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Communication

A New Chloroplast Protein Is Induced by Growth on Low CO_2 in *Chlamydomonas reinhardtii*[1]

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ABSTRACT

The biosynthesis of a 36 kilodalton polypeptide of *Chlamydomonas reinhardtii* was induced by photoautotrophic growth on low CO_2. Fractionation studies using the cell-wall-deficient strain of *C. reinhardtii*, CC-400, showed that this polypeptide was different from the low CO_2-induced periplasmic carbonic anhydrase. In addition, the 36 kilodalton polypeptide was found to be localized in intact chloroplasts isolated from low CO_2-adapting cultures. This protein may, in part, account for the different inorganic carbon uptake characteristics observed in chloroplasts isolated from high and low CO_2-grown *C. reinhardtii* cells.

The unicellular green alga *Chlamydomonas reinhardtii* can grow photoautotrophically at very low CO_2 concentrations due to its ability to concentrate C_i[2], internally to levels much higher than could be obtained by diffusion (2). This ability to concentrate C_i is a major photosynthetic adaptation that is seen in many unicellular algae, both eukaryotic and prokaryotic (1). However, the mechanism by which unicellular algae concentrate C_i is still poorly characterized.

In *C. reinhardtii*, the CO_2 concentrating mechanism is inducible, in that if the alga is grown on elevated CO_2 (1% [v/v] in air or higher), it exhibits a relatively low affinity for external C_i (2, 16). If, however, the alga is grown on low CO_2 concentrations, it acquires a very high affinity for C_i and has an extremely low CO_2 compensation point (2, 16). During adaptation to low CO_2, a number of proteins are preferentially made (3, 4, 10, 15). This induction requires both light and low CO_2 to be complete and involves an increase in transcription (19). To date, only the periplasmic carbonic anhydrase has been identified and characterized (1, 5, 19). Previous work has implied that a fundamental change in the C_i uptake characteristics of the chloroplast also occurs, as chloroplasts isolated from low CO_2 grown cells accumulate C_i to a greater extent than chloroplasts isolated from high CO_2 grown cells (12, 18). In this communication we describe an additional low CO_2 induced protein that is located within the chloroplast.

MATERIALS AND METHODS

Algal Strains and Culture Conditions

The wild-type strain of *Chlamydomonas reinhardtii*, 137 *mt*⁺, has been maintained in R. K. Togasaki's laboratory. The cell-wall-deficient mutant, CC-400 *cw-15 mt*⁺, was obtained from the Duke University *Chlamydomonas* Culture Collection. In liquid culture, the strains were grown in minimal medium (17), aerated with 5% CO_2 in air, and illuminated with 300 $\mu E\ m^{-2}\ s^{-1}$ of white light. For $^{35}SO_4{}^{-2}$ labeling, the algal cells were switched to minimal medium with one-tenth the normal amount of sulfur 48 h prior to the experiment.

^{35}S Labeling of Wild Type and CC-400 Cells

The labeling procedures used are similar to those previously published (10). In brief, harvested cells were resuspended in minimal medium lacking sulfate (Min-S), pelleted again by centrifugation, resuspended in Min-S (3 mL), and the Chl concentration was determined. The cells were adjusted to 25 μg Chl/mL and divided into six 150-mL flasks. Three flasks were bubbled with air and three with air supplemented with 5% CO_2. After a 30 min adaptation to the low and high CO_2 regimes, the cultures were labeled with carrier-free $^{35}SO_4{}^{-2}$ (1000 Ci/mmol) in the light (3 h for wild-type cells and 4 h for CC-400).

Cell Fractionation

All steps were carried out at 0 to 4°C. After incubating the cells for the appropriate time, triplicate samples were combined, and 100 mL of cells were withdrawn from each treatment (air *versus* CO_2). The cells were harvested by centrifugation, washed twice with 50 mL ice-cold 20 mM Tris-HCl (7.5), resuspended in 250 μL of 20 mM Tris-HCl (pH 7.5), quick-frozen, and stored at −20°C.

Cells from the remaining 350 mL of each sample were collected by centrifugation, and the culture supernatant was brought to 70% saturation with $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ precipitates were collected by centrifugation, and the pellets were taken up in 1 mL 20 mM Tris-HCl (pH 7.5) and dialyzed overnight against 20 mM Tris-HCl (7.5). The resulting dialy-

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[2] Abbreviations: C_i, inorganic carbon; low CO_2, air containing ambient (350 ppm) CO_2; high CO_2, air supplemented with CO_2 so that the final CO_2 concentration is 5% (v/v); Min medium, the phosphate buffered medium described by Sueoka (17) that contains no carbon source other than CO_2.

sate (extracellular protein fraction) was quick-frozen and stored at −20°C.

The pelleted cells from the 350 mL harvest were washed twice with 100 mL ice-cold 20 mM Tris-HCl (pH 7.5) and recollected by centrifugation. The pellets were resuspended in 12 mL cold fractionation buffer (20 mM Tris-HCl [pH 7.5], 150 mM sucrose, 50 mM NaCl, 0.4 mM benzamidine, and 0.4 mM aminocaproic acid), and passed two times through a cell disruption bomb (PARR 4639) at 1800 psi. Unbroken cells were pelleted by centrifugation in a Sorvall Hb-4 rotor for 2 min at 670*g* (2000 rpm) and discarded. The supernatant was centrifuged at 6800*g* for 10 min and the pellet was taken up in 250 μL 20 mM Tris-HCl and quick-frozen (low speed pellet). The supernatant was then centrifuged for 2 h at 156,000*g*. The pellet was resuspended up in 250 μL 20 mM Tris-HCl (pH 7.5) and quick-frozen (high speed pellet). The supernatant was brought up to 70% saturation with $(NH_4)_2SO_4$, collected by centrifugation and the pellet was resuspended in 1 mL 20 mM Tris-HCl (pH 7.5) and dialyzed overnight (intracellular soluble protein fraction).

^{35}S-Labeling of Intact Chloroplasts

Chlamydomonas strain CC-400 cultures were grown in Min medium (3.0 L) on 12 h light/12 h dark regime to synchronize growth. Cells switched to minimal one-tenth sulfur media were harvested in the middle of the third light period by centrifugation, and the pellets were washed two times in 100 mL of 20 mM Hepes-KOH (pH 7.5) and were resuspended in 5 mL breaking buffer (300 mM sorbitol, 50 mM Hepes-KOH [pH 7.2], 2 mM Na-EDTA, 1 mM $MgCl_2$, 1% BSA). The cell number was adjusted to 5×10^7 cells/mL and 20 mL aliquots were passed once through a cell disruption bomb (Parr 4639) at a pressure of 500 psi. The lysate was centrifuged in a Sorvall Hb-4 rotor for 2 min at 760*g* (2000 rpm) to pellet whole cells and intact chloroplasts. This pellet was resuspended in 2 mL breaking buffer and layered onto discontinuous Percoll gradients (20, 40, 60% Percoll) prepared according to Price and Reardon (14). A 15 min centrifugation of the gradients was carried out in a Sorvall Hb-4 rotor at 4200*g*. The 40 to 60% interface was collected and diluted fourfold with breaking buffer. Intact chloroplasts were collected by centrifugation at 670*g* for 1 min, and resuspended in 250 μL of 50 mM Hepes-KOH (pH 8.0), 0.3 M sorbitol. These intact chloroplasts retained galactosyltransferase activity and had similar physical and photosynthetic properties as *C. reinhardtii* chloroplasts previously reported (6, 9, 11, 12, 18).

Other Methods

Carbonic anhydrase was assayed as previously described (8). Chl concentrations were determined spectrophotometrically. Proteins estimations and SDS-PAGE was performed as previously described (10). The immunoblot assay was performed according to the protocol from Bio-Rad Laboratories except that 5% nonfat dry milk was used to block the nitrocellulose.

Materials

Goat anti-rabbit IgG(H+L) Horseradish Peroxidase conjugate and HRP color development reagent were purchased from Bio-Rad Laboratories. Carrier-free $H_2{}^{35}SO_4$ was obtained from ICN.

RESULTS

Chlamydomonas reinhardtii synthesizes at least four polypeptides preferentially when grown under limiting CO_2 (4, 10, 15). One of the labeled polypeptides had a molecular mass of 36 kD, similar to that of the 37 kD periplasmic carbonic anhydrase (5, 19). However, when fractionation studies were done with ^{35}S-labeled *C. reinhardtii* cells, the labeled 36 kD protein was found predominantly in the membrane fraction while the carbonic anhydrase activity was found mostly in the soluble protein fraction. Spalding and Jeffrey (15) had done fractionation studies on *cw-15 mt*$^+$ cells and reported membrane-associated proteins with molecular masses of 35 and 36 kD. This cell-wall deficient strain excretes the periplasmic carbonic anhydrase into the medium (5). We performed cell fractionation experiments on CC-400 cells (*cw-15 mt*$^+$) and recovered both the periplasmic carbonic anhydrase from the cell supernatant and isolated membrane fractions. Labeled proteins of about 36 kD were present in both the extracellular protein fraction (Fig. 1, A and B, lanes 9 and 10), and the low speed membrane fraction (Fig. 1, A and B, lanes 1 and 2). However, only the extracellular protein was recognized by the antibody raised against the periplasmic carbonic anhydrase indicating that the membrane-associated, ^{35}S-labeled band was distinct from the periplasmic carbonic anhydrase (Fig. 1C). That this polypeptide is distinct from the periplasmic carbonic anhydrase is further supported by the observation that greater than 95% of the cells carbonic anhydrase activity is in the extracellular protein fraction (1316 units mg Chl^{-1}) from CC-400 cultures, and less than 1% of the activity was in the low speed pellet (5 units mg Chl^{-1}). Hence, at least five distinct proteins (indicated with asterisks in Fig. 1B), including a 36 kD polypeptide that is membrane bound, are specifically induced in a low CO_2 environment.

We further localized this protein by isolating intact chloroplasts from both low and high CO_2-grown CC-400 cells. These cells were grown synchronously in Min ⅒ S medium for 2 d on high CO_2, and then labeled for 4 h on low or high CO_2 before isolating chloroplasts. Figure 2 shows that the 36 kD low-CO_2-induced protein is only present in isolated chloroplasts from the low-CO_2-adapted cultures. The soluble proteins induced by photoautotraphic growth on low CO_2 are not present in these chloroplast preparations (Fig. 2). Furthermore, an immunoblot of chloroplast proteins confirms the absence of the periplasmic carbonic anhydrase in these preparations (data not shown) and indicates that the 36 kD chloroplast protein is distinct from the periplasmic carbonic anhydrase. These data indicate that there is a low-CO_2-induced protein present on intact chloroplasts that may play a role in the ability of low-CO_2-grown cells to accumulate inorganic carbon.

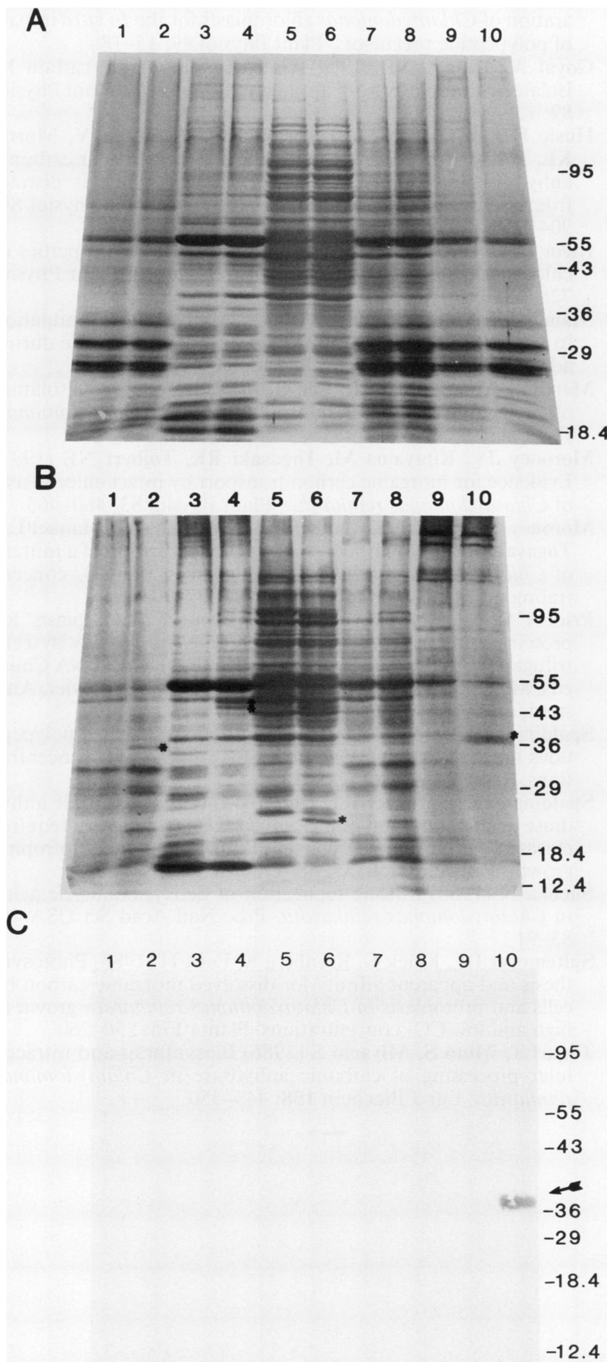

Figure 1. ^{35}S-Labeled protein and immunoblot analysis of protein fractions from CC-400 cells. The labeled cells were (100 μg/lane) subjected to SDS-PAGE, stained with Coomassie blue (A), subjected to autoradiography (B), or probed with an antibody raised against the periplasmic CA (C). In lanes 1, 3, 5, 7, and 9 cells were grown on high CO_2, whiie lanes 2, 4, 6, 8, and 10 were from cells grown on low CO_2. Lanes 1 and 2, low speed membrane fraction; lanes 3 and 4, high speed membrane fraction; lanes 5 and 6, intracellular soluble protein fraction; lanes 7 and 8, total cellular protein fraction; lanes 9 and 10, extracellular protein fraction. The stained proteins seen in lanes 9 and 10 in A also include proteins from cells that have lysed during the initial harvest. These lysed cells do not incorporate ^{35}S, however (B).

DISCUSSION

Chlamydomonas reinhardtii possesses a CO_2 concentrating mechanism that involves at least five inducible proteins. These proteins include the periplasmic carbonic anhydrase (5, 19; Fig. 1, B and C, lanes 9 and 10), and soluble proteins with molecular masses of 46 and 44 kD (4, 10, 15; Fig. 1B, lanes 5, 6, 7, and 8). In addition, Spalding and Jeffrey (15) reported on low CO_2-induced membrane associated proteins with molecular masses of 36 and 21 kD which we also observed (lanes 1 and 2, Fig. 1B). Since the periplasmic carbonic anhydrase is cell wall associated, we fractionated a wild-type strain and a cell wall deficient strain of *C. reinhardtii* to determine whether the membrane associated 36 kD polypeptide was different than the periplasmic carbonic anhydrase. Carbonic anhydrase activity measurements indicated that the periplasmic carbonic anhydrase was largely in the soluble protein fraction while the ^{35}S labeled 36 kD protein was primarily in the low speed pellet. The low CO_2-induced 36 kD protein was also shown to be distinct from the 37 kD periplasmic carbonic anhydrase by immunoblots analysis of fractions from the CC-400 cells (Fig. 1C).

We have also shown that this 36 kD polypeptide is located in intact chloroplasts (Fig. 2), while the other proteins induced by growth on low CO_2 are not. Intact chloroplasts from *C. reinhardtii* have been isolated and partially characterized by a number of laboratories (6, 9, 11, 12, 18). The chloroplast preparations reported here are free of intact cells, nuclei, and other internal organelles as judged by electron microscopy. An intact plasma membrane is not present since the chloroplasts are permeable to P_i and DHAP. We cannot rule out the possibility of some contamination with plasma membrane fragments. In addition, these chloroplasts do retain the stromal enzymes rubisco (9, 11, 12) and carbonic anhydrase

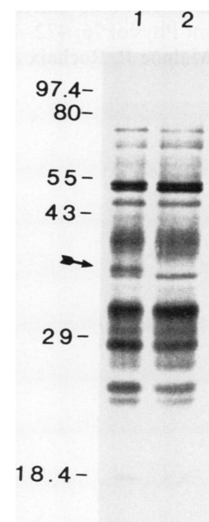

Figure 2. ^{35}S-Labeled polypeptides of intact chloroplasts fractionated on Percoll gradients. Intact chloroplasts (100 μg protein) isolated from ^{35}S-labeled cells were subjected to SDS-PAGE and autoradiography. Lane 1, intact chloroplasts isolated from low-CO_2 grown cells; lane 2, intact chloroplasts isolated from high-CO_2 grown cells.

(8), and the envelope enzyme galactosyl transferase (11). The fact that the stromal enzymes are retained while the low CO_2 induced soluble polypeptides are lost supports the contention that those soluble proteins are not chloroplast localized.

The role of the 36 kD polypeptide is still unclear although the observation that its biosynthesis is induced along with the periplasmic carbonic anhydrase implies that it is important in CO_2 acquisition. In addition, this protein is not synthesized by the high-CO_2-requiring mutant *CIA-5* (13). Moroney *et al.* (12) and Sültemeyer *et al.* (18) have demonstrated that chloroplasts isolated from low CO_2-grown cells of *C. reinhardtii* have the ability to accumulate C_i to higher levels than chloroplasts from high CO_2-grown cells. Goyal and Tolbert (7) have made the same observation with chloroplasts from *Dunaliella*. The 36 kD polypeptide is the first report of a low-CO_2-inducible chloroplast protein. This protein may, in part, be responsible for the physiological differences in C_i uptake observed between chloroplasts isolated from low and high-CO_2-grown cells.

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