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The Regulation of Carbonic Anhydrase and Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Activase by Light and CO_2 in *Chlamydomonas reinhardtii*[1]

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We have investigated the regulation of accumulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activase and the periplasmic carbonic anhydrase (CA) in *Chlamydomonas reinhardtii*. In algae, the periplasmic CA is required for efficient CO_2 fixation when the CO_2 concentration is low. These two proteins are affected differently by the CO_2 level in the environment. The steady-state level of the ribulose-1,5-bisphosphate carboxylase/oxygenase activase transcript was only slightly and transiently affected by a reduction in ambient CO_2 concentration, whereas the CA transcript level was strongly induced by air containing ambient (350 parts per million) CO_2 (low CO_2) conditions. The transcripts for both proteins showed strong oscillations when the alga was grown under a 12-h light/12-h dark growth regime, with the transcripts encoding these proteins present just before the onset of the light cycle. The observation that the CA transcript was made in the dark was surprising, since earlier reports indicated that active photosynthesis was required for the induction of the periplasmic CA. Further experiments demonstrated that the CA transcript was partially induced under low-CO_2 conditions even when the switch to low CO_2 was done in the dark. Our results suggest that *C. reinhardtii* might sense the CO_2 concentration in a more direct manner than through C_2 or C_3 cycle intermediates, which has been previously suggested.

In higher plants, a number of proteins are required for growth on low CO_2. These proteins include Rubisco activase, the enzymes of the C_2 or photorespiratory cycle, and enzymes involved in nitrogen assimilation. In addition to these proteins, unicellular algae also have a CO_2-concentrating mechanism that overcomes the slow diffusion of CO_2 in the aqueous environment under low-CO_2 conditions. The CO_2-concentrating mechanism in *Chlamydomonas reinhardtii* is influenced by the level of CO_2 in the environment (Badger et al., 1980; Aizawa and Miyachi, 1986). *C. reinhardtii* cells that are grown under high-CO_2 conditions have an apparent affinity for CO_2 similar to that of C_3 plants, requiring about 20 to 30 μM CO_2 for maximal rates of photosynthesis. However, when algal cells are placed in low CO_2, their apparent affinity for CO_2 increases and only 1 to 2 μM CO_2 is required for high rates of photosynthesis.

The induction of the CO_2-concentrating mechanism results in the synthesis of at least six proteins (Coleman et al., 1984; Manuel and Moroney, 1988). One component of the CO_2-concentrating mechanism that has been conclusively identified is a CA localized to the periplasmic space in *C. reinhardtii* and many other algae (Coleman et al., 1984). This protein is encoded by the *Cah*1 gene (Fujiwara et al., 1990), and it is strongly induced when the alga is grown on low-CO_2 conditions (Fukuzawa et al., 1990). The induction of CA is regulated at the transcriptional level (Toguri et al., 1984; Fukuzawa et al., 1990). The RNA transcript is present 1 h after transfer to low-CO_2 conditions, and it slowly increases in amount until 6 h, when it starts to decrease (Bailly and Coleman, 1988; Fujiwara et al., 1990). The effect of light on the induction and repression of *Cah*1 transcript has also been examined in *Chlamydomonas*. When high-CO_2-grown cells are transferred to low CO_2 in the dark, the 37-kD CA protein as well as the RNA transcript is not induced. Addition of DCMU, an electron transport inhibitor, to low-CO_2 cells immediately after their transfer from high-CO_2 conditions also inhibits the induction in protein levels and transcript levels of CA, implying that photosynthesis may be needed for induction to occur (Fukuzawa et al., 1990). The need for blue light (460 nm) has been demonstrated (Dionisio et al., 1989). *Chlamydomonas* cells illuminated with red light (620–680 nm) alone during low-CO_2 adaption did not show induction of the protein and transcript levels of CA, but when blue light (460 nm) was also used to illuminate the cells along with the red light, the induction of CA protein levels did occur (Dionisio et al., 1989).

The expression of Rubisco activase in organisms with a CO_2-concentrating mechanism has not been extensively studied. Rubisco activase was first identified in *Arabidopsis thaliana* as the enzyme missing in a high-CO_2-requiring *rca* mutant (Somerville et al., 1982). Since then, it has been shown that Rubisco activase promotes the activation of Rubisco in the presence of ribulose bisphosphate, as well as other inhibitory sugar phosphates in higher plants at atmospheric CO_2 concentrations (Portis et al., 1986; Robinson and Portis, 1989b). The exact mechanism by which Rubisco activase activates Rubisco is not known, although two nucleotide-binding domains have been identified in the nucleotide sequences of Rubisco activase genes from higher

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Abbreviations: CA, carbonic anhydrase; C_i, inorganic carbon; high CO_2, air supplemented with CO_2 so that the final CO_2 concentration is 5% (v/v); low CO_2, air containing ambient (350 ppm) CO_2.

plants. Rubisco activase is also known to have ATPase activity (Streusand and Portis, 1987; Robinson and Portis, 1989a).

Rubisco activase is present in *C. reinhardtii* (Salvucci et al., 1987), where it is localized in the pyrenoid along with Rubisco (Lacoste-Royal and Gibbs, 1987; McKay et al., 1991). Rubisco activase has been purified from *Chlamydomonas*, and the gene for Rubisco activase has been cloned from *C. reinhardtii* (Roesler and Ogren, 1990).

In this study we investigated the expression of Rubisco activase and CA in *C. reinhardtii*. When cells are grown under a 12-h light/12-h dark growth regime, the transcript levels for both the periplasmic CA and Rubisco activase vary in amount during the 24-h cycle. We have found that, unlike CA, Rubisco activase is not greatly affected by the external CO_2 concentration. We have also found that photosynthesis does not appear to be an absolute requirement for the induction of CA, implying that the CO_2 level may be sensed by *C. reinhardtii* even in the dark.

MATERIALS AND METHODS

Algal Cultures

The wild-type strain used in this study, 137 (mt^+), was obtained from Dr. R.K. Togasaki (Indiana University, Bloomington). The cultures were grown photoautotrophically 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% CO_2 in air (final C_i concentration = 2 mM) or ordinary air (final C_i = 4 μM). Cultures in the dark bubbled with ordinary air had a final C_i concentration of 21 μM. The C_i concentration of a culture was estimated by adding a known volume of the culture to a suspension of low-CO_2-adapted wild-type cells that had depleted their endogenous C_i and measuring the amount of O_2 evolved. This amount was calibrated by adding known concentrations of a 10 mM $NaHCO_3$ solution to the cell suspension.

Low-CO_2 Induction

In the low-CO_2-induction experiment, the cultures were grown on high CO_2 asynchronously until the cell density was approximately 5×10^6 cells/mL. At that time, the cells were concentrated by centrifugation at 5000 rpm (Beckman JA-10 rotor) for 5 min and resuspended in fresh media and either bubbled with high CO_2 or bubbled with air. The cultures were harvested at times indicated in the figure legends by centrifugation at 5000 rpm for 5 min. The cells were washed with 10 mM Tris (pH 7.5) and 5 mM EDTA. For protein analysis, the pellets were resuspended in 20 mM bis-Tris propane (pH 7.0), 5 mM MgCl, 1 mM EDTA, 5 mM DTT, 0.2 mM ATP, 1 mM PMSF, 10 μM leupeptin, and 2 mM benzamidine. For RNA analysis, the pellets were resuspended in RNA lysis buffer (50 mM Tris, 10 mM EDTA, 1% SDS) and recentrifuged. The pellets of this spin were snap frozen and stored at −80°C until extracted for RNA.

Light/Dark Experiment

In the light/dark experiment, wild-type cells were grown on either high or low levels of CO_2 in minimal medium and placed on a regime of 12 h of light and 12 h of dark for at least 3 d to cause the cells to become synchronous. The cells were harvested during the light period, 1, 5, and 9 h after the lights were turned on (at 9 AM, 1 PM, and 5 PM, respectively), and during the dark period, 1 and 5 h after the lights were turned off (9 PM and 1 AM, respectively) and 1 h before the lights were turned on (7 AM). These times were used again for another cycle of light and dark period for one set of samples. In another set, the second light period was replaced by a dark period, and cells were harvested at the same times. For both sets of samples, an additional harvest time was used, 1 h before the lights were turned on before the first light period. The cells were harvested in the same manner for protein and RNA analysis as in the low-CO_2-induction experiment. Also, as in the low-CO_2 experiment, the cell density ranged from 4×10^6 to 5×10^6 cells/mL during the course of the experiment.

Switching Cells on High CO_2 to Low CO_2 in the Dark

Wild-type cells were grown under the same conditions as in the light/dark experiment and bubbled with 5% CO_2 in air. Samples for RNA and protein analysis were harvested during the end of the dark period, at 1 h before the lights were turned on (7 AM) and 1, 5, and 9 h after the lights were turned on (9 AM, 1 PM, and 5 PM, respectively). After the lights were turned off, the cells were switched to bubbling with low CO_2 0.5 h later. When this protocol was used, low C_i levels were achieved 1 h after the light period has ended. Cells were then harvested 1 and 5 h after the the lights were turned off (9 PM and 1 AM, respectively) and and 1 h before the lights were turned on (7 AM). For one set of cells, the light was turned on and the cells were harvested 1 and 5 h after the lights were turned on. Another set of cells were left in the dark, and the samples were taken at the same time as when the lights would have been turned on. Also, as in the low-CO_2 experiment, the cell density ranged from 4×10^6 to 5×10^6 cells/mL during the course of the experiment.

To ensure that cultures were kept dark, we transferred the cultures to 2.8-L Nalgene flasks that had been completely coated with electrical tape and autoclaved. This resulted in a black coating on the flasks. We then covered these flasks with aluminum foil and masking tape. Using a Li-Cor (Lincoln, NE) light meter (model LI-185B) we were unable to detect any light within the flask at the most sensitive scale (3 μE m^{-2} s^{-1} full scale). In addition, the lights were kept off in the room, and the cells were harvested in the dark.

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 recombinant *Chlamy-*

domonas Rubisco activase (kindly provided by Dr. Bob Ramage, University of Illinois, Urbana) and antisera raised against periplasmic CA (kindly provided by Mr. Livingston Manuel, Louisiana State University, Baton Rouge).

RNA Extraction and Northern Analysis

RNA was extracted from *C. reinhardtii* cultures using the method of Smart and Selman (1991). Northern blots were performed essentially as described by Sambrook et al. (1989), using a full-length Rubisco activase cDNA clone (kindly provided by Dr. A. Portis, University of Illinois, Urbana), and a partial *Cah*1 cDNA clone (kindly provided by Dr. M. Spalding, Iowa State University, Ames). The *Cah*1 cDNA clone was cut with restriction enzymes *Spe*I and *Xho*I to obtain the 3′ untranslated end of the gene, and this fragment was used as the probe to avoid spurious detection of the *Cah*2 transcript.

Photosynthesis Assays

The photosynthetic rate of algal cells was measured with an oxygen electrode (Rank Brothers, Cambridge, UK). Algae were centrifuged at 5000 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 C_i 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 was measured during the next 30 s to 2 min. Chl concentrations were determined spectrophotometrically. The $K_{0.5}(CO_2)$ value is the CO_2 concentration required to give half-maximal rates of O_2 evolution.

CA Assays

The CA activity was assayed electrometrically using a modification of the Wilbur-Anderson method (Wilbur and Anderson, 1948). The samples were assayed at 3°C by adding intact cells equivalent to 200 μg of Chl to 3 mL of 15 mM 4-(2-hydroxyethyl)-1-piperazine-propanesulfonic acid, pH 8.0. The reaction was initiated by addition of 2 mL of ice-cold CO_2-saturated water. The time required for the pH to decrease from 7.7 to 6.3 was measured. The activity of the test sample was calculated using the equation: units = $T_o/T - 1$, where T is the time required for the pH change when the test sample is present and T_o is the time required for the pH change when the CA inhibitor acetazolamide (50 μM final concentration) was also added to the solution.

RESULTS

The Effect of External CO_2 Concentration on the Levels of Rubisco Activase and the Periplasmic CA

In this experiment algal cultures were grown in minimal medium with high CO_2 (5% CO_2 in air). The cultures were then transferred to air levels of CO_2, and the levels of Rubisco activase and the periplasmic CA were estimated by RNA blots and immunoblots. Figure 1 shows that

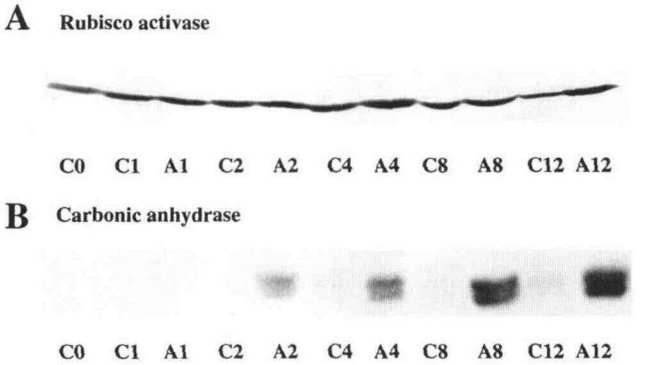

Figure 1. Protein analysis of low-CO_2-adapted and high-CO_2-grown cells. Lanes C0, C1, C2, C4, C8, and C12, High-CO_2-grown cells at various times (in hours) after resuspension into fresh media. Lanes A1, A2, A4, A8, and A12, Cells switched to low CO_2 (air levels) for various times (in hours) after resuspension into fresh medium. A, Immunoblot of total cell protein probed with antibody to *C. reinhardtii* Rubisco activase. All lanes contained 100 μg of protein. B, Immunoblot of total cell protein probed with antibody to *C. reinhardtii* periplasmic CA. All lanes contained 50 μg of protein.

Rubisco activase was not induced by low CO_2. The protein levels of Rubisco activase remained constant after the transfer of cultures from high CO_2 to low CO_2 (Fig. 1A). The level of the Rubisco activase transcript showed a transient decline when cells were placed in air, but after 4 h the transcript levels were equal to the transcript levels in the high-CO_2-grown cells (Fig. 2A). In contrast, the transfer to low CO_2 resulted in the synthesis of CA within 2 h (Fig. 1B). The increase in the periplasmic CA protein was reflected in the increase in its *Cah*1 transcript level, which reached its maximum in the 1st h after transfer to air (Fig. 2B). Figure 2C shows that the amount of total RNA loaded in each lane was equal, and thus the increase in the amount of *Cah*1 transcript is a reflection of the relative *Cah*1 transcript level at different times in the light/dark cycle.

The Effect of a Light and Dark Cycle on the Level of Rubisco Activase and the Periplasmic CA

In higher plants, Rubisco activase is influenced by the circadian clock (Martino-Catt and Ort, 1992). Figure 3A shows that when *C. reinhardtii* was grown on a 12-h light/12-h dark regime Rubisco activase protein levels oscillated during the 24-h period. Rubisco activase protein levels were lower during the dark phase of the cycle and more abundant during the light period. The Rubisco activase mRNA transcript level also varied throughout the 24-h period (Fig. 4A). The oscillation pattern differed from that of *C. reinhardtii Cab* mRNA transcript, which codes for the Chl *a*/*b*-binding proteins of PSII (Jacobshagen and Johnson, 1994). The *Cab* transcript levels were highest early in the light period, whereas the Rubisco activase transcript level was highest just before the start of the light period and was low throughout the light period. The Rubisco activase protein level oscillation seemed to be staggered with respect to the mRNA transcript. The accumulation of the Rubisco activase protein was low in the dark in contrast to the

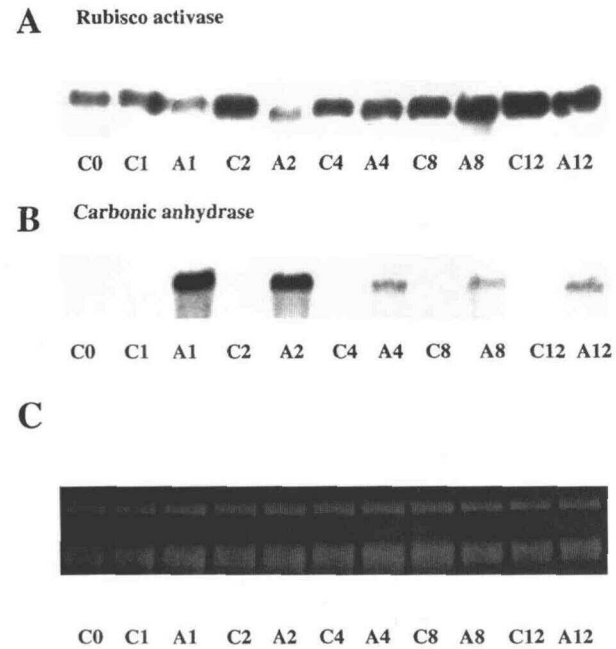

Figure 2. RNA analysis of total RNA extracted from low-CO_2-adapted and high-CO_2-grown cells. The lanes refer to the same samples as described in the legend to Figure 1. All lanes contained 5 μg of RNA. A, RNA blot probed with a Rubisco activase cDNA clone of *C. reinhardtii*. B, RNA blot probed with a partial *Cah*1 cDNA clone of *C. reinhardtii*. C, Formaldehyde agarose gel of total RNA stained with ethidium bromide.

maximum levels of Rubisco activase transcript, which occurred before the light was turned on in the dark period. The difference in timing between the transcript and protein levels might indicate that Rubisco activase was synthesized near the very beginning of the light period and was degraded very slowly throughout the light period. It should

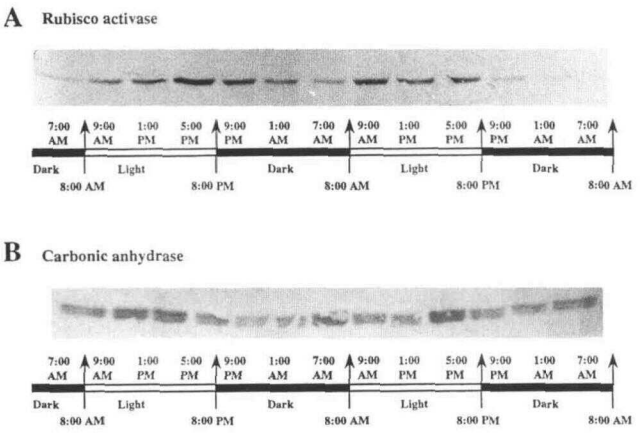

Figure 3. Protein analysis of low-CO_2-grown cells under a 12-h light/12-h dark regime. The lights came on at 8 AM and went off at 8 PM. The 7 AM harvest was in the dark. The points at 9 AM, 1 PM, and 5 PM were from illuminated samples. Darkened samples also were harvested at 9 PM, 1 AM, and 7 AM. All lanes contained 100 μg of protein. A, Immunoblot of total cell protein probed with antibody to *C. reinhardtii* Rubisco activase. B, Immunoblot of total cell protein probed with antibody to *C. reinhardtii* periplasmic CA.

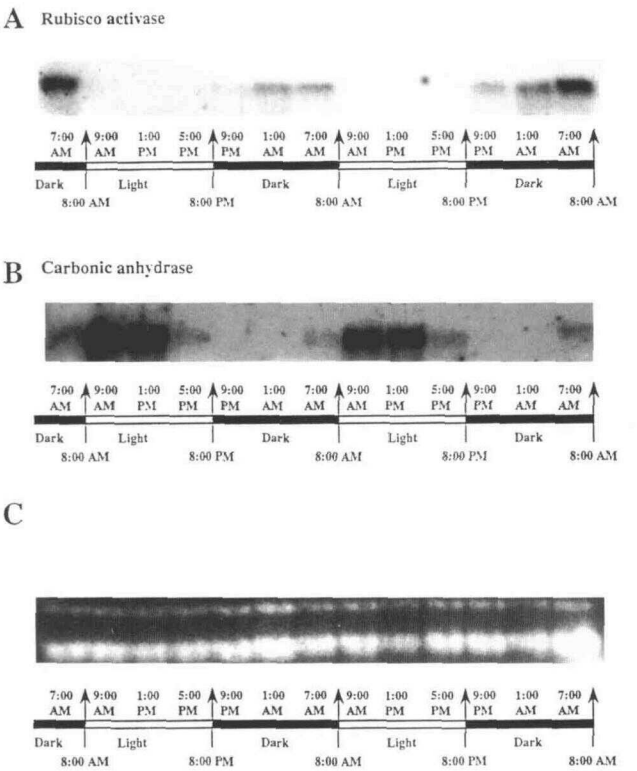

Figure 4. RNA analysis of total RNA extracted from low-CO_2-grown cells under a 12-h light/12-h dark regime. The times and growth conditions are the same as described in the legend to Figure 3. All lanes contained 10 μg of total RNA. A, RNA blot probed with a *ra* cDNA clone of *C. reinhardtii*. B, RNA blot probed with a partial *Cah*1 cDNA clone of *C. reinhardtii*. C, Formaldehyde agarose gel of total RNA stained with ethidium bromide.

be noted that the protein was present at all times during the cycle, and it is only the amount of the protein that varied. Furthermore, *C. reinhardtii* cells grown under high-CO_2 conditions showed the same Rubisco activase protein and mRNA transcript oscillation patterns (data not shown).

When *C. reinhardtii* was grown under low-CO_2 conditions and a 12-h light/12-h dark cycle, the *Cah*1 transcript coding for the periplasmic CA also appeared to undergo oscillations in levels (Fig. 4B). Like the Rubisco activase transcript, the level of the *Cah*1 transcript began to increase during the dark period before the light was turned on. The level of the message continued to increase during the early light period and then declined late in the light period; therefore, the level of the transcript was very low for most of the dark cycle. As shown in Figures 1 and 2, low CO_2 was required for induction of the *Cah*1 transcript. If the cells were grown with elevated CO_2, the level of the *Cah*1 transcript remained undetectable throughout the light/dark cycle (data not shown). These blots were probed with a noncoding 3′ end of a *Cah*1 clone, which recognizes only the *Cah*1 transcript (Fujiwara et al., 1990). The *Cah*1 and *Cah*2 genes are so similar in their nucleotide sequences in the coding region that the *Cah*1 probe would bind to the *Cah*2 transcripts. Although the *Cah*2 message is known to be present in lesser amounts than the *Cah*1 transcript (Fu-

jiwara et al., 1990; Rawat and Moroney, 1991), the *Cah*2 transcript is still present in the dark, which would confuse the pattern of *Cah*1 message.

In low-CO_2-grown cells, the periplasmic CA protein level did not show an obvious oscillation (Fig. 3B). This is not surprising, because it is known that the periplasmic CA is a very stable protein. Recent work from our laboratory indicates that the CA protein is detectable in cells even 2 d after they have been switched to high-CO_2 growth conditions, where they stop making the protein (Ramazanov et al., 1994).

Switching Cells from High CO_2 to Low CO_2 in the Dark

One surprising result of these studies was that the *Cah*1 transcript could be detected in the dark in the synchronous cultures, since the results of earlier studies indicated that photosynthesis was required for the induction of CA. However, in cultures grown on light/dark cycles, the *Cah*1 transcript was evident at the 7 AM time points (Fig. 4B), 1 h before the light cycle began. We therefore wanted to determine whether light was absolutely necessary for the induction of CA when cells were grown on a light/dark cycle. For these experiments, the cells were grown synchronously on high CO_2 and then were switched to low-CO_2 conditions after the start of the dark period. Some of the cells were kept on the 12-h light/12-h dark cycle, and others were left in continuous darkness. Figure 5 shows that the periplasmic CA protein was induced when the cells were placed in low-CO_2 conditions, even though the switch to low CO_2 was done in the dark. The protein appeared just before the beginning of the light period and continued increasing in amount during the light period. The CA protein was also present in cells that were kept in constant darkness after being switched to low CO_2 (Fig. 5). This appearance of protein in the dark just before the light period correlated with the appearance of the *Cah*1 transcript in the dark period (Fig. 6A). The transcript was also present in the cells left in continuous darkness (Fig. 6B).

Further evidence that these cells were adapting to low-CO_2 conditions in the dark is shown in Figure 7. In this case cells were assayed for the presence of the low-CO_2-inducible periplasmic CA and for their affinity for added C_i. Cells switched to low CO_2 in the dark period had an increase in CA activity and an increase in their apparent affinity for C_i (Fig. 7). Although the CA activity of the dark-adapted cells was less than the control cells (switched to low CO_2 and allowed to enter the light cycle), the activity of the low-CO_2, dark-adapted cells was significantly higher than the cells left on elevated CO_2 (Fig. 7). In addition, the cells switched to low CO_2 in the dark partially adapted to low CO_2 as judged by their increase in apparent affinity for C_i (Fig. 7). The $K_{0.5}(CO_2)$ of the cells placed on low CO_2 in the dark was 9 μM compared to about 30 μM for the cells left on high CO_2 in the dark or in the light (Fig. 7). Control cells that remained on high CO_2 in the light or in the dark did not induce the protein or the *Cah1* message. Cells left on high CO_2 also did not induce CA activity or increase their apparent affinity for C_i (Fig. 7).

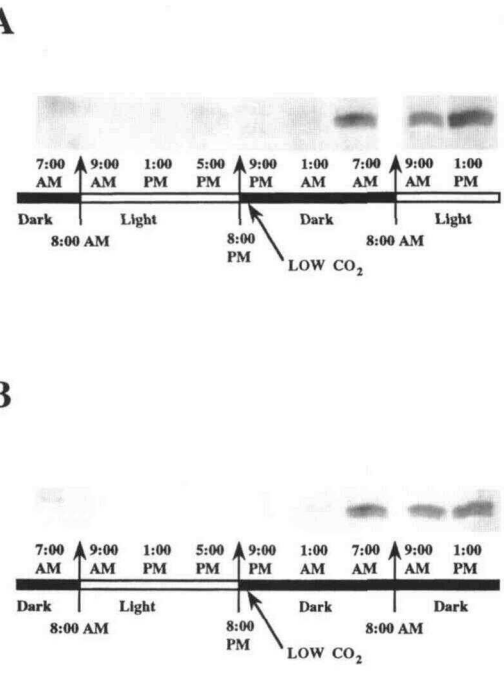

Figure 5. Protein analysis of cells grown synchronously under high-CO_2 conditions and switched to low-CO_2 conditions in the dark. The cells were grown under a 12-h light/12-h dark regime. The lights were turned on at 8 AM and turned off at 8 PM. The cells were switched from high CO_2 to low CO_2 at 8:15 PM. The harvest time at 7 AM was in the dark and under high-CO_2 conditions. The points at 9 AM, 1 PM, and 5 PM were from illuminated samples and under high-CO_2 conditions. Darkened samples under low-CO_2 conditions were harvested at 9 PM, 1 AM, and 7 AM. A, One set of samples was placed in light and samples at 9 AM and 1 PM were harvested. B, Another set was left in the dark and samples at 9 AM and 1 PM were harvested. All lanes contained 100 μg of protein, were electrophoresed on the same gel, and were probed with antibody to *C. reinhardtii* periplasmic CA.

The CA activity measured in Figure 7 was due to the expression of the *Cah1* gene, since immunoblots of whole cells revealed that the CA had an apparent molecular mass of 37 kD and was the same protein that was expressed by cells in the light (Fig. 8, lanes 1 and 2). The *Cah*2 gene product that is expressed in the dark (Fujiwara et al., 1990) has an apparent molecular mass of 39 kD (Rawat and Moroney, 1991; Fig. 8, lane 3) and can be distinguished from the *Cah1* gene product. These results provide additional evidence that light is not an absolute requirement for the induction of the *Cah*1 transcript in cells growing on a light/dark cycle.

DISCUSSION

In higher plants, Rubisco activase has been shown to be required for optimal growth under low-CO_2 conditions. In algae, the periplasmic CA is part of a CO_2-concentrating mechanism that increases the efficiency of CO_2 fixation by increasing the CO_2 level at the site of Rubisco. We have studied the regulation of the accumulation of these proteins by light/dark cycles and by the CO_2 level in the medium.

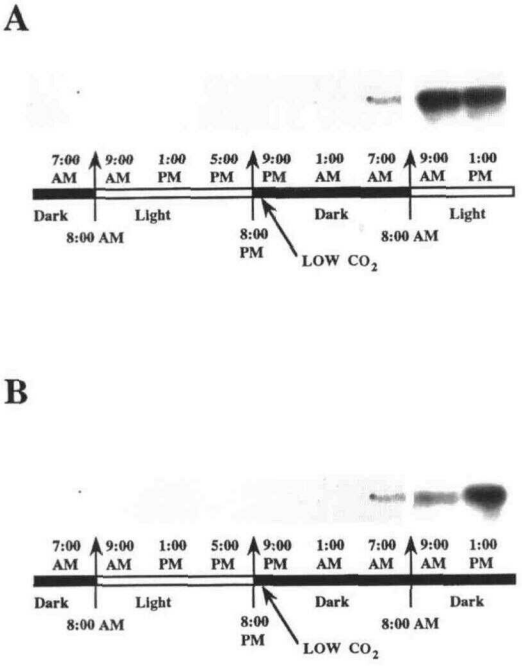

Figure 6. RNA analysis of cells grown synchronously under high-CO_2 conditions and switched to low-CO_2 conditions in the dark. The times and growth conditions are the same as those described in the legend to Figure 5. All lanes contained 10 μg of RNA, were electrophoresed on the same gel and probed with a partial *Cah*1 cDNA clone.

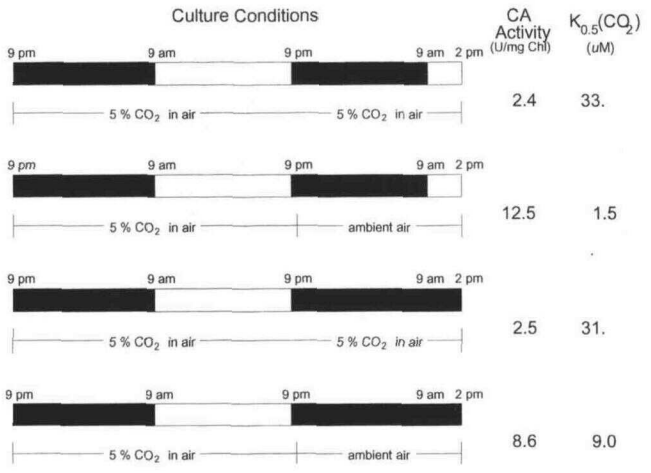

Figure 7. The induction of CA activity and increase in apparent cell affinity for C_i in cells switched to low CO_2 in the dark. Cells were grown on elevated CO_2 and a 12-h light/12-h dark regime for 3 d. Cells were then subjected to one of four treatments. The four treatments included (a) elevated CO_2 and the same light/dark cycle, (b) switch the cells to ambient air and leave them on the same light/dark cycle, (c) elevated CO_2 and continuous darkness, and (d) switch to ambient air and continuous darkness. Where indicated cells were switched to ambient air 1 h into the dark cycle. CA assays and the $K_{0.5}(CO_2)$ were determined as described in "Materials and Methods." U, Units.

When cells were grown on a 12-h light/12-h dark regime, the transcript for Rubisco activase showed a strong oscillation. In higher plants, Rubisco activase appears to be controlled in a circadian fashion (Martino-Catt and Ort, 1992). The oscillation in Rubisco activase transcript levels reported here (Fig. 4A) is consistent with what is observed in higher plants, with the transcript peaking right before the start of the light cycle. As in higher plants, the Rubisco activase protein oscillation is staggered with respect to the transcript in *C. reinhardtii*. In *C. reinhardtii* the oscillation pattern of Rubisco activase is different from that of *Cab*, another gene whose protein product is involved in photosynthesis (Jacobshagen and Johnson, 1994). In addition, the Rubisco activase oscillations appear to be weaker in intensity when compared to the *Cab* gene. We observed this oscillation of the Rubisco activase transcript in low-CO_2-grown cells as well as high-CO_2-grown cells.

In higher plants, there is evidence that Rubisco activase is required for optimal growth under limiting CO_2 conditions (Somerville et al., 1982; Salvucci et al., 1987). Rubisco activase from *C. reinhardtii* has been cloned and sequenced. The mature polypeptide has a 65% amino acid sequence identity with Rubisco activase from Arabidopsis. Unlike higher plants, there is no evidence of alternative splicing and it is coded by a single gene (Roesler and Ogren, 1990). Since one of the proposed functions of Rubisco activase is to allow full activation of Rubisco at atmospheric CO_2 conditions in higher plants, we looked at the level of Rubisco activase in low-CO_2- and high-CO_2-grown cells in *C. reinhardtii*. We found that the level of Rubisco activase transcript and protein was unaffected by the amount of CO_2 supplied to the *C. reinhardtii* cells. The increase in apparent affinity for CO_2 that occurs as a result of the CO_2-concentrating mechanism is not reflected in a change in the Rubisco activase protein and transcript levels (Figs. 1 and 2). The physiological role of Rubisco activase in organisms that possess a CO_2-concentrating mechanism has not been determined at this time.

Like Rubisco activase, the level of the transcript for the periplasmic CA also showed significant oscillations over the light/dark cycle. Previously, there had been no evidence that the periplasmic CA showed this type of oscillation, although Marcus et al. (1986) reported an oscillation in CA activity when cells were grown on low CO_2 and a light/dark cycle. In addition to the requirement for low CO_2, light was thought to be required for transcription of

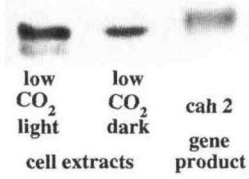

Figure 8. Immunoblot of cell extracts and purified periplasmic CA. The CA expressed by cells adapted to low CO_2 in the light (lane 1) and dark (lane 2) were compared to the *Cah2* (lane 3) gene product. The cell extracts contained 20 μg of protein each, and the blot was probed with antibody to *C. reinhardtii* periplasmic CA.

the *Cah1* gene. Toguri et al. (1989) reported that the CA protein level did not vary significantly over a light/dark cycle. They, however, did not look at the CA transcript levels. We also observed that CA protein levels did not vary much over the light/dark cycle (Fig. 3B), but there was a strong oscillation in the *Cah1* transcript level over the same period (Fig. 4B). These observations are not contradictory, since it is known that the CA protein is very stable (Ramazanov et al., 1994); therefore, the protein level would be expected to remain fairly constant even though the transcript level fluctuated. These studies also indicated that transcription of the *Cah1* gene occurred in low-CO_2 conditions in the dark, which is in apparent disagreement with earlier studies. However, previous experiments on the induction of CA in low CO_2 were done with *C. reinhardtii* cells that had been grown under constant light in asynchronous conditions. To determine whether light is needed along with low-CO_2 conditions for this induction in synchronous conditions, we first grew *C. reinhardtii* synchronously under high CO_2 in which induction of the *Cah1* transcript does not take place. Then the cells were switched to low-CO_2 conditions immediately after the lights were turned off. The cells were in complete darkness when they were switched to low-CO_2 conditions. Samples taken at the end of the dark period showed that the CA protein and transcript were induced before the lights were turned on (Figs. 5 and 6). These results imply that, at least in synchronous cells, photosynthesis is not absolutely required for the induction of the *Cah1* transcript in *C. reinhardtii*. This is in marked contrast to asynchronous cells, in which experiments using photosynthetic mutants (Spalding and Ogren, 1982), the addition of DCMU (Fukuzawa et al., 1990), and light of different wavelengths (Dionisio et al., 1989) led researchers to believe that light and active photosynthesis were required for the induction of CA.

We further determined the CO_2 affinity and CA activity of cells grown synchronously and switched to low CO_2 in the dark. When these darkened cultures were switched to low CO_2 they partially adapted to the low CO_2, which is shown by the increase in CA activity and the increase in the cells' apparent affinity for C_i (Fig. 7). Thus we have shown that darkened cells not only transcribe the *Cah1* gene but synthesize mature periplasmic CA. In addition, the fact that the cells' affinity for C_i increases supports the hypothesis that other components of the CO_2-concentrating mechanism are regulated in a manner similar to that of the *Cah1* gene.

It is not clear why there is a dark induction of the *Cah1* gene in synchronously grown cells as opposed to asynchronously grown cells, but we have described a few possible reasons below. First, it is important to emphasize that the dark induction reported here, although easily detectable, is less than induction seen in the light. Apparently, there are multiple factors that affect the transcription of the *Cah1* gene; therefore, a partial dark induction of the gene under low-CO_2 conditions might occur during a specific portion of the cell cycle through the mediation of one or more such factors. In asynchronous cells the cell cycle would be randomized and this effect might not be detected. Second, an additional explanation is that we observe this induction toward the end of the dark cycle (11 h). Most studies with asynchronous cells measured *Cah1* transcript or CA protein levels 2 to 6 h after a switch to low CO_2 in the dark. We found the transcript level to be very low 5 h into the dark period. Third, since the transcript appears to increase just before the light period, it is possible that the *Cah1* gene is under at least partial circadian control, which is presumably less of an influence in asynchronous cells. The oscillations in CA activity reported by Marcus et al. (1986) support this latter explanation. Such hypotheses will be tested in future studies.

The previous studies with asynchronous cells also raised the possibility that the alga might sense the level of CO_2 in the environment indirectly by sensing a balance of photosynthetic metabolites or by sensing the level of a photorespiratory cycle intermediate such as phosphoglycolate. Since the transcription of the *Cah1* gene is induced by low CO_2 in the dark, it is unlikely that active photosynthesis is an absolute requirement for *Cah1* gene transcription. Therefore, it appears that the alga can sense the CO_2 level in the growth medium in a way other than through photosynthetic intermediates such as Pi or triose phosphates. It is even more unlikely that a metabolite of the photorespiratory cycle is the sole signal for the transcription of the *Cah1* gene, since the levels of these metabolites are low unless the Rubisco oxygenase activity is high. The evidence that a photosynthetic metabolite may not be the "sensor" is further supported by the fact that *C. reinhardtii* cells grown in low-CO_2 conditions in the presence of acetate induce the CA to a lesser extent. These same cells exhibit high rates of respiration, which would result in increased CO_2 inside the cell, mimicking high-CO_2 conditions (Fett and Coleman, 1994). All of these results suggest that *C. reinhardtii* may be able to sense the CO_2 level in a way other than through photosynthetic intermediates. How the cell senses the level of CO_2 remains to be determined.

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