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Carbonic Anhydrase Activity in Isolated Chloroplasts of Wild-Type and High-CO_2-Dependent Mutants of *Chlamydomonas reinhardtii* as Studied by a New Assay[1]

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In an assay of carbonic anhydrase (CA), $NaH^{14}CO_3$ solution at the bottom of a sealed vessel releases $^{14}CO_2$, which diffuses to the top of the vessel to be assimilated by photosynthesizing *Chlamydomonas reinhardtii* cells that have been adapted to a low-CO_2 environment. The assay is initiated by illuminating the cells and is stopped by turning the light off and killing the cells with acid. Enzyme activity was estimated from acid-stable radioactivity. With bovine CA, 1.5 Wilbur-Anderson units (WAU) was consistently measured at 5- to 6-fold above background. Sonicated whole cells of air-adapted wild-type *C. reinhardtii* had 740 ± 12.4 WAU/mg chlorophyll (Chl). Sonicated chloroplasts from a mixotrophically grown wall-less strain, cw-15, had 35.5 ± 2.6 WAU/mg Chl, whereas chloroplasts from wall-less external CA mutant strain cia-5/cw-15 had 33.8 ± 1.9 WAU/mg Chl. Sonicated chloroplasts from the wall-less mutant strain cia-3/cw-15, believed to lack an internal CA, had 2.8 ± 3.2 WAU/mg Chl. Sonicated whole cells from cia-3/cw-15 had 2.8 ± 7.8 WAU/mg Chl. Acetazolamide, ethoxyzolamide, and *p*-aminomethylbenzene sulfonamide (Mafenide) at 100 μM inhibited CA in sonicated chloroplasts from cia-5/cw-15. Treatment at 80°C for 10 min inhibited this CA activity by 90.8 ± 3.6%. Thus, a sensitive ^{14}C assay has confirmed the presence of a CA in cw-15 and cia-5/cw-15 chloroplasts and the lack of a CA in cia-3/cw-15 chloroplasts. Our results indicate that HCO_3^- is the inorganic carbon species that is accumulated by chloroplasts of *Chlamydomonas* and that chloroplastic CA is responsible for the majority of internal CA activity.

The unicellular, eukaryotic green alga *Chlamydomonas reinhardtii*, grown photoautotrophically at air levels of CO_2 (0.03%), has a much higher affinity for CO_2 than cells grown photoautotrophically under high CO_2 or mixotrophically in acetate medium (Badger et al., 1980; Spalding and Ogren, 1982; Tsuzuki, 1983; Moroney and Tolbert, 1985; Moroney et al., 1985; Aizawa and Miyachi, 1986; Badger, 1987). C_I accumulation in these cells has been attributed to a C_I-concentrating system composed of abundant CA activity (Spalding et al., 1983a; Spencer et al., 1983), as well as an active transport system for C_I (Spalding et al., 1983b). The appearance of an external CA within one of the outermost layers of the cell wall (Moroney et al., 1985; Yagawa et al., 1986) in response to adaptation to low levels of CO_2 correlates well with the appearance of the C_I-concentrating mechanism (Kimpel et al., 1983; Spencer et al., 1983; Coleman et al., 1984; Yang et al., 1985).

Several *Chlamydomonas* mutants have been isolated that require elevated CO_2 for growth and are defective in some component of the C_I-concentrating system (Spalding et al., 1983a, 1983b; Moroney et al., 1986, 1987b). These mutants have been assigned to three complementation groups (Moroney et al., 1986), one of which appears to define an internal CA function. An additional mutant, cia-5, appears to be a lesion in a regulatory component of the system (Moroney et al., 1989). cia-5 requires high CO_2 for growth, has no detectable external CA by immunoreactive or enzymic measurements, does not accumulate C_I above that expected from diffusion, and does not synthesize any of four ^{35}S-labeled polypeptides made by wild-type *C. reinhardtii* when cells have been switched to low-CO_2 conditions. However, the presence of chloroplastic CA has been difficult to determine because of limitations of the electrometric method (G.L. Katzman, unpublished data).

Studies have shown correlative evidence that a chloroplastic CA is present in *C. reinhardtii* (Spalding et al., 1983a; Moroney et al., 1985, 1987b; Husic et al., 1989). Theoretically, a chloroplastic CA would be necessary to provide CO_2 substrate for Rubisco, prevent O_2 inhibition of CO_2 fixation, and thus permit maximal rates of photosynthesis at low external CO_2 concentrations. Overall total CA activity increases upon lysis of cells (Spalding et al., 1983a, 1985; Tsuzuki et al., 1984). Intact chloroplasts isolated from air-adapted cells of *Chlamydomonas, Dunaliella,* and *Euglena* are capable of C_I accumulation, but the possibility of extrachloroplastic C_I transport still remains (Moroney et al., 1987a; Suzuki et al., 1987; Goyal and Tolbert, 1989).

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Abbreviations: AZ, acetazolamide; CA, carbonic anhydrase; C_I, inorganic carbon (CO_2 plus HCO_3^- plus CO_3^{2-}); EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid); EZ, ethoxyzolamide; Mafenide, *p*-aminomethylbenzene sulfonamide; WAU, Wilbur-Anderson unit.

In this paper, a sensitive assay for CA activity is presented which uses light-dependent $^{14}CO_2$ uptake by air-adapted *C. reinhardtii* wild-type cells. These cells act as a trap for gaseous $^{14}CO_2$ released by CA from an aqueous mixture of [^{14}C]-$Na^+HCO_3^-$ and the unknown sample. Although MS has been used by several laboratories to measure CA activity, this paper presents an alternative that is simpler and less expensive.

MATERIALS AND METHODS

Algal Strains, Culture Conditions, and Chemicals

The *Chlamydomonas reinhardtii* wild-type strain used was 137c (mt+). The double mutant cia-5/cw-15 (mt+) was derived from a cross of cia-5 (mt+) and cw-15 (mt−) in the laboratory of Robert K. Togasaki. The cell wall-less mutant cw-15 (mt−) was obtained from the Chlamydomonas Genetics Center at Duke University (Durham, NC). The double mutant cia-3/cw-15 (mt−) was derived form a cross of cia-3 (mt+) and cw-15 (mt−) in the laboratory of James V. Moroney. All cells were grown mixotrophically in 300 mL of Tris-acetate phosphate (growth) medium (Gorman and Levine, 1965) in 500-mL Erlenmeyer flasks, constantly shaken at 22°C under daylight fluorescent light with an incident light intensity of 2200 lux. Wild-type cells were then washed twice in minimal medium (Sueoka, 1960) and induced overnight. The chemicals and their manufacturers are as follows: [^{14}C]$Na^+HCO_3^-$, ICN (Costa Mesa, CA); Bio Safe II, Research Products International (Mount Prospect, IL); Percoll, Pharmacia; and bovine CA (catalog No. C-7500), Mafenide, and AZ, Sigma. EZ was a generous gift from Upjohn Co. (Kalamazoo, MI).

Chloroplast Isolation

Chloroplasts were prepared from cw-15, cia-3/cw-15, and cia-5/cw-15 essentially as described by Belknap (1983). Cultures at a cell density of 3×10^6 to 5×10^6 cells/mL were harvested at 2500*g* briefly (by hand-braking the rotor as soon as the centrifuge reached the desired speed), washed in an equal volume of medium, and harvested again in the same manner. The cells were then washed with 35 mL of Hepes buffer (pH 7.0), centrifuged briefly at 2500*g*, and resuspended in 6 mL of chloroplast isolation buffer (300 mM sorbitol, 50 mM Mes-Tris buffer [pH 7.5], 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 3 mM KH_2PO_4, and 1% [w/v] BSA [Sigma A-4503], 25°C, final pH 7.2). This cell suspension was disrupted in a Yeda pressure cell by rapidly releasing it into a 50-mL plastic Oak Ridge tube after equilibrating for 3 min under nitrogen at 65 psi (except cia-3/cw-15, which was equilibrated at 35 psi) and subsequently retained on ice as the pressate. A gradient of two steps of 70 and 45% (v/v) Percoll in chloroplast isolation buffer was prepared by loading 4 mL of each step into a 15-mL Corex tube, followed by 3 mL of pressate. Gradients were then centrifuged at 12,000*g* for 10 min at 4°C. The gradient pattern was: whole cells pelleted at the bottom, intact chloroplasts banding at the 70/45% interface, and broken chloroplasts banding at the pressate/45% interface. The middle, intact chloroplast band was extracted, diluted 50-fold with chloroplast isolation buffer minus BSA, and centrifuged briefly at 2500*g* as described above. Each chloroplast pellet was resuspended in 1.5 to 3.0 mL of 22 mM EPPS buffer (pH 8.2) prior to assay.

Sample Preparation

Resuspended chloroplasts from cw-15, cia-3/cw-15, and cia-5/cw-15 were kept on ice and sonicated twice for 15 s each time with a Fisher Sonic Dismembrator, model No. 150 set at 60%, using a chilled microtip.

To assay external CA activity, wild-type cells were shifted from growth medium to minimal medium, after washing twice with minimal medium, and allowed to induce overnight. These cells were taken directly from culture and sonicated as described above except the number of sonications was increased from two to four for 15 s each time.

Controls consisted of deionized, distilled water (0 WAU or blank) and bovine CA calibrated to 150 WAU/mL (described below) with deionized, distilled water as the diluent. Every round of the assay was accompanied by 0 and 1.5 WAU controls.

CA Assay

Bovine CA (150 WAU/mL) was calibrated using the electrometric method of measuring the rate of H^+ production by CO_2 at 2°C, with an initial pH of 8.1 (Wilbur and Anderson, 1948). The remaining measurements were obtained using the ^{14}C assay method. Induced wild-type cells in minimal medium were harvested at 4000*g* for 5 min and resuspended in minimal medium to a final cell concentration of 5×10^8 cells/mL. The best results were obtained with an early log-phase wild-type suspension culture initiated from 3- to 4-d-old cells. Discs of 1.3-cm diameter were cut from Whatman GF/B glass microfiber filters using a No. 7 cork borer and placed at the bottom of a scintillation vial (Fig. 1). Fifty microliters of the induced wild-type cell suspension (2.5×10^7 cells/disc) was then placed onto the center of the fiberglass disc to act as a CO_2 trap (trap vial). A second scintillation vial contained 100 μL of 22 mM EPPS (pH 8.2) and 100 μL of sample (in 22 mM EPPS, pH 8.2). In the AZ, EZ, and Mafenide inhibitor studies, 90 μL of EPPS buffer and 10 μL of inhibitor were used. The trap vial was inverted over the sample vial, and the vials were sealed together with rubber tubing (Fig. 1). The entire assembly was placed on ice with the sample side down, in the dark, until use.

The assay apparatus is shown in Figure 2, except two blocks yielding eight positions were actually used (one block

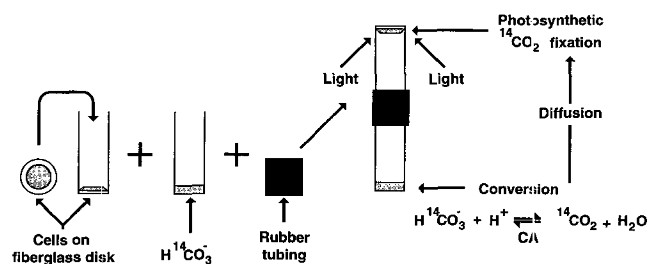

Figure 1. Preparation of sample vials for CA assay.

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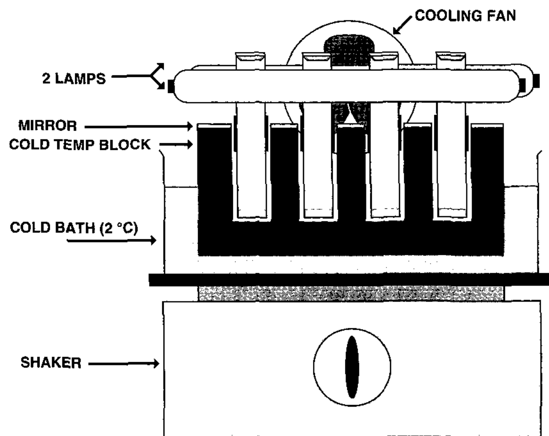

Figure 2. CA assay system. Sample assemblies shown in Figure 1 were placed into the temperature blocks upon which a mirror has been fixed. The blocks rest in a cold bath kept at 1 to 4°C, and the bath is rotated at approximately 150 rpm by a gyratory shaker. Two lamps, lowered on both sides of the sample assemblies, were turned on to produce an incident light intensity of at least 2000 lux. Simultaneously, two fans placed perpendicular to, and behind, the apparatus were switched on to assure that heat produced by the lamps was quickly dissipated. There were holes in the block so that eight assays could be run simultaneously. Assays were performed as described in "Materials and Methods."

has been omitted from the figure for the sake of simplicity), and is as follows. Sample assemblies were placed into the temperature blocks upon which a mirror had been fixed. The blocks rested in a dish half-filled with water/ethylene glycol maintained at 1 to 4°C by a Lauda circulating water bath (model K-2/RD; Westbury, NY), and the dish was rotated at approximately 150 rpm by a Sarstedt TPM-2 gyratory shaker (Newton, NC). A commercial version of this apparatus is now available from Jordan Scientific (Bloomington, IN). For illumination, two Sylvania Cool White F20T12/CW lamps, lowered on both sides of the sample assemblies, were turned on, producing an incident light intensity of at least 2000 lux. Two Dayton 4.5-inch fans (model No. 4C550; Chicago, IL) placed perpendicular to, and behind the apparatus, assured that heat produced by the lamps was quickly dissipated.

The assay procedure was begun by moving the sample assemblies from dark storage into a position in a block. The lamps were turned on for a 3-min pre-illumination period, after which the lamps were turned off and removed from the apparatus. Each sample assembly was successively removed from the block, the trap vial was detached, and 50 μL of [^{14}C]-$Na^+HCO_3^-$ (50 μmol 25 μCi^{-1} mL^{-1}) were added to the sample vial. The vials were then reassembled and placed back in the same position in the block. The lamps were lowered back onto the apparatus and immediately turned on for a 6-min CO_2 fixation period (except where noted otherwise), after which the lamps were turned off and removed. Each sample assembly was quickly buried in ice and covered with a black cloth. The sample assemblies were then disassembled in a hood, and 50 μL of glacial acetic acid was added to both vials, which were then placed onto a hot plate until dry. Bio-Safe II (4 mL) was added to each trap vial, and acid-stable radioactivity was measured for 10 min in a Beckman LS 9000 scintillation counter.

For each round of the assay, four sample vials were prepared for each sample to be tested, and 1.5- and 0-WAU controls were always included as reference points for electrometrically calibrated CA and background data, respectively. Values for the four vials were used to calculate a mean and SD for each sample.

Calculations

After each round, the blank cpm mean was subtracted from the sample cpm mean and the precalibrated 1.5-WAU cpm mean, and a ratio of 1.5-WAU cpm to sample cpm was established, yielding the WAU present in the sample. This number was then corrected for mg of Chl used, yielding a final WAU/mg Chl number comparable to numbers found using the electrometric method of assaying. Each sample type was tested on at least three occasions, after which an overall mean of the individual sample means was established, and the SE was calculated (SE = SD of the means/square root of n, where n is the number of the means; Ott, 1977).

RESULTS

Electrometric Calibration of Bovine CA

Bovine CA was dissolved with water and calibrated to 150 WAU/mL using the Wilbur-Anderson electrometric method of CA assay (Wilbur and Anderson, 1948). This bovine CA was then stored in 100-μL aliquots at −20°C in 1.5-mL Eppendorf tubes. Before use, the aliquots were thawed and diluted to 15 WAU/mL using 900 μL of water, and 100 μL (1.5 WAU) was used in each individual assay. This was done to ensure comparability between the two assays.

Determination of ^{14}C Assay Conditions

Bovine CA at 5.0, 1.5, 0.5, and 0 WAU was assayed for 3, 6, and 12 min, and the sample cpm results were graphed versus time, as was the (sample cpm/blank cpm). All WAU tested displayed linear fixation for the full 12 min, and 6 min was chosen as the optimum time of fixation for future assay work. This point was found to have a large difference between the sample cpm and the blank cpm, yet retained a fairly low signal-to-noise ratio (data not shown).

C. reinhardtii cells that act as the CO_2 trap in the sample vials (Fig. 1) were tested at cell concentrations of 0.28×10^7, 0.83×10^7, 2.5×10^7, and 5.0×10^7 cells/disc for their ability to fix $^{14}CO_2$ released by 5.0 WAU of bovine CA. A density of 2.5×10^7 cells/disc was found to fix the greatest amount of $^{14}CO_2$, with no increase apparent at 5.0×10^7 cells/disc (data not shown). Therefore, 2.5×10^7 cells/disc was chosen as the trap-cell density for the assay. Uniform labeling of the cell traps in all eight positions of the assay apparatus was subsequently shown by testing equal-WAU bovine CA samples (data not shown).

All testing of these assay conditions was carried out using a 22 mM EPPS (pH 8.2) buffer to retain uniformity with the electrometric method and to help maintain the bicarbonate form of the [^{14}C]$Na^+HCO_3^-$ added to the samples tested.

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EPPS (22 mM) buffers at pH 8.6 and 9.0 were found to decrease significantly the signal-to-noise ratio by decreasing the difference between the cpm fixed by the blank and the sample (data not shown). Therefore, 22 mM EPPS (pH 8.2) was chosen as the buffer for all subsequent assays.

The sensitivity of the new assay was determined using the above conditions by assaying bovine CA at 10.0, 5.0, 3.0, 1.5, 0.5, and 0 WAU (Fig. 3). Saturation of the assay was evident at 3.0 WAU. The results were linear from 0 to 1.5 WAU, and subsequent assay samples were thus measured in this range. Denatured bovine CA had no activity (data not shown).

Chloroplast Isolation

Optimal pressure used during the Yeda press breakage was found to be 65 psi for both cw-15 and cia-5/cw-15 and 35 psi for cia-3/cw-15. At these pressures, microscopic observation revealed the highest ratio of cup-shaped chloroplasts to whole cells, broken cells, and broken chloroplasts in the pressate (data not shown). After the Percoll was removed from the chloroplasts, they were resuspended in 22 mM EPPS (pH 8.2); Chl determination showed a 0.2- to 0.6-mg yield per preparation (approximately 10–30% recovery).

CA Assay Results

Sonicated whole cells of wild-type *C. reinhardtii* that had been induced overnight in minimal medium yielded 740 ± 12.4 WAU/mg Chl (Table I), which is comparable to results obtained using the electrometric method of CA activity measurement. Therefore, we can directly compare data collected by our new assay with the data base currently available in the literature.

Chloroplast sonicate from cw-15 grown on growth medium gave 35.5 ± 2.6 WAU/mg Chl (Table I) and demonstrates the presence of a chloroplastic CA in *C. reinhardtii* cells. cw-15 was used as the source of wild-type chloroplasts because external CA was easily and efficiently washed away in this wall-less mutant and because chloroplasts are exceedingly difficult to isolate from wild-type *C. reinhardtii* cells.

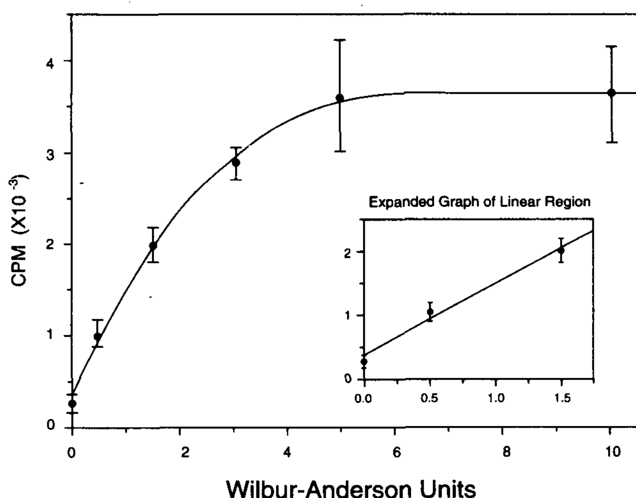

Figure 3. Saturation curve of assay. Sensitivity of the assay was determined by assaying bovine CA at 10.0, 5.0, 3.0, 1.5, 0.5, and 0 WAU. Results were graphed as cpm versus bovine CA activity (WAU).

Table I. *Assay results for wild-type and mutant strains of C. reinhardtii*

[^{14}C]CA assays were performed on the following samples: sonicated chloroplasts isolated from cw-15, cia-3, and cia-5 cells from growth medium; sonicated whole washed cells of cia-3/cw-15 from growth medium; and sonicated whole cells from wild-type cells induced overnight in minimal medium.

Strain	Sample Preparation	Enzyme Activity
		WAU/mg Chl
Wild type	Whole-cell sonicate	740 ± 12.4
cw-15	Whole-chloroplast sonicate	35.5 ± 2.6
cia-5/cw-15	Whole-chloroplast sonicate	33.8 ± 1.9
cia-3/cw-15	Whole-chloroplast sonicate	2.8 ± 3.2
cia-3/cw-15	Whole-cell sonicate	2.8 ± 7.8

The high-CO_2-dependent mutant, cia-3, which is allelic to the mutant ca-1 described by Spalding et al. (1983a), was crossed with cw-15 to yield the double mutant cia-3/cw-15 (mt−). cia-3 requires high CO_2 for growth despite the fact that it accumulates C_I to very high levels when exposed to low-CO_2 conditions (Moroney et al., 1987b). This ability to accumulate C_I implies that cia-3 can make a functional C_I transport system when adapting to low CO_2. This idea was supported by the observations that cia-3 made normal levels of the external CA (Moroney et al., 1987b). A deficiency of chloroplastic CA, which would normally draw upon the C_I pool within the chloroplast to supply CO_2 to Rubisco, could explain this overaccumulation of C_I. Chloroplast sonicate of cia-3/cw-15 had 2.8 ± 3.2 WAU/mg Chl (Table I), thus confirming the absence of chloroplastic CA activity.

Previous work with the putative regulatory mutant cia-5 suggested that it may contain some CA activity, but the amount was low and the measurements were not reproducible (data not shown). To study the mutant further and to determine the cellular location of any CA activity, cia-5 was crossed to cw-15. The chloroplast sonicate of this strain showed 33.8 ± 1.9 WAU/mg Chl (Table I). Thus, cia-5/cw-15 contains a chloroplastic CA activity that is essentially equivalent to the wild-type level found in chloroplasts isolated from cw-15.

To determine whether the activity was due to a CA, chloroplast sonicate of cia-5/cw-15 was assayed in the presence of 100 μM AZ, EZ, or Mafenide. The percentage inhibition values were as follows: AZ, 88.7 ± 7.9; EZ, 68.6 ± 9.2; Mafenide, 93.3 ± 2.2. Additionally, cia-5/cw-15 chloroplast sonicate was incubated at 80°C for 10 min, resulting in a 90.8 ± 3.6% inhibition. We can conclude that the activity we are measuring is heat labile and sensitive to classic CA inhibitors.

DISCUSSION

Although the existence of an external CA in *C. reinhardtii* residing in the periplasmic space or cell wall is firmly estab-

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lished and has been extensively characterized, the evidence for a chloroplastic CA has been indirect. Total CA activity seems to increase when *C. reinhardtii* whole cells are sonicated and CA activity has been measured upon the lysis of well-washed cw-15 cells (Husic et al., 1989). Unfortunately, these measurements have approached the limits of the electrometric method of CA assay.

Further suggestive evidence for a chloroplastic CA comes from inhibitor studies. The membrane-permeable CA inhibitor EZ increases the concentration of CO_2 required for 50% of the maximal rate of photosynthetic O_2 evolution of low- and high-CO_2-grown wild-type cells to the same high level of 60 μM, very similar to the 55 μM value of *C. reinhardtii* Rubisco, whereas the membrane-impermeable inhibitor AZ had almost no effect. EZ also causes accumulation of C_I in high-CO_2-grown cells at pH 5.1 to higher levels than normally observed, because of the passive diffusion of CO_2 into the more basic cell interior. cia-3 strongly mimics the physiological characteristics of EZ-treated wild-type cells (Spalding et al., 1983a; Moroney et al., 1986, 1987b).

cia-3, a high-CO_2-dependent mutant, displays the phenotype expected of a chloroplastic CA mutant. It contains a normal photosynthetic electron transport chain and C_3 carbon fixation cycle and normal levels of external CA, and it overaccumulates C_I. This suggests that the lack of a chloroplastic CA to supply Rubisco with CO_2 causes this mutant phenotype and C_I overaccumulation. We have found no chloroplastic CA activity in cia-3/cw-15 chloroplasts grown on growth medium (2.8 ± 3.2 WAU/mg Chl), consistent with the hypothesis that cia-3 is a chloroplastic CA mutant. Since the capacity for CO_2 to HCO_3^- conversion is greatly reduced within the chloroplast, if CO_2 were the primary species transported across the chloroplast envelope, the absence of chloroplastic CA should have little effect or increase the rate of photosynthesis. Instead, the observed rate of photosynthesis is greatly reduced in the cia-3 mutant (Moroney et al., 1987b). The data presented in this paper are more consistent with the HCO_3^- ion being the primary C_I species transported. We have found no evidence for an active cytosolic CA. A well-washed whole-cell sonicate of growth medium grown cia-3/cw15 had little or no activity with 2.8 ± 7.8 WAU/mg Chl. In addition, the level of chloroplastic CA found in cw-15 and cia-5/cw-15 is comparable to the previously reported activity in lysed whole cells of cw-15 (Husic et al., 1989). From this we can conclude that there is no detectable cytosolic CA activity in growth-medium-grown cells and that the phenotype of cia-3 is actually that of a chloroplastic CA mutant.

Moroney et al. (1989) characterized a high-CO_2-requiring mutant, cia-5, which appears to be deficient for some regulatory element of the CO_2-inducing system because it is unable to induce any of the characteristics that make up the system. Using the ^{14}C assay, we measured normal levels of chloroplastic CA from isolated growth-medium-grown cia-5/cw-15 chloroplasts (33.8 ± 1.9 WAU/mg chl). The presence of wild-type levels of chloroplastic CA in this putative induction-deficient mutant suggests that chloroplastic CA is under a different regulation than are other components of the CO_2-concentrating system. Because this mutant does not have the 20-, 37-, 45-, or 46-kD polypeptides associated with the CO_2-inducing system in the wild-type *C. reinhardtii* described above, yet contains normal levels of chloroplastic CA activity, the chloroplastic CA must be a different, still unidentified protein. These four polypeptides are also seen in low-CO_2-grown cia-3 cells (data not shown). Since the classic CA inhibitors AZ, EZ, and Mafenide at 100 μM or treatment at 80°C for 10 min inhibited this CA activity, it seems to be a bona fide CA protein.

In this paper, we have shown statistically significant evidence that growth-medium-grown wild-type chloroplasts contain a chloroplastic CA with activity of 36.5 ± 2.6 WAU/mg Chl. With this sensitive [^{14}C]CA assay, with an effective range of measurement of 0.5 to 1.5 WAU, chloroplastic CA can be measured in noninduced *C. reinhardtii* cells. Determination of external CA activity, from induced wild-type cells, of 740 ± 12.4 WAU/mg Chl demonstrates that the assay is comparable to the electrometric assay and allows a direct comparison with published data using the electrometric assay.

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