerated by plants with C_3-type photosynthesis.

The intracellular localization of ribulose bisphosphate carboxylase oxygenase (Rubisco) appears to be critical in the operation of C_4-photosynthesis and the CCM. In plants with C_3 photosynthesis, Rubisco is distributed throughout chloroplast stroma

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TABLE 1. – The localization of Rubisco in plants using different photosynthetic pathways.

Photosynthesis Type	Type of Organism	CO_2 affinity	Rubisco location
C-3 photosynthesis	most land plants	low	chloroplast stroma-leaf mesophyll cells
C-4 photosynthesis	diverse land plants	high	chloroplast stroma-bundle sheath cells
CO_2 concentrating mechanism (CCM)	most algae and cyanobacteria	high	unicellular green algae-chloroplast pyrenoid cyanobacteria - carboxysomes

of the mesophyll cells of the leaf (Table 1). For plants with C_4 photosynthesis, Rubisco is still soluble in the chloroplast but now is localized only within the bundle sheath cells of the leaf. Finally in algae, Rubisco is packaged within the cell or chloroplast. In cyanobacteria Rubisco is localized in proteinaceous structures called carboxysomes. In eukaryotic algae Rubisco is localized in the pyrenoid. Recent research summarized below indicates that the carboxysome and the pyrenoid may be essential to the functioning of the CCM in algae.

In cyanobacteria Rubisco is localized within the carboxysome (Codd and Marsden, 1984). Kaplan and his colleagues (Friedberg *et al.*, 1989; Lieman-Hurwitz *et al.*, 1991) as well as Badger and his colleagues (Price *et al.*, 1993) used insertional mutagenesis to select for cyanobacterial mutants that have a defective CCM. The phenotype of these mutant strains is that they are unable to grow on low levels of CO_2. One class of mutant that they have identified are cells that have aberrant carboxysomes or have no carboxysomes at all. Mutants identified have point mutations or insertions in genes known as ccmL or just identified as an open reading frame (ORF). Recently English *et al.* (1994) have identified the gene encoding the carboxysome coat protein of the chemolithotroph *Thiobacillus neapolitanus* and this gene has significant homology to the ORFII identified in *Synechococcus* (Friedberg *et al.*, 1989). A second type of carboxysome mutant has an insertion within the Rubisco small subunit gene. This results in a cell that has very long rod-shaped carboxysomes and also requires high CO_2 for growth. The implication of these results is that the carboxysome must be intact for the CCM to operate.

In eukaryotic algae Rubisco is found in pyrenoids. The pyrenoid is a large protein complex found within the chloroplast sometimes surrounded by a sheath of carbohydrate such as starch, amylose or paramylon (Gibbs, 1962a, 1962b; Griffiths, 1980; Kuchitsu *et al.*, 1988a; Okada, 1992). In the past the most common physiological role suggested for the pyrenoid was that of a protein or carbohydrate storage body (Griffiths, 1980; McKay and Gibbs, 1991; Okada, 1992). However recent immunolocalization studies have indicated that most, if not all, of the Rubisco is localized in the pyrenoid (Lacoste-Royal and Gibbs, 1987; Osafune *et al.*, 1990; Kuchitsu *et al.*, 1991; McKay and Gibbs, 1991; Okada *et al.*, 1991), indicating that the pyrenoid is the active location of CO_2 fixation and not simply a storage structure.

Light microscopy studies have shown that cells adapted to low CO_2 conditions are more likely to have starch around the pyrenoid than cells adapted to high CO_2 conditions (Miyachi *et al.*, 1986; Kuchitsu *et al.*, 1988b; Kuchitsu *et al.*, 1991). In *Chlamydomonas reinhardtii*, as well as *Chlorella*, *Scenedesmus* and *Dunaliella* species, the CCM is inducible; only algal cells grown on air levels of CO_2 (0.03%) have an active CCM (Badger *et al.*, 1980; Aizawa and Miyachi, 1986). Recent work from our laboratory has shown that the formation of the starch sheath around the pyrenoid correlates well with the induction of the CCM in *Chlamydomonas* (Ramazanov *et al.*, 1994). This is further evidence that the pyrenoid may play an important role in the CCM in these eukaryotic algae, a role similar to that of the carboxysome in cyanobacteria. In this report we have looked at pyrenoid morphology in other unicellular green algae growing in an environment low in CO_2. In addition we report on the pyrenoid structure of some high CO_2-requiring mutants of *Chlamydomonas*.

MATERIAL AND METHODS

Algal culture and growth conditions

Chlamydomonas reinhardtii 137 wild type was obtained from Dr. R. K. Togasaki, Indiana University, Bloomington IN, USA, and maintained in this laboratory. *Chlamydomonas acidophila* was

obtained from Prof. J. Kalina of the Czech culture collection. *Chlorella spK* was obtained from the Russian algal collection (Moscow). *C. reinhardtii* and *Chlorella spK* were grown in minimal media (Sueoka, 1960) while *C. acidophila* was grown in Murashige and Skoog media without sucrose (Murashige and Skoog, 1962) with the pH adjusted to 4.5. In liquid culture, the strains were inoculated at a cell density of 4 x 10^4 cells ml^{-1} and aerated with 5% CO_2 in air with continuously illumination at 100 µmol $m^{-2}s^{-1}$ of white light for 3 days. The cultures were then switched to air levels of CO_2, where indicated, and maintained at that level of CO_2 until they were harvested.

Electron Microscopy

For transmission electron microscopy two methods of fixation and embedding were used. In the first procedure, cells were fixed for 2 h at 4° C in 2.5% glutaraldehyde added to Hepes-KOH (pH 7.5) buffer. Samples were rinsed in the same buffer, then postfixed overnight at 4° C in 1% osmium tetroxide. After a rinse in distilled water, the samples were dehydrated in a graded series of ethanol, with 2% uranyl acetate added to the 80% step and held overnight at 4° C, to a final concentration of 100% ethanol. Samples were pelleted by centrifugation between each change. The samples were slowly infiltrated at 4° C with Spurr's resin (Spurr, 1969), with seven changes over a period of several days, then polymerized for 12 h at 60° C.

In the second procedure, cells were fixed in a mixture of 2% glutaraldehyde and 2% osmium tetroxide in half-strength growth medium for 15 min. The cells were then filtered and further fixed in the same fixative mixture in 0.05M cacodylate buffer (pH 7.1), for 20 min, rinsed for 5 min, stained *en bloc* with 0.5% uranyl acetate for 30 min, dehydrated in ethanol, then infiltrated and embedded in LR White medium resin. Thin sections were cut using a diamond knife mounted on a Sorvall Porter-Blum MT-2 or MT 5000 Ultramicrotome, poststained with uranyl acetate and or lead citrate, and examined and photographed on a JEOL JEM 100CX Transmission Electron Microscope.

Photosynthesis assays

The photosynthetic rate of algal cells was measured with an oxygen electrode (Rank Brothers, Cambridge, England). Algae were centrifuged at 5,000 rpm for 5 min and the pelleted algae were resuspended at 25 µg Chl ml^{-1} in 4 ml of 25 mM Hepes-KOH (pH 7.3) and transferred to the electrode chamber, where they were allowed to consume the inorganic carbon of the buffer and intracellular pool of C_i until no net O_2 exchange was observed which took between 3 and 10 min. Bicarbonate at the indicated concentrations was added and the rate of O_2 evolution measured over the next 0.5-2 min. Chlorophyll concentrations were determined spectrophotometrically.

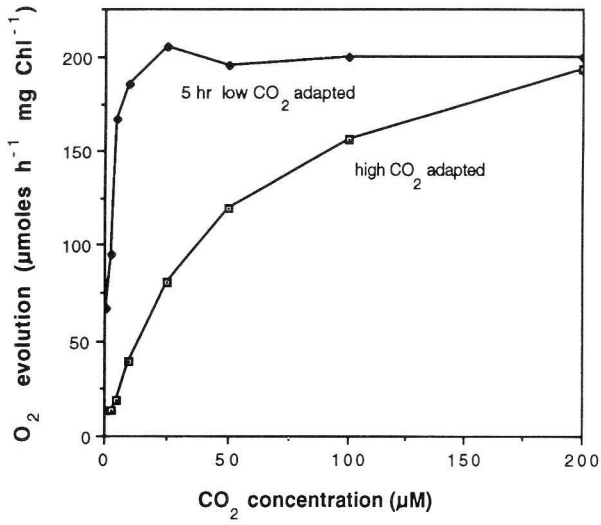

FIG. 1. – The rate of photosynthesis versus inorganic carbon concentration for wild-type *C. reinhardtii* cells grown under high CO_2 concentrations (□) or adapted to low CO_2 for five hours (•).

RESULTS

In unicellular green algae, the CCM is inducible. When these cells are grown on elevated CO_2 they do not induce the CCM and the cells' affinity for CO_2 remains low. Cells grown under these conditions require about 25 to 40 µM CO_2 for half maximal rates of CO_2 fixation (Fig. 1). However when these cells are placed under limiting CO_2 they induce the CCM and they now require about 1 to 3 µM CO_2 for half maximal rates of photosynthesis (Fig. 1). This inducibility has been seen in a large number of unicellular algae (Aizawa and Miyachi, 1986; Merret, 1991). The induction of the CCM requires about five hours. The pyrenoid starch sheath also is formed within this period of time. Figure 2 shows that *Chlamydomonas* cells that have adapted to low CO_2 have a complete starch sheath while those on elevated CO_2 have only the stromal form of starch.

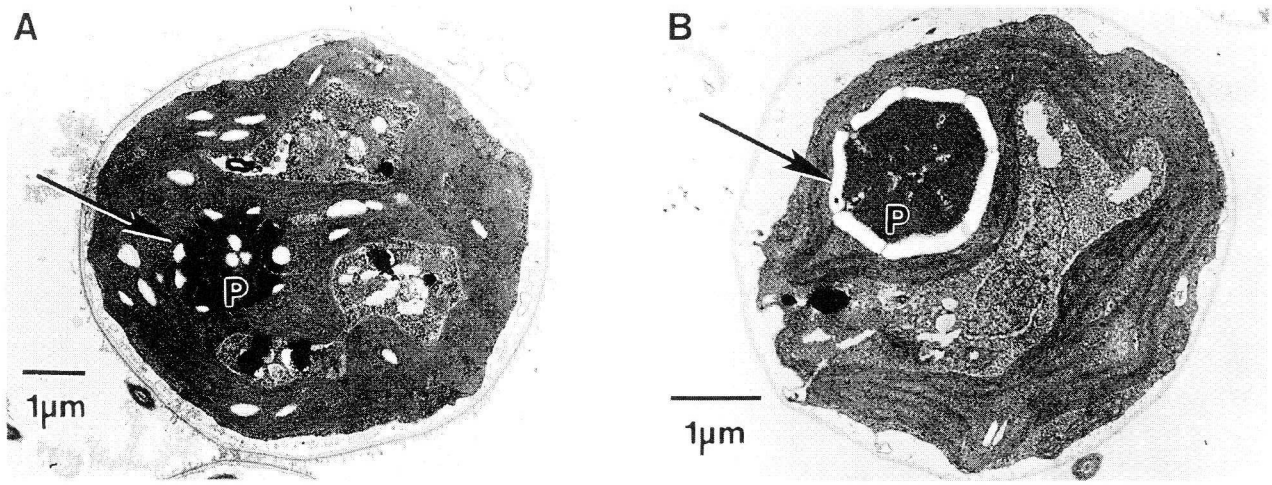

FIG. 2. – Electron micrographs of *C. reinhardtii* cells grown on elevated CO_2 (A) or adapted for five hours on low CO_2 (B).

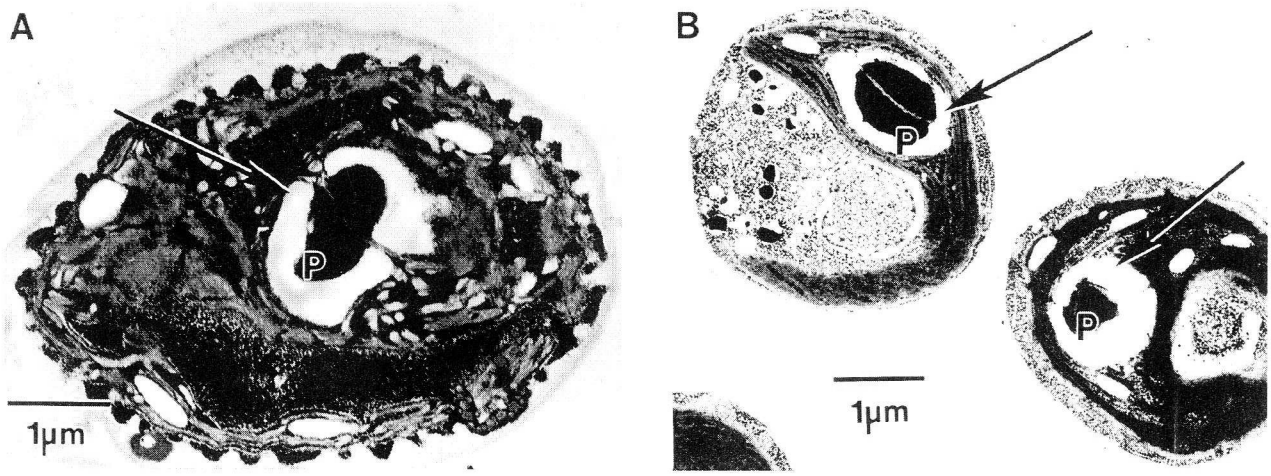

FIG. 3. – Electron micrographs of *Chlamydomonas acidophila* (A) and *Chlorella spK* (B) grown on low CO_2.

Recent work from this laboratory has shown that the biosynthesis and breakdown of the starch sheath coincides well with the induction and loss of the CCM in *C. reinhardtii* (Ramazanov *et al.*, 1994).

This thick starch sheath is also seen in other unicellular green algae when they are grown on limiting CO_2. *Chlamydomonas acidophila* is an acid tolerant alga which has a different type of pyrenoid than *C. reinhardtii* with fewer thylakoid membranes within the pyrenoid. The *Chlorella spK* has also been used in the study of CCM organization and carbonic anhydrase activity (Pronina *et al.*, 1981). These two algae both induce a CCM as does *C. reinhardtii*. In addition, both make the pyrenoid starch sheath when exposed to low CO_2 conditions (Fig. 3). These results support the contention that the pyrenoid starch sheath may be playing a role in the CCM in a variety of green algae.

If the pyrenoid is playing a role in the CCM then one might expect that mutants that have a disrupted pyrenoid might also have a less efficient CCM. With this in mind we investigated the pyrenoid structure of some high CO_2 requiring *C. reinhardtii* mutants. In Table 2 we show the apparent CO_2 affinity of three of these high CO_2 requiring strains and in Figure 4 we show the pyrenoid morphology of these strains. Cia-5 is a strain that adapts poorly to low CO_2 conditions (Moroney *et al.*, 1989). When placed in a low CO_2 environment this strain has a lower affinity for CO_2 than wild-type cells and fails to make any of the low CO_2 inducible proteins normally seen in wild type cells (Moroney *et al.*, 1989). Its starch sheath is typically thin when grown on low CO_2 which follows its generally weak adaptation to low CO_2 conditions. Cia-3 is a strain that is thought to be deficient in a chloroplast carbonic anhydrase (Moroney *et al.*,

TABLE 2. – Apparent affinity for CO_2 of wild-type cells and the high CO_2 requiring mutants cia-5, cia-3 and ca-1. Cells were switched from a high CO_2 environment to one low in CO_2 for five hours prior to measuring their rates of O_2 evolution in the presence of differing concentrations of inorganic carbon. The $K_{0.5}(CO_2)$ is the CO_2 concentration which supports half-maximal rates of photosynthesis.

Strain	$K_{0.5}(CO_2)$ (μM)
wild-type	2.5
cia-5	17
cia-3	65
ca-1	52

1986; Husic and Marcus, 1994; Katzman *et al.*, 1994). This strain has a very poor affinity for CO_2 (Table 2) and fails to synthesize the pyrenoid starch sheath (Fig. 4). Ca-1 is a high CO_2 requiring mutant selected by Spalding *et al.* (1983) which is also thought to be deficient in the chloroplast carbonic anhydrase. Like cia-3 it has a very low affinity for CO_2 (Table 2) but its pyrenoid morphology is quite different than cia-3 (Fig. 4). Ca-1 makes a lot of starch around the pyrenoid under low CO_2 conditions but the shape of the pyrenoid is abnormal (Fig. 4). Unlike wild type cells one often observes irregularly shaped pyrenoids and starch that is deposited unevenly around the pyrenoid (Fig. 4). From these data it appears that some of the high CO_2-requiring *C. reinhardtii* strains have abnormal pyrenoid development similar to the carboxysome mutants of cyanobacteria.

Earlier genetic studies indicated that ca-1 and cia-3 were allelic (Moroney *et al.*, 1986). Since both of these strains were deficient in the chloroplast carbonic anhydrase, there might be a link between the chloroplast carbonic anhydrase and pyrenoid structure. To test this we added two carbonic anhydrase inhibitors, acetazolamide and ethoxyzolamide, to *C. reinhardtii* cells and looked at the pyrenoid morphology in these cells. Acetazolamide is a less permeant inhibitor thought to only inhibit the periplasmic carbonic anhydrase (Moroney *et al.*, 1985). This inhibitor had little affect on the affinity of the cells for CO_2 or pyrenoid structure (Fig. 5B). However cells treated with the membrane permeant inhibitor, ethoxyzolamide, failed to make a starch sheath (Fig. 5C). This result resembles the results obtained with cia-3 (Fig. 4B). One additional effect of ethoxyzolamide was observed in cells grown on elevated CO_2 (Fig. 5E). Cells treated with ethoxyzolamide made a lot of starch that was distributed throughout the chloroplast stroma. These results indicate that there might be a link between the chloroplast carbonic anhydrase activity and starch deposition by these algal cells.

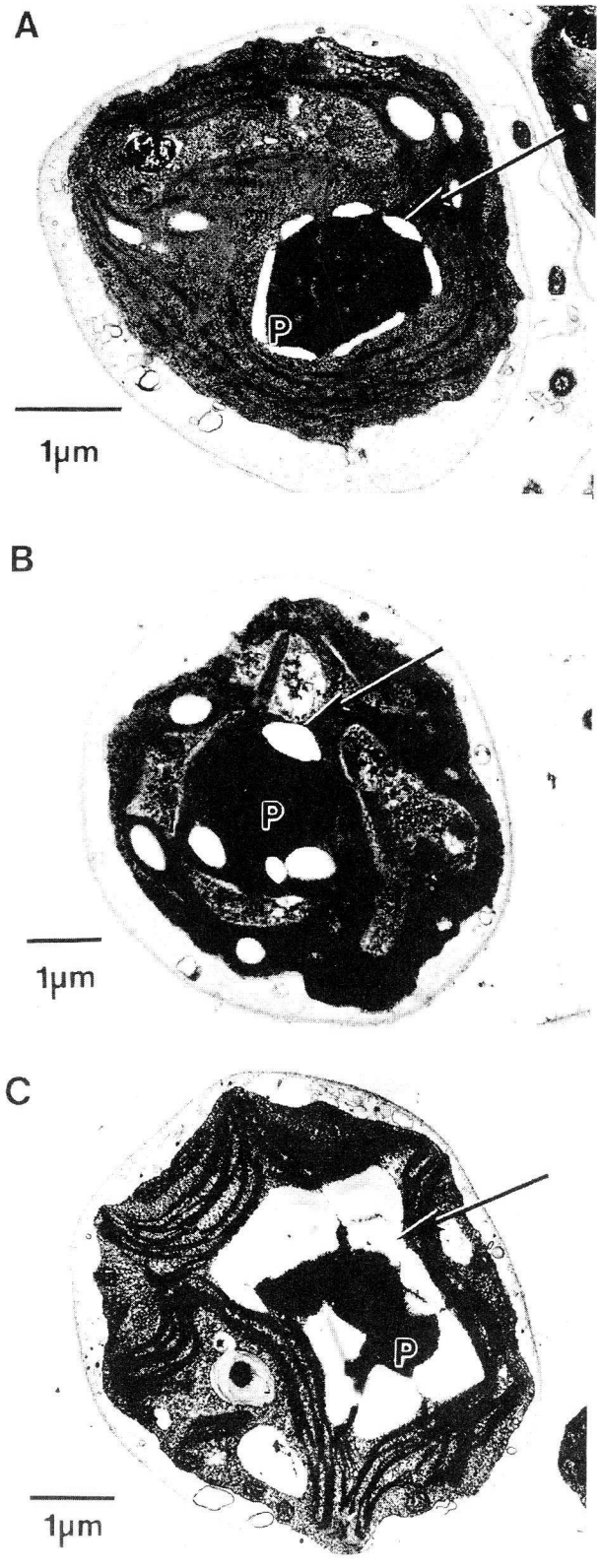

FIG. 4. – The pyrenoid morphology mutant strains of *C. reinhardtii* adapted to low CO_2 for five hours. The cells are cia-5, (A); cia-3, (B) and ca-1, (C).

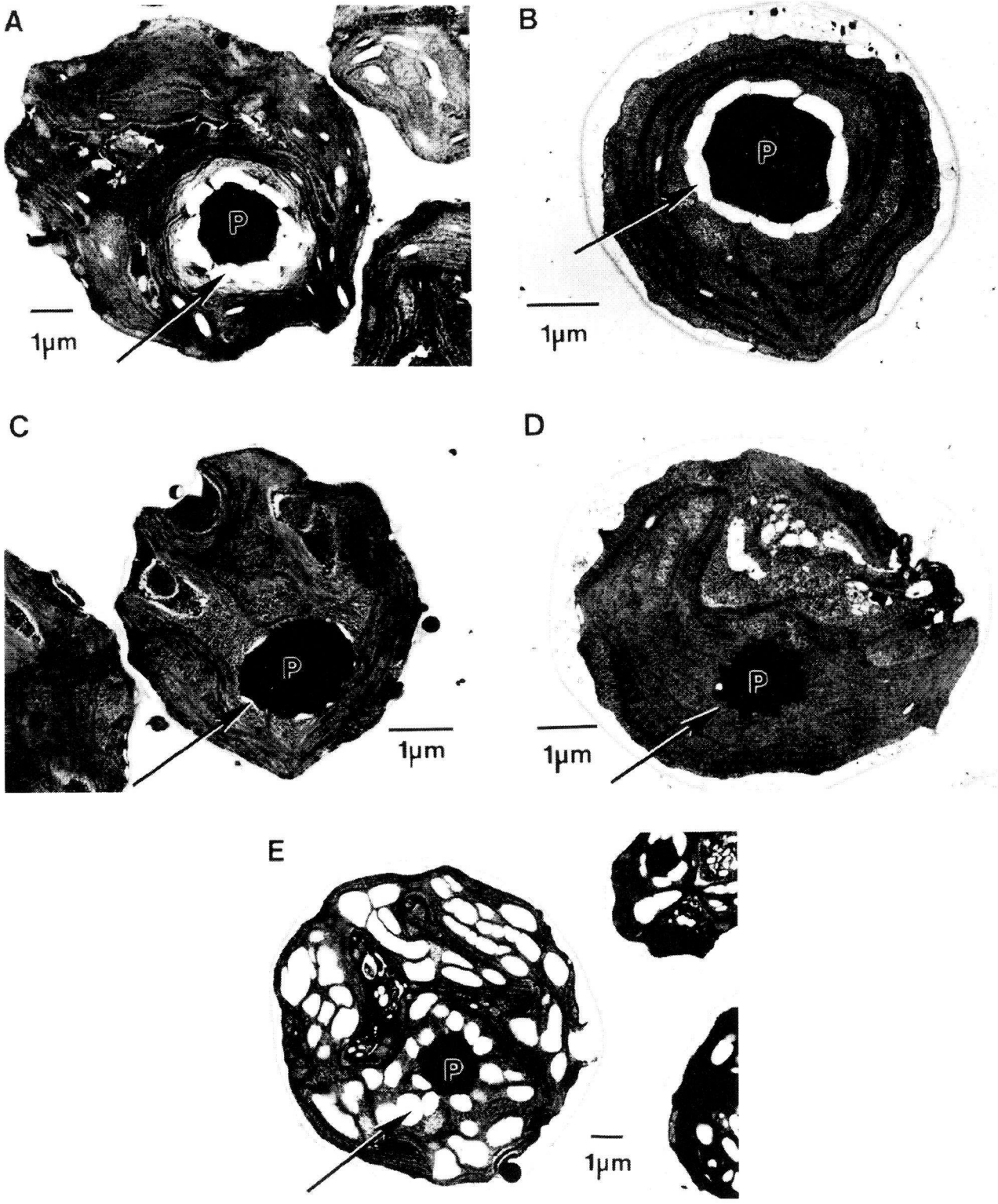

FIG. 5. – The pyrenoid morphology wild type *C. reinhardtii* cells treated with carbonic anhydrase inhibitors. The treatments are no inhibitor, 0.035% CO_2 (A); acetazolamide, 0.035% CO_2 (B); ethoxyzolamide, 0.035% CO_2 (C); no inhibitor, 5% CO_2 (D); and ethoxyzolamide, 5% CO_2 (E).

DISCUSSION

The intracellular packaging of Rubisco is very common among photosynthetic organisms that possess a CCM. In cyanobacteria Rubisco is localized in carboxysomes while in eukaryotic unicellular algae it is localized to the pyrenoid. It is thought that the carboxysome and the pyrenoid are the sites where the bicarbonate accumulated by the cell is dehydrated to CO_2 causing a localized elevated CO_2 concentration. This mechanism would allow the Rubisco to take advantage of the higher CO_2 concentration increasing the carboxylation reaction while decreasing photorespiration (Badger and Price, 1994).

The evidence supporting this hypothesis is strong in cyanobacteria. First it is clear that these organisms take up bicarbonate from the medium (Miller *et al.*, 1990; Reinhold *et al.*, 1991). Recently, a carbonic anhydrase has been localized in or at the carboxysome (Price *et al.*, 1992). In addition, the introduction of a human carbonic anhydrase into the cytoplasm causes the dehydration of bicarbonate to occur in an inappropriate location which disables the CCM (Price and Badger, 1989). Finally a number of carboxysome mutants have been discovered (Friedberg *et al.*, 1989; Lieman-Hurwitz *et al.*, 1991; Price *et al.*, 1993), including a strain mutated in the carboxysome coat protein (English *et al.*, 1994). All of these strains exhibit the high CO_2 growth requirement.

In this communication we provide evidence that the pyrenoid is playing a similar role in the eukaryotic algae. This chloroplast structure is almost ubiquitous among eukaryotic unicellular algae (Bold and Wynne, 1985). In cyanobacteria there is an increase in the number of carboxysomes when cells are placed in a low CO_2 environment (Turpin *et al.*, 1984; McKay *et al.*, 1993). In *C. reinhardtii*, there is a rapid biosynthesis of a starch sheath when the cells are placed in low CO_2 (Ramazanov *et al.*, 1994 and Fig. 2). Other algae also make a starch sheath in response to low CO_2 growth conditions (Fig. 3 and Kuchitsu *et al.*, 1988). In addition, some of the high CO_2-requiring strains of *C. reinhardtii* contain abnormal pyrenoids (Table 2 and Fig. 4). These strains are reminiscent of the cyanobacterial carboxysome mutants in which disruption of the carboxysome structure leads to a high CO_2-requiring phenotype. It appears that the correct packaging of Rubisco might be an essential component of the operation of the CCM in algae.

The data presented in this paper also suggest that there might be a link between the chloroplast carbonic anhydrase and the functioning of the pyrenoid. In two cases, mutants with defects in the chloroplast carbonic anhydrase exhibit abnormalities in their pyrenoid structure. Ca-1 has pyrenoids that are not spherical while cia-3 fails to produce a pyrenoid starch sheath (Fig. 4).

Under low CO_2 conditions, the effects of ethoxyzolamide mimic the cia-3 mutants, namely a pyrenoid starch sheath is not produced. Under high CO_2 the chloroplast deposits a lot of starch throughout the stroma when ethoxyzolamide is present.

At this point it is unclear whether the altered pyrenoid morphology observed in the high CO_2 requiring mutants is directly due to the carbonic anhydrase defect or is due to an additional mutation. We are presently addressing this question by crossing cia-3 and ca-1 with wild-type cells to determine whether there is a linkage between the chloroplast carbonic anhydrase and the pyrenoid. In addition a number of researchers are working at purifying the chloroplast carbonic anhydrase to determine whether or not it is localized to the pyrenoid. While the data presented in this manuscript are consistent with the carbonic anhydrase being localized to the pyrenoid, this important point remains to be proven.

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