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Regulation of Periplasmic Carbonic Anhydrase Expression in *Chlamydomonas reinhardtii* by Acetate and pH[1]

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The effects of mixotrophic growth with acetate and growth medium pH on expression of extracellular carbonic anhydrase (CA) in *Chlamydomonas reinhardtii* were evaluated. Addition of 10 mM acetate to the culture medium resulted in reduction of CA activity that was parallel to the reduction generated by growth of the algae in high external CO_2 concentrations. This reduction in activity is a consequence of lower levels of the CA protein as determined by western analysis. Transcript abundance of *cah-1*, the gene encoding the low CO_2-induced CA, is also reduced by the addition of acetate as verified by northern analysis. Measurements of photosynthesis and respiration suggest that the acetate-induced reduction of CA expression is not a function of lowered photosynthetic capacity, but may be the result of increased internal CO_2 concentration generated by high, acetate-stimulated respiratory rates. Growth medium pH can also influence extracellular CA expression. The induction of CA activity, protein abundance, and transcript levels by exposure to limiting inorganic carbon (C_i) concentrations is much more pronounced at higher than at lower pH values. The relationship between pH regulation of CA expression and its role in the C_i-concentrating mechanism are discussed.

Many species of eukaryotic algae and cyanobacteria are able to grow and photosynthesize efficiently at low C_i concentrations by inducing the expression of CCMs (Aizawa and Miyachi, 1986; Coleman, 1991). The activity of these CCMs results in the formation of a large intracellular C_i pool from which CO_2 is obtained for fixation by Rubisco. It is generally presumed that it is the abundance and availability of intracellular C_i that results in the "C_4-like" photosynthetic phenotype exhibited by cells grown at limiting C_i concentrations. This phenotype includes little or no photorespiration or O_2 inhibition of photosynthesis, very low CO_2 compensation points, and a cellular affinity for C_i that is at least 1 order of magnitude greater than that exhibited by purified algal or cyanobacterial Rubisco. The enhanced expression of the enzyme CA has also been associated with induction of the CCMs. CA catalyzes the interconversion of CO_2 and HCO_3^- and is an important component in the intracellular mobilization of the HCO_3^- pool, by catalyzing the production of CO_2 for Rubisco.

In the eukaryotic green alga *Chlamydomonas reinhardtii*, although intracellular isoforms are present, the majority (greater than 90%) of the CA activity is found in the periplasmic space, external to the cell, and is obviously not involved in intracellular HCO_3^- pool mobilization (Kimpel et al., 1983; Coleman et al., 1984). The expression of extracellular CA activity is regulated by the C_i concentration of the medium with the abundant *cah*-1 gene product induced by growth at limiting C_i (Bailly and Coleman, 1988; Fukuzawa et al., 1990). A small amount of extracellular CA activity (less than 5% of total cellular CA activity) has been associated with the *cah*-2 gene product, which is expressed at high C_i concentrations and repressed at C_i levels that induce *cah*-1 gene expression (Fujiwara et al., 1990). A well-defined role for the extracellular CA activity has yet to be conclusively shown, although it is generally presumed that catalysis of CO_2/HCO_3^- equilibration in the periplasmic space is useful for the provision of CO_2 and/or HCO_3^- to plasmalemma-localized C_i transport proteins of the CCM (Coleman, 1991). It has been shown that both CO_2 and HCO_3^- are actively transported by *C. reinhardtii* cells acclimated to limiting C_i concentrations, with CO_2 being preferentially removed from the medium (Sultemeyer et al., 1989; Palmqvist et al., 1990). Extracellular CA activity would ensure that transport of either C_i species would not be limited by the rate of C_i interconversion.

Expression of extracellular *cah-1* gene product in *C. reinhardtii* appears to be regulated at the transcriptional level (Bailly and Coleman, 1988). Both the protein and mRNA levels increase after transfer of cells from high C_i concentrations to limiting levels of C_i. Transfer of cells acclimated to limiting C_i levels to a high-C_i environment results in the elimination of the *cah-1* transcript within 60 min. A major question in the regulation of CA (and CCM) expression in algae and cyanobacteria concerns the nature of the inducing signal. It has been suggested that C_i-regulated CA expression could be induced by changes in photosynthetic capacity or metabolite levels following transfer from one C_i environment to another. In earlier studies, limiting C_i-acclimated *C. reinhardtii* grown mixotrophically on media supplemented with acetate showed decreases in CA activity when compared with autotrophic cultures (Spalding and Ogren, 1982; Coleman et al., 1991). These decreases were believed to reflect the decline in photosynthetic capacity of the mixotrophic cultures, since these cells are able to use the acetate supple-

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Abbreviations: CA, carbonic anhydrase; *cah-1*, gene encoding the low-CO_2-induced carbonic anhydrase; CCM, high-affinity C_i-concentrating mechanism; C_i, inorganic carbon.

ment as a carbon source. The extent of CA repression, however, seemed to be variable, and changes in photosynthetic capacity had not been correlated with CA activity. It has also been shown that the presence of extracellular CA activity in *C. reinhardtii* is pH dependent, with higher activities expressed in cells grown at alkaline pH than at acid pH (Patel and Merret, 1986; Williams and Turpin, 1987). However, it was not determined if lower pH values of the growth medium were inactivating the enzyme or if the pH effect was at the level of CA induction.

In this study we investigated the effects of acetate and pH on *C. reinhardtii* periplasmic CA expression by determining CA activity, protein abundance, and *cah-1* transcript levels in cells exposed to different CO_2 concentrations following addition of acetate to the culture media and exposure to different pH values. Photosynthetic and respiratory rates of mixotrophic and autotrophic cultures were also measured and these provide initial information on the mechanism by which acetate may influence CA expression.

MATERIALS AND METHODS

Growth of Algae

Chlamydomonas reinhardtii WT strain 2137 mt+ was maintained on TAP agar (Sueoka, 1960) and grown in 300 mL of minimal medium as described by Spreitzer and Mets (1981) with the following modifications: Tris buffer was replaced by 20 mM Mops, pH 7.5, or 20 mM Mes, pH 5.5, as required; the microelements were as according to Hutner et al. (1956); and 1% (w/v) ferric ammonium citrate was used at a final concentration of 1 mL/L media. For mixotrophic growth, sodium acetate was added to the minimal medium for a final concentration of 10 mM. Gas dispersion tubes were used to bubble all cultures (flow rates of 750 mL min^{-1}) with air containing different levels of CO_2 and to ensure maximum equilibration between the liquid and gas phases. These included high CO_2 cultures (bubbled with air containing 20 mL L^{-1} [v/v] CO_2), air cultures (bubbled with air containing 350 μL L^{-1} [v/v] CO_2) and low CO_2 cultures (bubbled with air containing 30 μL L^{-1} [v/v] CO_2). The algae were grown at 30°C at a light intensity of 300 μmol photons m^{-2} s^{-1} (400–700 nm), and all experiments were performed with cells at mid-phase exponential growth (2–3 μg Chl mL^{-1} culture medium).

CA Activity Assays and Western Analysis

CA activity in cell lysates was determined electrometrically as described previously (Wilbur and Anderson, 1948; Kimpel et al., 1983). Cells were harvested by centrifugation (8000*g*, 10 min), resuspended in cold Veronal buffer (20 mM, pH 8.3), and lysed by passage through a prechilled French press (20,000 p.s.i.), and the CA activity was immediately determined at 2°C. For western analysis of soluble proteins, cells were harvested by centrifugation, resuspended in extraction buffer (20 mM Mops, pH 7.5, 10 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, and 5 mM DTT), lysed by passage through the French press, and centrifuged for 30 min at 26,000*g*. The proteins in the supernatant were precipitated by the addition of TCA (10% [v/v] final concentration), collected by centrifugation, and prepared for SDS-gel electrophoresis as previously described (Bailly and Coleman, 1988). Equal aliquots of total soluble proteins were separated by electrophoresis on denaturing 12% (w/v) polyacrylamide gels and electrotransferred to nitrocellulose according to the manufacturer's specifications (Pharmacia Multiphor II Systems Handbook 18-1013-42). Blots were subjected to western analysis by incubating with a polyclonal antibody against *C. reinhardtii* CA (Bailly and Coleman, 1988) and an alkaline phosphatase-conjugated secondary antibody (GIBCO-BRL).

Northern Analysis

For isolation of total RNA, cells were harvested by centrifugation and resuspended in RNA extraction buffer (0.1 M Tris-HCl, pH 9.0, 0.2 M NaCl, 10 mM Mg acetate, 16 mM EDTA, 5 mM DTT, 1% [w/v] SDS). Following a 5-min lysis period, the preparation was extracted twice with phenol:chloroform (1:1) and once with chloroform alone. The nucleic acids were precipitated from the aqueous phase by the addition of 0.2 M NaCl, 0.6 volume of isopropanol at −20°C, followed by sequential precipitation steps with 2 M LiCl and finally with 0.2 M NaCl, 2.5 volumes of ethanol. The isolated RNA was resuspended in sterile distilled water and stored at −70°C. For northern analysis, equal aliquots of 10 μg of RNA (determined by spectrophotometry) were denatured, eletrophoresed on 1.5% (w/v) agarose gels containing 0.66 M formaldehyde, and transferred to nitrocellulose as described by Fourney et al. (1988). Northern blots were prehybridized overnight and hybridized for 36 h at 42°C in a solution containing 50% (v/v) deionized formamide, 5× SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 6.3 mM EDTA, pH adjusted to 7.4 with NaOH), 0.1% (w/v) nonfat milk powder, and 0.1% (w/v) SDS. A 2.5-kb *Eco*RI fragment of genomic DNA containing a portion of the *C. reinhardtii cah-1* gene (Coleman et al., 1991) was labeled with [^{32}P]dCTP by the random primer labeling procedure (Feinberg and Volgestein, 1984) and used as probe in all hybridizations. The hybridized blots were washed twice for 15 min at room temperature in 2× SSPE, 0.1% (w/v) SDS and twice for 20 min at 50°C in 0.1× SSPE, 0.1% SDS. The hybridization patterns were determined by autoradiography.

Determination of Photosynthesis and Respiration Rates

Cells were collected by centrifugation and resuspended in fresh medium at a final Chl concentration of 20 μg mL^{-1}. Oxygen evolution rates were determined with the use of a Clark-type oxygen electrode at a light intensity of 1000 μE m^{-2} s^{-1} in the presence of saturating concentrations of $NaHCO_3^-$ and at 30°C. Respiration rates were also determined by measuring oxygen consumption in the dark at 30°C. The rates of photosynthesis and dark respiration were calculated as μmol O_2 mg^{-1} Chl h^{-1}. Chl concentrations were determined as described previously (Porra et al., 1989).

RESULTS AND DISCUSSION

Acetate and CO_2 Effects on CA Expression

Time-course experiments were performed in which air-grown cells were transferred to conditions of high CO_2, air

levels of CO_2 plus acetate, or low CO_2, or were maintained at air levels of CO_2 for the length of the experimental period. During acclimation periods, a uniform Chl concentration was maintained for all treatments by dilution with fresh medium every 12 h. This prevented depletion of nutrients (including acetate) and eliminated potential confounding effects of differing growth rates resulting in self-shading. As expected, air-grown cells contained high levels of periplasmic CA activity, and cells transferred to low-CO_2 conditions exhibited a slight increase in CA activity above normal levels (Fig. 1) The transfer of air-grown cells to high-CO_2 conditions, as expected, caused a decrease in CA activity with time. Interestingly, the addition of acetate to air-grown cells resulted in a similar rate of decrease in CA activity (Fig. 1). Western analysis of soluble proteins obtained from cells isolated at the same time points showed that the changes in activity were due to changes in CA protein abundance (Fig. 2). Both high-CO_2 and acetate treatments resulted in lower CA protein levels with time, whereas the protein levels remained constant with exposure to air levels of CO_2 and increased under low-CO_2 conditions. To verify whether or not those changes in activity and protein levels were reflecting changes in *cah-1* transcript level, RNA from cells grown at either high or air levels of CO_2 and transferred to the various alternate treatments for 3 h was isolated and subjected to northern analysis. Autoradiograms of these blots clearly show the accumulation of the *cah-1* transcript when cells were transferred from high to low CO_2 or to air levels of CO_2, whereas there was little accumulation of the transcript when cells were transferred to air levels of CO_2 in the presence of acetate (Fig. 3). Transfer of air-grown cells to medium containing acetate resulted in a significant decrease in *cah-1* transcript levels and the virtual elimination of the transcript following transfer to high-CO_2 conditions (Fig. 3). Transcript levels were maintained when cells were transferred to low-CO_2 conditions or kept at air levels of CO_2 (Fig. 3).

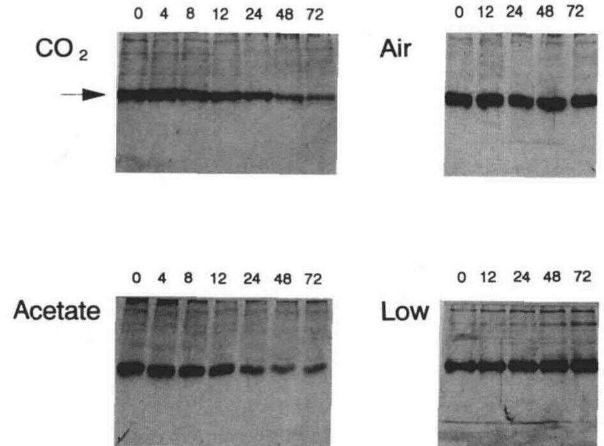

Figure 2. Expression of the periplasmic CA protein in air-grown *C. reinhardtii* cells transferred to high-CO_2, acetate, air, and low-CO_2 treatments for 0, 4, 8, 12, 24, 48, and 72 h (CO_2 and Acetate) and 0, 12, 24, 48, and 72 h (Air and Low). Total soluble proteins were extracted, equal aliquots were separated by SDS-PAGE, and the abundance of the CA protein was determined by western analysis using a polyclonal antibody generated against the 37-kD monomer of the periplasmic CA protein (Bailly and Coleman, 1988). The arrow indicates the position of the 37-kD CA monomer.

The effect of acetate as a repressor of CA expression could be a result of acetate reducing the photosynthetic capacity of the *C. reinhardtii*, since the cells can use acetate as an alternative source of carbon. It has been shown that CA induction following transfer to limiting concentrations of CO_2 is light and photosynthesis dependent (Spalding and Ogren, 1982;

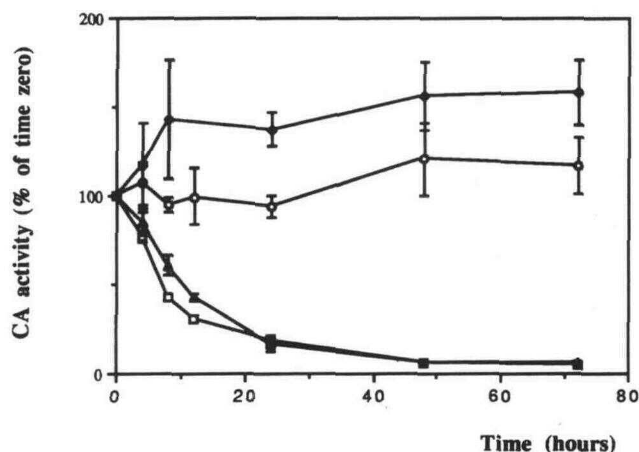

Figure 1. CA activity of air-grown cells of *C. reinhardtii* transferred to high (20 mL L^{-1}) CO_2 (□), air (350 μL L^{-1}) plus sodium acetate (10 mM) (△), air (○), and low (30 μL L^{-1}) CO_2 (◇) treatments. Aliquots of cells were removed at 0, 4, 8, 12, 24, 48, and 72 h after transfer and assayed for CA activity. Data are expressed as a percentage of initial activity at time of transfer. Each data point is the mean of three replicates, obtained in separate experiments, with SD values shown as vertical bars.

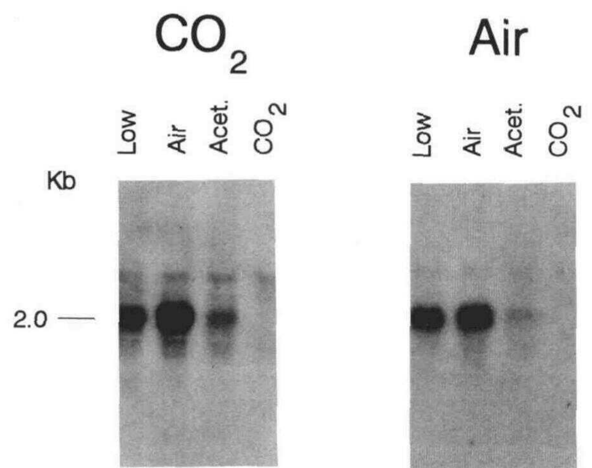

Figure 3. Expression of the *cah-1* gene in *C. reinhardtii* cells grown under high CO_2 (left) and under normal air (right) and transferred for 3 h to low-CO_2, air, acetate, and high-CO_2 treatments. Total RNA was extracted from cells and 10 μg of RNA from each treatment was subjected to northern analysis, using a radiolabeled 2.5-kb fragment of the *cah-1* gene as a probe. The position of the 2.0-kb *cah-1* transcript is indicated.

Dionisio et al., 1989), and, therefore, lower photosynthetic rates could result in lower CA expression. It is also possible that acetate metabolism results in the release of increased amounts of respiratory CO_2 and that the intracellular generation of high amounts of CO_2 mimics growth under high external C_i concentrations. To investigate those possibilities, we determined the photosynthetic and respiratory rates of air-grown cells exposed to the different treatments for 3 and 24 h (Table I). Using cells maintained at air levels of CO_2 as a standard, we identified an increase of over 100% in respiration when acetate was added after either 3 or 24 h. In contrast, there was only a 20 and 30% decline in photosynthesis after 3 and 24 h, respectively, of acetate exposure. The significant increase in respiration and the relatively minor reduction in photosynthesis generated by acetate addition suggest that acetate repression of CA is not a direct function of reduced photosynthetic carbon assimilation capacity but could be a response to increased intracellular CO_2 levels. The increased intracellular CO_2 levels are the result of elevated respiratory production and reduced demand by Calvin cycle activity.

As well as being assimilated via the TCA cycle, photoassimilation of acetate is also known to induce the glyoxylate cycle in many species of green algae. As one of the products of this cycle, glyoxylate could be reduced to glycolate, which is a product of photorespiration. It has been suggested that the induction of CCMs and CA may be stimulated by photosynthetic or photorespiratory intermediates (Spalding and Ogren, 1982), and, more recently, that one metabolite from the photorespiratory glycolate pathway is the effector of CA induction (Ramazanov and Cardenas, 1992). In our experiments, however, it is apparent that acetate metabolism and the presumed synthesis of glyoxylate has an inhibitory effect, as opposed to a positive effect, on CA induction.

Mixotrophic growth of *C. reinhardtii* on acetate has been shown to reduce the abundance of at least one of the transcripts encoding the Chl *a/b* binding protein (*cabII-1*; Kindle, 1987) and to modify the ratio of the two transcripts encoding the small subunit of Rubisco (Goldschmidt-Clermont, 1986). However, there is no evidence to suggest that mixotrophic growth results in a dramatic down regulation of genes required for photosynthetic carbon metabolism, and certainly not to the extent of the rapid decline in the *cah-1* transcript observed in this study.

Previous studies of CA and CCM activity using mixotrophic *C. reinhardtii* cultures have produced variable results. Spalding and Ogren (1982) were able to show that growth with acetate in the light resulted in CA activities that were approximately 20 to 25% of air-grown levels, but these were significantly higher than CA levels of a number of acetate-grown, photosynthesis-deficient *C. reinhardtii* mutants. They also observed that mixotrophic cultures were able to transport and accumulate C_i at rates that were comparable to those found in air-grown cells, whereas the photosynthesis-deficient mutants did not express CCM activity.

We were able to repress CA activity to a greater extent than that reported by Spalding and Ogren (1982). In preliminary studies (data not shown) it became apparent that frequent replenishment of the medium with acetate was required to fully repress CA expression. It is quite possible that the cells used in the earlier study were acetate limited and had initiated CA and CCM expression as air-grown cells. Another report using mixotrophic cultures presented data that are more consistent with the results of our study. Moroney et al. (1987) showed that isolated chloroplasts from acetate-grown cells were unable to transport C_i, whereas chloroplasts isolated from air-grown cells had some capacity for C_i accumulation (Moroney et al., 1987). It was also reported in that study that acetate-grown, intact cells had a much reduced affinity for C_i when compared with air-grown *C. reinhardtii* cultures. Irrespective of the conclusions made about the site of active C_i transport, these data clearly show that mixotrophic growth represses CCM activity.

pH Effects on CA Expression

Time-course experiments were performed to investigate the effect of medium pH on CA activity and protein abundance. Cells grown at high CO_2 concentrations were harvested, resuspended in new medium at the appropriate pH level, and transferred to air levels of CO_2. Following transfer from high-CO_2 conditions to air levels of CO_2, the induction of CA activity was much more pronounced at pH 7.5 than at pH 5.5 (Fig. 4). Moreover, the abundance of CA protein visualized on the western blots correlated with the activities, with faster induction at pH 7.5 compared with pH 5.5 at all time points (Fig. 5). These results indicate that the effect of hydrogen ion concentration on *C. reinhardtii* periplasmic CA expression was not simply due to inactivation of the enzyme at the lower pH. RNA was extracted from high-CO_2-grown *C. reinhardtii* cells transferred to air levels of CO_2 for 3 h in media adjusted to pH 7.5 or 5.5. Northern blot analysis

Table I. *Effect of acetate and CO_2 concentration on photosynthesis and respiration of C. reinhardtii*

	3 h[a]		24 h[a]	
	Photosynthesis	Respiration	Photosynthesis	Respiration
High CO_2	188 ± 24[b] (97%)[c]	23 ± 0.1 (161%)	183 ± 20 (116%)	24 ± 0.6 (104%)
Air ± acetate	154 ± 36 (80%)	41 ± 2.8 (205%)	112 ± 7 (71%)	48 ± 1.4 (209%)
Air	193 ± 9 (100%)	20 ± 3.5 (100%)	158 ± 12 (100%)	23 ± 2.0 (100%)
Low CO_2	ND[d]	ND	144 ± 8 (91%)	24 ± 4.9 (104%)

[a] Time of exposure of air-grown cells to various treatments. [b] Data expressed as μmol O_2 mg^{-1} Chl h^{-1} and represent the mean ± SD of three independent replicates for each treatment. [c] Values expressed as percentages of data obtained for air-grown cells. [d] Not determined.

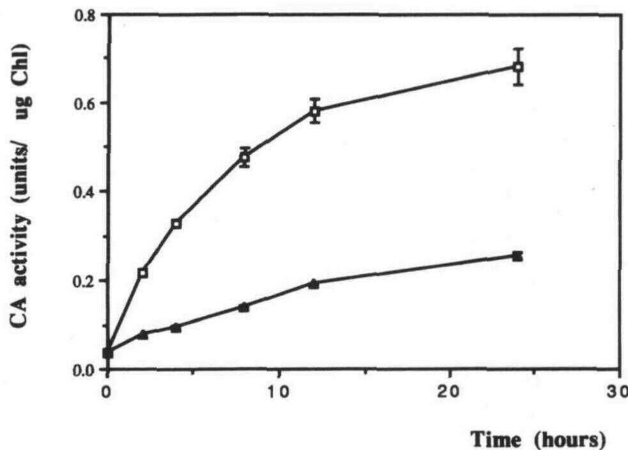

Figure 4. Effect of medium pH on induction of CA activity. *C. reinhardtii* cells were grown under high-CO_2 conditions and transferred to air levels of CO_2 in medium at pH 7.5 (□) or 5.5 (Δ). CA activity was assayed at 0, 2, 4, 8, 12, and 24 h after transfer and is expressed as Wilbur-Anderson units μg^{-1} Chl. Each data point is the mean of three independent replicates, with SD values shown as vertical bars.

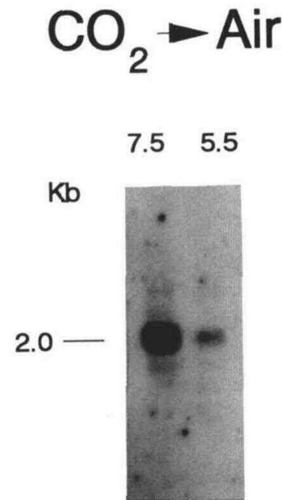

Figure 6. Effect of pH on induction of the *cah-1* transcript. *C. reinhardtii* cells were grown at high CO_2 concentrations and transferred to air levels of CO_2 in medium at pH 7.5 or 5.5. Total RNA was extracted after 3 h and 10 μg of each sample was subjected to northern analysis as described in Figure 3. The position of the 2.0-kb *cah-1* transcript is indicated.

verified that the *cah-1* transcript levels were also affected by pH. High-CO_2 cells, when transferred to air for 3 h, had significantly higher levels of *cah-1* transcript at pH 7.5 compared with pH 5.5 (Fig. 6).

The impact of pH on CA expression is in concert with many studies on the effects of pH and CA activity on photosynthesis of *C. reinhardtii*. In these earlier reports, elimination of extracellular of CA activity at alkaline pH significantly reduced whole-cell affinity for C_i, whereas little effect of CA inhibition was observed under acidic conditions (Tsuzuki, 1983; Moroney et al., 1985; Husic, 1991). These observations suggest that at acidic pH, when the predominant form of C_i in equilibrium is CO_2, there is less (or no) limitation of CO_2 availability for transport and/or diffusion into the cells, and therefore, little need for external CA activity. At higher pH levels, however, the predominant form in equilibrium is HCO_3^- and CO_2 availability is limited. As such, the periplasmic CA would play a significant role by speeding the reacquisition of C_i species equilibrium when external CO_2 levels are reduced by active transport into the cell. Although it has been shown that cells can transport HCO_3^-, the preferred form of C_i that is taken up by *C. reinhardtii* is CO_2 (Sultemeyer et al., 1989; Palmqvist et al., 1990). Our data on pH regulation of CA expression support these earlier observations and clearly show that the pH effect takes place by modification of transcript abundance.

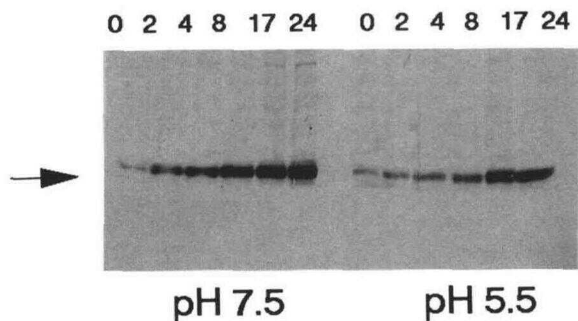

Figure 5. Effect of pH on induction of CA protein. *C. reinhardtii* cells were grown at high CO_2 levels and transferred to air in medium at pH 7.5 or 5.5. Total soluble proteins were extracted at 0, 2, 4, 8, 17, and 24 h after transfer and subjected to western analysis as described in Figure 2. The arrow indicates the position of the 37-kD CA monomer.

CONCLUSIONS

The data indicate that both acetate addition to the medium and low pH levels can reduce *cah-1* transcript abundance, and this results in lower CA protein levels and activity. The effect of acetate addition parallels the effect of increasing extracellular CO_2 concentration and is correlated with a significant increase in respiration and a minor decrease in photosynthesis. The increased respiratory activity in concert with reduced photosynthetic demand may raise intracellular CO_2 concentrations and thus repress the induction of CA expression. The increased CA activity at high pH appears to be required for the maintenance of the HCO_3^-/CO_2 equilibrium. Again, induction of CA seems to be a function of the rate of CO_2 supply available for transport. Without CA, the amount of CO_2 for transport is lower at alkaline pH when compared with levels obtained at acidic pH values. The mechanism(s) by which CO_2 regulates CA expression seems to be central for the regulation of activity of this enzyme in *C. reinhardtii*. Analysis of the upstream regions of the CA genes and a concomitant investigation of the transduction pathway between CO_2 and the response at the transcriptional level are required for a better understanding of this process.

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