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Molecular and Structural Changes in *Chlamydomonas* under Limiting CO_2[1]

A Possible Mitochondrial Role in Adaptation

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When *Chlamydomonas reinhardtii* cells are transferred to limiting CO_2, one response is the induction of a CO_2-concentrating mechanism (CCM) with components that remain to be identified. Characterization of membrane-associated proteins induced by this transfer revealed that synthesis of the 21-kD protein (LIP-21) was regulated at the level of translatable message abundance and correlated well with the induction of CCM activity. Phase partitioning of LIP-21 and the previously characterized LIP-36 showed that both appeared to be peripherally associated with membranes, which limits their potential to function as transporters of inorganic carbon. Ultrastructural changes that occur when cells are transferred to limiting CO_2 were also examined to help form a model for the CCM or other aspects of adaptation to limiting CO_2. Changes were observed in vacuolization, starch distribution, and mitochondrial location. The mitochondria relocated from within the cup of the chloroplast to between the chloroplast envelope and the plasma membrane. In addition, immunogold labeling demonstrated that LIP-21 was localized specifically to the peripheral mitochondria. These data suggest that mitochondria, although not previously incorporated into models for the CCM, may play an important role in the cell's adaptation to limiting CO_2.

During adaptation of *Chlamydomonas reinhardtii* cells to limiting CO_2 conditions, a CCM is induced (reviewed by Coleman, 1991; Kaplan et al., 1991; Badger and Price, 1992). Inorganic carbon is actively transported and accumulated within the air-adapted cells, elevating the CO_2 concentration at the site of Rubisco and allowing for increased carboxylation rates and decreased oxygenase activity of Rubisco. The mechanism by which this occurs has yet to be fully characterized. In an earlier review of the CCM, Badger (1987) proposed four essential components: (a) a pumping mechanism, (b) an energy supply, (c) a mechanism to reduce efflux of CO_2, and (d) a mechanism to provide rapid interconversion between CO_2 and HCO_3^-. Although the existence of most of these components has been clearly demonstrated, the only components that have been specifically identified are carbonic anhydrases.

De novo synthesis of several proteins coincident with the CCM has been reported. The well-characterized periplasmic carbonic anhydrase, with subunits of 4 and 37 kD (Kamo et al., 1990), is synthesized and secreted into the periplasmic space of the cells (Coleman et al., 1984), where it helps to maintain an equilibrium between CO_2 and HCO_3^-. There are two soluble proteins induced in the 44- to 52-kD range (Manuel and Moroney, 1988; Spalding and Jeffrey, 1989). There have been reports of one or two proteins induced in the 19- to 22-kD range, sometimes identified as membrane-associated (Spalding and Jeffrey, 1989) and sometimes identified as soluble (Bailly and Coleman, 1988; Manuel and Moroney, 1988). One or two membrane-associated proteins in the 35- to 36-kD range have also been identified (Spalding and Jeffrey, 1989; Geraghty et al., 1990; Ramazanov et al., 1993). The work presented here concentrates on two membrane-associated proteins, one at 21 kD and one at 36 kD, referred to as LIP-21 and LIP-36, respectively. The nature of their association with membranes was investigated to help clarify their potential for involvement in transport of inorganic carbon or other aspects of the CCM.

In a previous study (Geraghty et al., 1990), LIP-36 antibodies were used to confirm the air-specific nature of the protein and its strict association with membranes, as well as the time course of its appearance, which correlates well with the induction of the CCM. The antibodies were also used to demonstrate that regulation occurred at the level of translatable mRNA. In this work, LIP-21 has been purified and used to produce polyclonal antibodies, which were used to characterize the protein and its expression and to determine its subcellular location.

Although much attention has been devoted to physiological and molecular aspects of the adaptation of *Chlamydomonas* to limiting CO_2, there have been few reports of the structural changes occurring during adaptation. These mainly have been limited to pyrenoids and associated starch. The pyrenoid is a proteinaceous body found in the chloroplasts of most eukaryotic algae, the physiological

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Abbreviations: CCM, CO_2-concentrating mechanism; LR White, London Resin White.

function of which is not well understood. Reported responses to limiting CO_2 include an increase in development of the pyrenoid and its starch sheath in *Chlamydomonas* (Kuchitsu et al., 1988, 1991; Ramazanov et al., 1994) and other algal species (Miyachi et al., 1986; Tsuzuki et al., 1986).

Within *C. reinhardtii* cells, mitochondria have been observed to vary greatly in size, number, and shape, either throughout the cell cycle (Blank et al., 1980) or during specific phases of the cell cycle (Osafune et al., 1972, 1975; Ehara et al., 1995). Mitochondrial changes in response to changes in CO_2 have not been reported in *Chlamydomonas*, although there have been reports of mitochondrial relocation in other algal species (Kramer and Findenegg, 1978; Tsuzuki et al., 1986).

There have been few other reports of structural responses to limiting CO_2, even though such observations may aid in understanding the CCM and other aspects of the adaptation to limiting CO_2. Structural observations made here pertain to mitochondria, pyrenoids, starch distribution, and vacuoles.

MATERIALS AND METHODS

Cell Culture

Chlamydomonas reinhardtii strains 2137 mt+ (Spreitzer and Mets, 1981) and CW15 mt+ (from Dr. R. Togasaki, Indiana University, Bloomington, IN) were grown as previously described (Geraghty et al., 1990). CO_2-enriched cells were aerated with 5% CO_2 in air, and air-adapted cells were not aerated. For timed inductions, cells were grown under CO_2-enriched conditions, collected by centrifugation, resuspended in air medium, and grown under air-adapted conditions.

Cells induced for 24 h were grown for 2 d under CO_2-enriched conditions and then for 24 h under air-adapted conditions. Air-grown cells were maintained on plates for multiple generations under air conditions prior to inoculation of liquid cultures and continued growth under air-adapted conditions. Cells grown with either of these protocols are referred to as air-adapted.

Cell Fractionation, Electrophoresis, Antibodies, and RNA

Cell fractionation, electrophoresis, staining, fluorography, western blotting, and immunodetection were all as previously described (Geraghty et al., 1990). LIP-21 was purified by carbonate extraction of membranes, followed by ammonium sulfate fractionation and excision of the 21-kD band from an SDS-PAGE gel. Polyclonal antibodies were raised against LIP-21 as previously described (Geraghty et al., 1990), using approximately 25 μg of protein for both the initial immunization and the boost in each of two rabbits. RNA isolation, poly(A)$^+$ RNA purification, in vitro translation of poly(A)$^+$ RNA, and immunoprecipitation of LIP-21 and LIP-36 translation products were as previously noted (Geraghty et al., 1990).

Triton X-114 Phase Partitioning

Total membrane fractions were prepared as previously described (Geraghty et al., 1990) and extracted with either 100 mM Na_2CO_3 or 100 mM NaOH in 10 mM EDTA, 5 mM amino caproic acid, and 2 mM benzamidine for 2 h on an Adams Nutator (model 1105, Clay Adams, Becton Dickinson) at 4°C. Membranes were removed by centrifugation (Sorvall SS34 rotor, 19,000 rpm, 30 min). Proteins were then separated, based on their hydrophobic or hydrophilic nature, using the detergent Triton X-114 as described by Bordier (1981). The extracted proteins and unextracted membrane samples were solubilized in 1% (v/v) precondensed Triton X-114 in buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) at 0°C for 30 min, and the membranes were centrifuged to remove any unsolubilized material. During a 5-min incubation at 30°C to cause condensation and phase separation, hydrophilic proteins partitioned into the aqueous phase, and hydrophobic proteins partitioned into the detergent phase. After re-extraction and washing of the phases (Bordier, 1981), the proteins from each phase were precipitated overnight in 80% acetone at −20°C. The precipitate was pelleted, dried under vacuum, and resuspended for SDS-PAGE.

Fixation, Embedding, Immunolocalization, and Microscopy

Cells used for microscopy alone were embedded in Spurr's resin (Spurr, 1969), and those used for immunolocalization were embedded in L.R. White to better maintain antigenicity (Roth et al., 1978). Cells were harvested by centrifugation (5 min, 750 rpm), rinsed with phosphate buffer (100 mM sodium phosphate, pH 7.2, 4°C), and fixed with 1% glutaraldehyde and 2% paraformaldehyde in the same buffer. Samples were dehydrated in a graded ethanol series, and then either switched to acetone, embedded in Spurr's resin, and polymerized at 60°C, or embedded directly in LB White and polymerized at 50°C.

Thin sections embedded in LR White were cut on glass knives and mounted on nickel grids. Grids were then floated section side down on drops of the following solutions: 1% BSA in PBS (200 mM sodium phosphate and 150 mM NaCl, pH 7.2) for 30 min; anti-LIP-21 antiserum diluted 1:100 in PBS/BSA for 60 min; 1% BSA in PBS, four times for 3 min each time; protein A-gold diluted 1:25 in PBS/BSA for 30 min; and PBS, four times for 3 min each time (McKay et al., 1991). BSA (fraction V) was obtained from Sigma (product A-7906) and 15-nm gold particles coupled to protein A were obtained from EY Laboratories (San Mateo, CA; product GP-01–15). Grids were poststained with aqueous uranyl acetate and lead citrate for 15 min each. For Spurr's resin-embedded cells, thin sections were mounted on copper grids and stained for 1 h each in uranyl acetate and lead citrate.

Sections were viewed in a JEOL 1200EX-II scanning transmission electron microscope at 80 kV. Structural observations were based on analysis of 90 medial or near-medial cell cross-sections from six different preparations. Micrographs shown were selected as being representative of observed trends. Calculations of cross-sectional areas of

LR White-embedded cells were corrected for distortion caused by holes in the sections where the resin failed to infiltrate and preserve starch deposits.

RESULTS

LIP-21 and LIP-36 Phase Partitioning and LIP-21 Purification

During phase partitioning in Triton X-114, hydrophilic proteins are recovered in the aqueous phase, and integral membrane proteins with an amphiphilic nature are recovered from the detergent phase (Bordier, 1981). In the present study untreated membranes were subjected to phase partitioning, and LIP-21 partitioned into the aqueous phase and LIP-36 partitioned mainly into the detergent phase (Fig. 1, lanes 5 and 6). LIP-21 was easily extracted by either sodium carbonate or sodium hydroxide, and in each case the extracted LIP-21 was found to partition into the aqueous phase following phase separation (Fig. 1, lanes 8 and 11). LIP-36 was extracted only by sodium hydroxide and not by sodium carbonate (Fig. 1, lanes 7 and 10). Following the NaOH extraction, however, the extracted LIP-36 was found to partition into the aqueous phase (Fig. 1, lane 11) rather than the detergent phase, as was seen with untreated membranes. This finding was confirmed by western blot analysis (data not shown).

LIP-21 purification (described in "Materials and Methods") was based on the finding that LIP-21 was highly enriched in the carbonate extract of membranes (Fig. 1, lane 7). Purified LIP-21 was used to raise polyclonal antibodies in two rabbits, both of which produced antisera sufficiently specific for use without further purification.

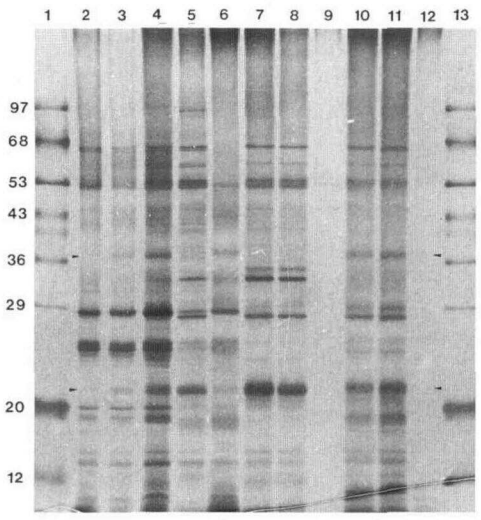


Figure 1. Triton X-114 phase separation of total membrane fraction (lanes 4–6) and sodium carbonate (lanes 7–9) and sodium hydroxide (lanes 10–12) extracts of total membrane fractions of air-adapted *C. reinhardtii* cells. Lanes 4, 7, and 10 are samples before separation. Lanes 5, 8, and 11 are polypeptides from the hydrophilic phase, and lanes 6, 9, and 12 are polypeptides from the hydrophobic phase. Total membrane fractions from CO_2-enriched cells (lane 2) and air-adapted cells (lane 3) are also shown. Lanes 1 and 13 contain molecular mass markers. Arrowheads indicate LIP-21 and LIP-36.

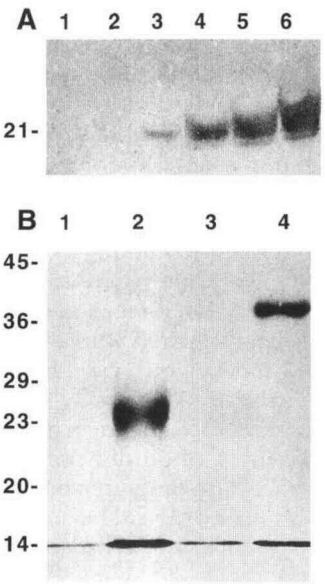


Figure 2. A, Immunoblot of SDS-PAGE of whole-cell samples from *C. reinhardtii* CO_2-enriched cells (lane 1) and CO_2-enriched cells transferred to air for 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), 8 h (lane 5), and 24 h (lane 6). B, Fluorograph of in vitro translation products immunoprecipitated with LIP-21 antiserum (lanes 1 and 2) and LIP-36 antiserum (lanes 3 and 4) from poly(A)$^+$ RNA isolated from CO_2-enriched cells (lanes 1 and 3) and air-adapted cells (lanes 2 and 4) of *C. reinhardtii*. Molecular masses are indicated on the left.

LIP-21 Expression during Adaptation

Anti-LIP-21 antiserum 1 was used in immunoblots to investigate the appearance of LIP-21 when cells were switched from CO_2-enriched conditions to normal air conditions. The immunoblot showed that, although there was no detectable LIP-21 or other cross-reacting protein present in CO_2-enriched cells, a small amount of LIP-21 was present 2 h after the cells were induced. The level continued to increase, reaching a near-maximal level by 8 h (Fig. 2A). Immunoblot results with antiserum 2 were essentially identical (data not shown).

The regulation of LIP-21 and LIP-36 (as a control) expression was investigated in induced and noninduced cells using immunoprecipitation from in vitro translation products. In each case, a specific precipitate was obtained from the in vitro translation products of the poly(A)$^+$ RNA from air-adapted cells but not from the CO_2-enriched cells (Fig. 2B). Each of the immunoprecipitated in vitro translation products appeared similar in size to its respective mature protein.

Structural Changes in Pyrenoids and Vacuoles during Adaptation

It is not clear what type of air-adapted cells are best for comparison with cells grown under CO_2-enriched conditions. One approach is to split a culture grown with enriched CO_2 and expose one of the subcultures to limiting

CO_2 to induce the adaptive response. However, transfer to limiting CO_2 is a shock to the cell's metabolism, so it may be difficult to differentiate between adaptive responses and transient changes due to shock. It is also a possibility that the sudden CO_2 limitation might cause the cells to arrest at a particular stage in the cell cycle, inducing synchrony in an otherwise nonsynchronous culture. Because of these possibilities, both cells adapting to limiting CO_2 and cells that had been grown under limiting CO_2 conditions continuously for many generations were used.

Little if any difference can be seen in pyrenoid development of CO_2-enriched compared with 24-h-induced or air-grown cells (Figs. 3 and 4), which were representative of 90 cells that were analyzed. There were significant differences, however, in starch accumulation and distribution. In air-adapted cells, only 1 of 55 did not have a complete starch sheath surrounding the pyrenoid; however, in the CO_2-enriched cells only 5 of 35 did have a complete sheath. The rest of the CO_2-enriched cells had varying amounts of pyrenoid starch, ranging from a nearly full sheath to a few grains dispersed around the periphery of the pyrenoid. Stromal starch, on the other hand, was generally abundant in CO_2-enriched cells (Figs. 3A and 4A), although results were somewhat variable. In 24-h-induced cells (Fig. 3B), there was consistently very little, if any, stromal starch. In air-grown cells (Fig. 4B), the amount of stromal starch was, as in CO_2-enriched cells, more abundant but variable.

Both 24-h-induced and air-grown cells consistently had a higher degree of vacuolization than CO_2-enriched cells (Figs. 3 and 4). The degree of vacuolization was quantified as the percentage of cross-sectional area of the cell occupied by the vacuole (Figs. 3 and 4), which were considered representative of all of the cells observed. There was approximately a 3-fold increase in vacuolization in the air-adapted cells (Table I). Furthermore, a large, central vacuole was observed in cells that had been induced for only 24 h (Fig. 3B), whereas in air-grown cells (Fig. 4B), there were several vacuoles, usually three to five, of a more

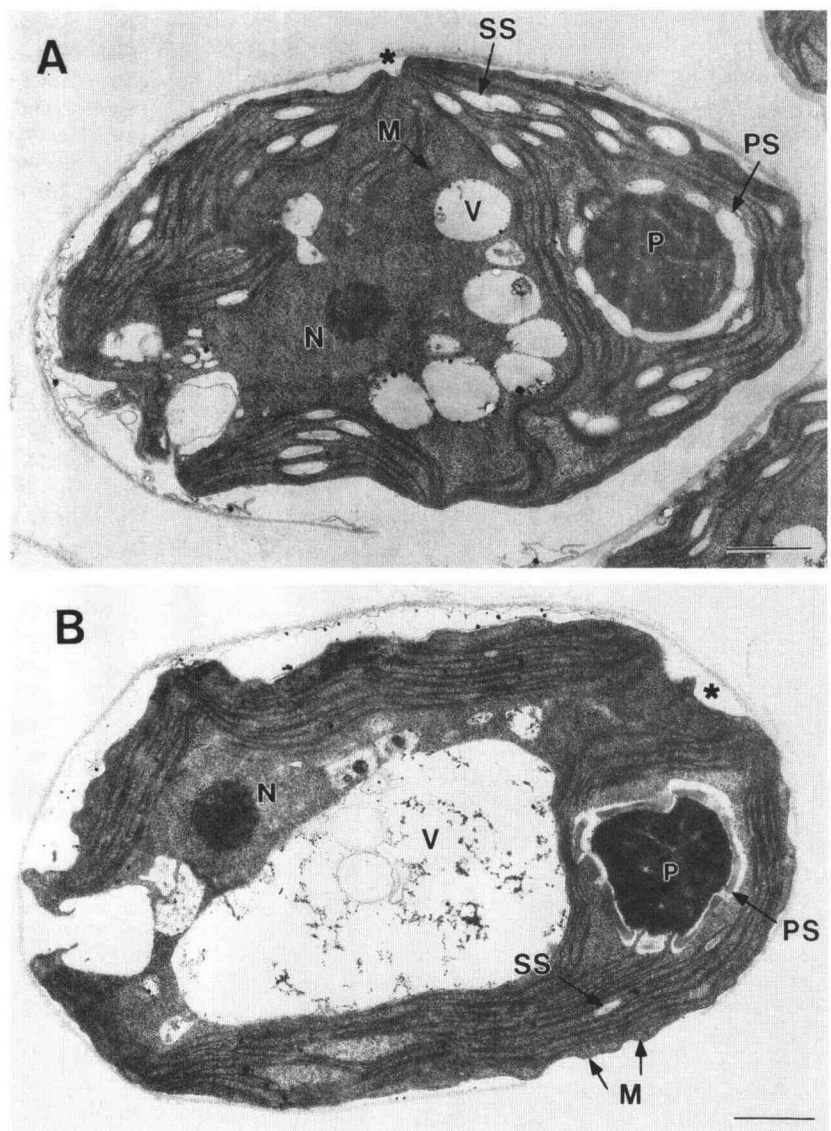


Figure 3. Cross-sections of CO_2-enriched (A) and 24-h-induced (B) *C. reinhardtii* cells embedded in Spurr's resin. M, Mitochondrion; P, pyrenoid; PS, pyrenoid starch; SS, stromal starch; V, vacuole; N, nucleus; *, chloroplast gap; bars = 1 μm.

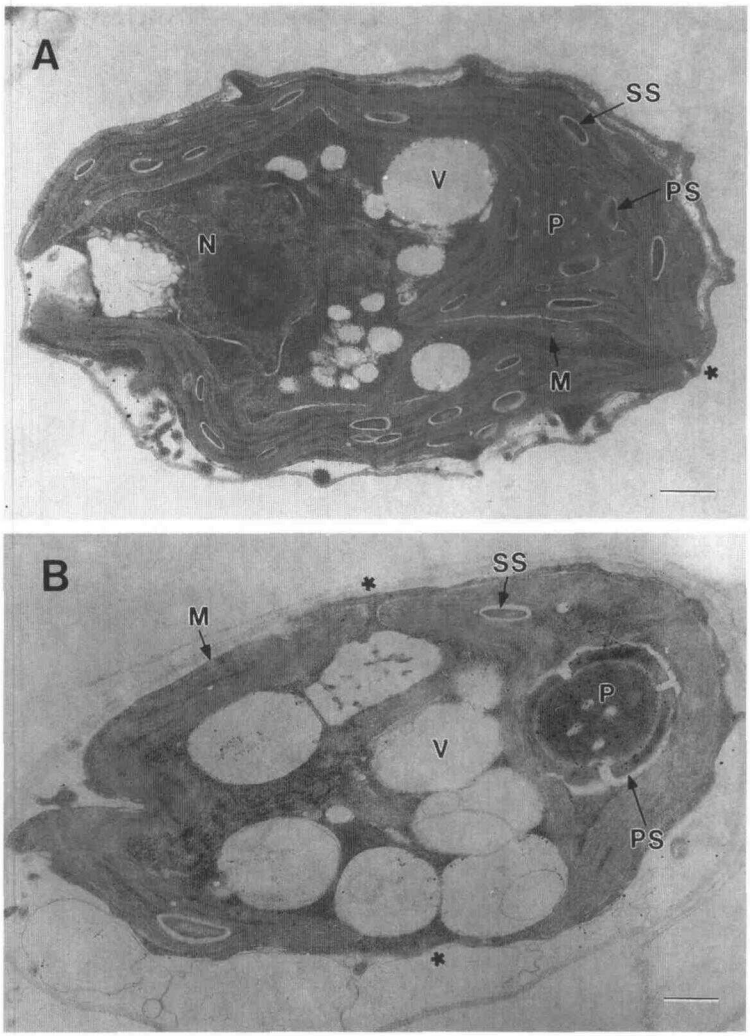


Figure 4. Cross-sections of CO_2-enriched (A) and air-grown (B) *C. reinhardtii* cells embedded in Spurr's resin. M, Mitochondrion; P, pyrenoid; PS, pyrenoid starch; SS, stromal starch; V, vacuole; N, nucleus; *, chloroplast gap; bars = 500 nm.

median size, approximately 1 to 2 μm in diameter or similar in size to the pyrenoid. The CO_2-enriched cells (Figs. 3A and 4A) tended to have smaller, more numerous vacuoles of less than 1 μm in diameter.

Mitochondrial Relocation and LIP-21 Immunolocalization

There is an apparent relocation of the mitochondria in *Chlamydomonas* during adaptation to limiting CO_2, which is retained in air-adapted cells. When cells were grown under CO_2-enriched conditions (Figs. 3A, 4A, and 5A), a single, medial cell cross-section revealed an average of five mitochondrial cross-sections located in a central position within the cup of the chloroplast, whereas only one or two there typically were found in a peripheral position between the chloroplast envelope and the plasma membrane. In air-adapted cells (Figs. 3B, 4B, and 5B), however, the opposite was found. Nearly all of the mitochondrial cross-sections were located in peripheral positions. Only in a few instances was a single mitochondrion observed in a central position. This was a consistent observation in cells that were induced for 24 h (Fig. 3B) and in cells that were air-grown (Fig. 4B). In addition, the mitochondrial cross-sections in air-adapted cells were smaller and more numerous. Medial sections of air-adapted cells showed an average of 20 mitochondrial cross-sections per cell in an external position. This could represent numerous small mitochondria or a few highly reticulated mitochondria. The apparent smaller size of the mitochondria in air-adapted cells could also be a sectioning artifact, since an oblique or longitudinal section of an elongated mitochondrion would appear larger than a cross-section; however, over the numerous

Table I. *Cell vacuolization*

Percentage of vacuolization in CO_2-enriched cells versus air-adapted cells of *C. reinhardtii* was calculated as the total percentage of cross-sectional area of a medial or near-medial section of the cell occupied by vacuoles for the cells shown in Figures 3, 4, and 6.

Figure	Cell Type	Vacuolization	Ratio of Air/CO_2
		%	
3	CO_2 enriched	8.1	3
	24 h induced	27.4	
4	CO_2 enriched	9.0	3
	Air grown	28.1	
6	CO_2 enriched	2.8	4
	Air grown	12.1	

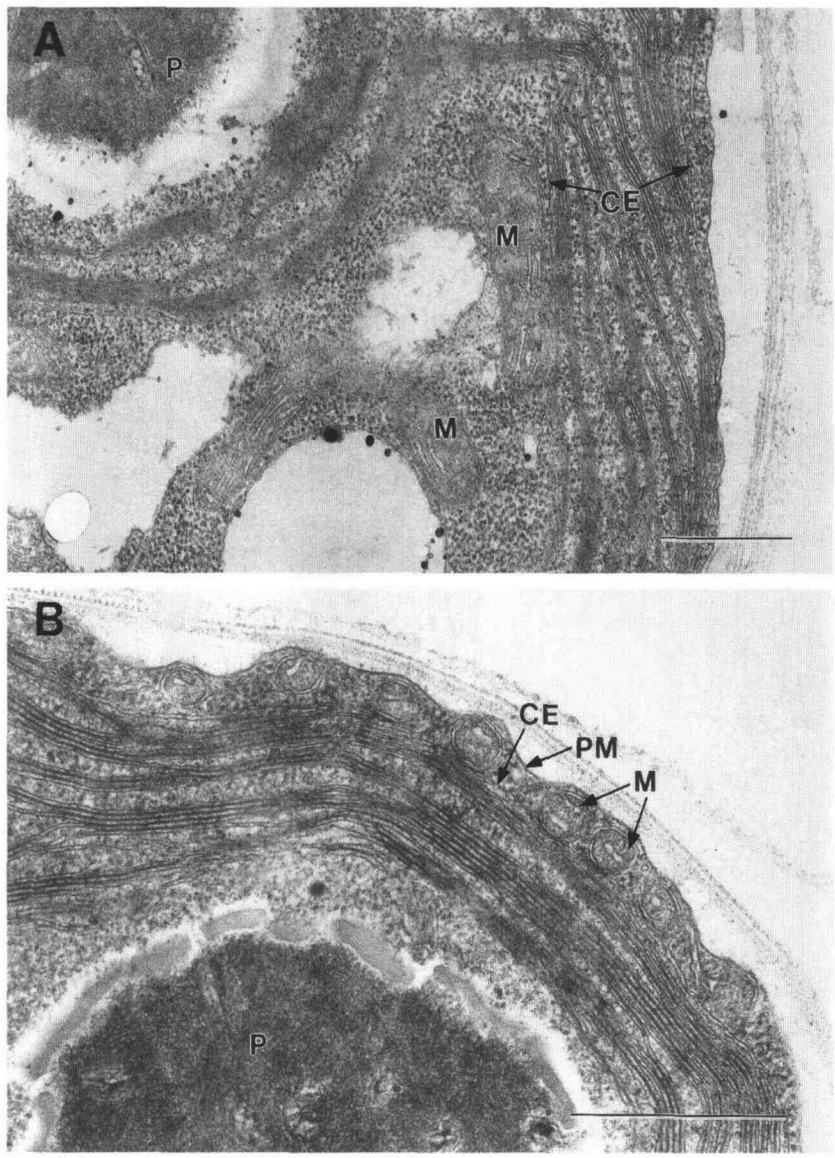


Figure 5. Mitochondrial detail within cross-sections of CO_2-enriched (A) and 24-h-induced (B) *C. reinhardtii* cells embedded in Spurr's resin. M, Mitochondrion; P, pyrenoid; CE, chloroplast envelope; PM, plasma membrane; bars = 500 nm.

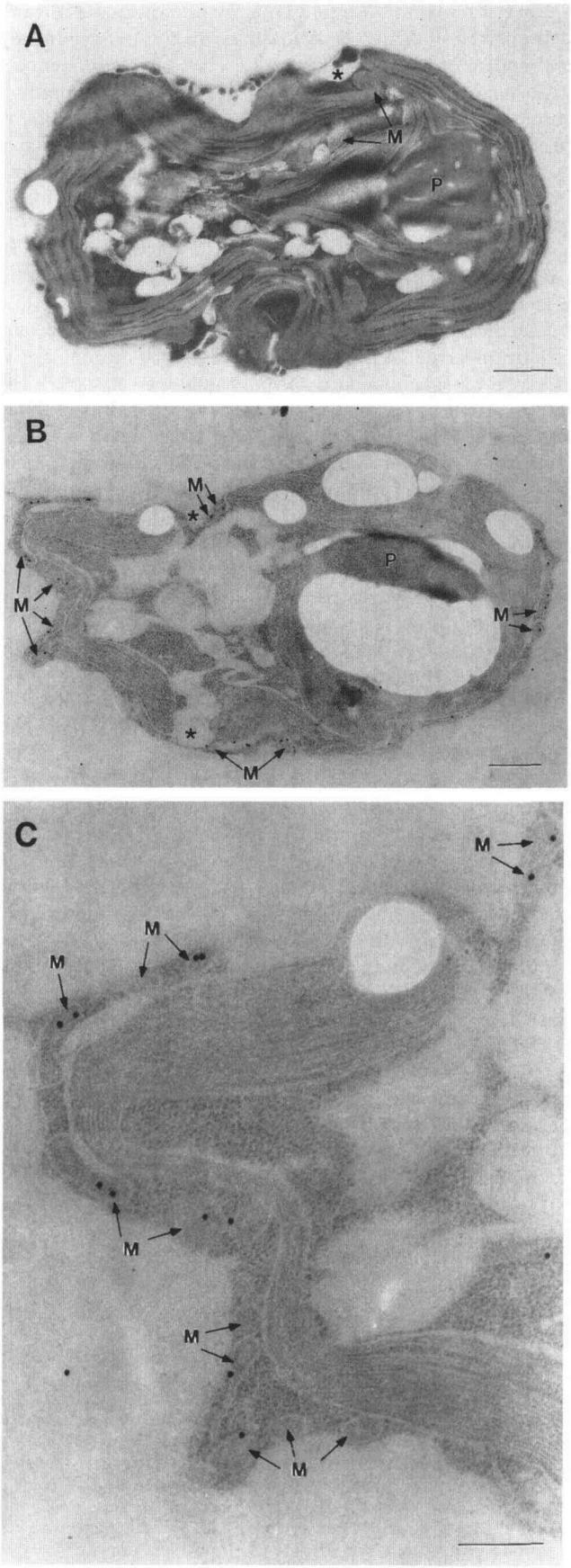
cells analyzed, the observation was consistent. Apparent gaps in the chloroplasts were present in many of the cross-sections (Figs. 3, 4, and 6), and mitochondria were occasionally observed within these gaps (Fig. 6).

Each of the two antiserum preparations raised against LIP-21 reacted specifically with the peripherally relocated mitochondria of air-grown *C. reinhardtii*. Antiserum 2 reacted only with the peripheral mitochondria (Fig. 6). Antiserum 1 also reacted with the peripheral mitochondria, but additional gold particles were observed over the pyrenoid region in both air-grown and CO_2-enriched cells (data not shown). However, when preimmune serum was used, the labeling over the pyrenoid was also observed (data not shown). The pyrenoid labeling, therefore, is not attributable to the LIP-21 antibodies. No labeling of mitochondria was observed with preimmune serum, nor was there any labeling of central or peripheral mitochondria by either antiserum in CO_2-enriched cells. Therefore, immunogold labeling using anti-LIP-21 antibodies is specific to the relocated peripheral mitochondria of air-grown cells.

DISCUSSION

To assess potential roles for the induced LIP-21 and LIP-36 proteins in adaptation to limiting CO_2, a phase separation was done to determine the nature of their association with membranes. LIP-21 was extracted easily from membranes and had an overall hydrophilic nature and, therefore, is not likely to be an integral membrane protein. It is probably a peripheral membrane protein, although possibly LIP-21 is not strictly associated with membranes in vivo. It might adhere to membranes only during cell fractionation, or it could associate and dissociate with a membrane complex, depending on the state of the cell.

Extraction of LIP-36 from membranes required sodium hydroxide; it was not removed by either carbonate or



cholate (data not shown). LIP-36 from untreated membranes partitions with the hydrophobic proteins, but following strong alkaline treatment, it partitions with the hydrophilic proteins. One possibility is that LIP-36 has a hydrophobic posttranslational modification, such as acylation, that is labile to strong alkaline treatment. Another possible explanation is that LIP-36 is a hydrophilic component of a hydrophobic protein complex that resists dissociation except under strong alkaline conditions. In either case, it seems likely that LIP-36 is also a peripherally associated membrane protein.

Neither LIP-21 nor LIP-36 seems to be an integral membrane protein that could span the membrane as a transporter; however, this does not preclude their involvement in inorganic carbon transport or other aspects of the CCM. LIP-21 and LIP-36 could still be peripheral components of a transport complex in the membrane, where they could be involved in the conversion of inorganic carbon, or in the regulation or energy supply for CCM activity.

Although LIP-21 was tentatively identified as an induced polypeptide in ^{35}S-labeling experiments, it has been difficult to unambiguously ascertain the presence or absence of LIP-21 in stained gels because of similarly sized constitutive proteins. The immunoblots presented here show that LIP-21 is synthesized de novo upon induction of the CCM. Its appearance correlates well with the time course of induction of periplasmic carbonic anhydrase (Fukuzawa et al., 1990), LIP-36 (Geraghty et al., 1990), and CCM activity (Spalding and Jeffrey, 1989) in *Chlamydomonas*, which would be consistent with LIP-21 involvement with the CCM.

Precipitation of an in vitro translation product from air-adapted cells but not CO_2-enriched cells shows that expression of LIP-21 is regulated by translatable mRNA abundance. Both the limiting CO_2-induced periplasmic carbonic anhydrase (Fujiwara et al., 1990; Fukuzawa et al., 1990) and LIP-36 (Geraghty et al., 1990) are regulated at the level of transcript abundance, which allows for the possibility that one signal may be responsible for transcriptional regulation of all three proteins.

In addition to changes occurring at the molecular level, observation of changes occurring at the ultrastructural level may also contribute to our understanding of the cell's adaptation to limiting CO_2. The presence of a well-developed pyrenoid with a starch sheath has been correlated with the growth of cells under normal air conditions, but the pyrenoid has been reported to be less developed or absent in cells grown with enriched CO_2, both at the level of light microscopy with *Chlamydomonas* (Kuchitsu et al., 1988) and at the level of electron microscopy with other algal species (Miyachi et al., 1986; Tsuzuki et al., 1986). Contrary to these published results, however, little if any difference was seen in the pyrenoid development of CO_2-

Figure 6. Cross-sections of CO_2-enriched (A) and air-grown (B and C) *C. reinhardtii* cells embedded in LR White and immunogold labeled using anti-LIP-21 antiserum 2. Mitochondrial detail (C) of air-grown cell (B) is shown. M, Mitochondrion; P, pyrenoid; *, chloroplast gap; bars = 500 nm (A and B) and 200 nm (C).

enriched versus air-adapted cells. There is not even a transient change in 24-h-induced cells that could be attributed to the cell cycle. Since the pyrenoid is primarily composed of Rubisco (Kuchitsu et al., 1988), and there is no significant change in the amount of Rubisco between air-adapted and CO_2-enriched cells, pyrenoid development should not be expected. It is not clear why the other reports differ; some used different species, and the observations of Kuchitsu et al. (1988) may have been based on changes in the starch sheath rather than the pyrenoid.

The increased pyrenoid starch sheath in air-adapted cells is consistent with previous reports in *C. reinhardtii* and other algae (Miyachi et al., 1986; Tsuzuki et al., 1986; Kuchitsu et al., 1988; Ramazanov et al., 1994). Observed variability in stromal starch abundance is also compatible with previous observations (Miyachi et al., 1986; Kuchitsu et al., 1988). The decrease in stromal starch during adaptation probably results from the depletion of starch reserves as the cell becomes limited for carbon, although it could also be attributed to the cell cycle if synchrony was induced. Either explanation would be consistent with the observed recovery of high but variable stromal starch in nonsynchronous, air-adapted cells.

The large, central vacuole observed in 24-h-induced cells probably cannot be attributed to a cell cycle effect, since it was never seen in the nonsynchronous CO_2-enriched cultures. It appears more characteristic of 24-h-induced cells than of air-grown cells, but both cell types were more highly vacuolated than the CO_2-enriched cells. The increased vacuolization could be attributed to any of the large changes in the cell's metabolism brought on by transfer to limiting CO_2.

The most intriguing structural change observed was the change in mitochondrial distribution in air-adapted cells. Following transfer to limiting CO_2 conditions, the mitochondria move from a central position in the cell within the cup of the chloroplast to a peripheral position between the chloroplast envelope and the plasma membrane. These observations were consistent in both 24-h-induced cells and in nonsynchronous, air-grown cells and, therefore, are not related to the cell cycle. Although such observations have not been previously reported in *Chlamydomonas*, Kramer and Findenegg (1978) reported a similar relocation of mitochondria and a 4-fold increase in the apparent number of mitochondria in air-adapted *Scenedesmus obliquus* cells. Miyachi et al. (1986) found no such increase in the number of mitochondria in *S. obliquus*, and Tsuzuki et al. (1986) reported the opposite relocation of mitochondria in *Dunaliella tertiolecta*.

As for the migration route, a cup-shaped chloroplast extending along the periphery from the basal end nearly to the apical end of the cell, if continuous, would necessitate migration through the apical end of the cell. Kramer and Findenegg (1978) suggested that *S. obliquus* mitochondria might move between "branches" of the chloroplast. The *Chlamydomonas* chloroplast also appears to have many such gaps through which mitochondria could migrate and, in fact, mitochondria were often observed in these gaps.

Data from immunogold-labeling experiments clearly demonstrate that LIP-21 is localized to the peripheral mitochondria of air-adapted cells. Resolution was inadequate to localize LIP-21 specifically to the inner or outer mitochondrial membranes. However, since it is a peripheral protein only loosely associated with membranes, as discussed above, in vivo it may not be strictly associated with membranes and may function within the matrix or in the intermembrane space.

The functional implications of the mitochondrial relocation are speculative. Kramer and Findenegg (1978) hypothesized that the mitochondrial changes in *S. obliquus* could reflect an increase in glycolate metabolism. *Chlamydomonas* cells transferred to limiting CO_2 experience an increase in ribulose-1,5-bisphosphate oxygenase activity, which initially results in glycolate excretion from the cells (Kaplan and Berry, 1981). Induction of CCM activity and up-regulation of photorespiratory enzymes (Nelson and Tolbert, 1969; Marek and Spalding, 1991) eliminates any detectable glycolate excretion, although glycolate pathway activity remains somewhat higher than in the cells growing in 5% CO_2. Since glycolate dehydrogenase (Beezley et al., 1976) and the subsequent enzymes of the photorespiratory pathway are found in the mitochondria of *C. reinhardtii* and *S. obliquus*, the peripheral positioning of mitochondria could serve to scavenge glycolate.

It is also possible that the mitochondria are involved in the supply of energy for active transport of inorganic carbon and that they are moved closer to the transporters. The phosphorylating pathway (respiration) could be involved directly in ATP synthesis, or the nonphosphorylating pathway (alternative respiration) could be involved indirectly in oxidizing excess reductant resulting from the export of ATP from the chloroplast via a phosphoglyceraldehyde-triose phosphate shuttle. In support of a possible role for the alternative pathway, Goyal and Tolbert (1989, 1990) reported salicylhydroxamic acid inhibition of inorganic carbon accumulation and a higher level of alternative oxidase activity in air-adapted cells.

The localization of the induced protein LIP-21 in the relocated mitochondria supports a role for mitochondria in the cell's adaptation to limiting CO_2. Further studies of LIP-21, including the cloning of its gene and determination of its function, should help to assess the possible role of the mitochondria in the *Chlamydomonas* CCM.

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