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Periplasmic Carbonic Anhydrase Structural Gene (*Cah1*) Mutant in *Chlamydomonas reinhardtii* [1]

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To survive in various conditions of CO_2 availability, *Chlamydomonas reinhardtii* shows adaptive changes, such as induction of a CO_2-concentrating mechanism, changes in cell organization, and induction of several genes, including a periplasmic carbonic anhydrase (pCA1) encoded by *Cah1*. Among a collection of insertionally generated mutants, a mutant has been isolated that showed no pCA1 protein and no *Cah1* mRNA. This mutant strain, designated *cah1-1*, has been confirmed to have a disruption in the *Cah1* gene caused by a single *Arg7* insert. The most interesting feature of *cah1-1* is its lack of any significant growth phenotype. There is no major difference in growth or photosynthesis between the wild type and *cah1-1* over a pH range from 5.0 to 9.0 even though this mutant apparently lacks *Cah1* expression in air. Although the presence of pCA1 apparently gives some minor benefit at very low CO_2 concentrations, the characteristics of this *Cah1* null mutant demonstrate that pCA1 is not essential for function of the CO_2-concentrating mechanism or for growth of *C. reinhardtii* at limiting CO_2 concentrations.

Aquatic and soil-borne photosynthetic organisms, including *Chlamydomonas reinhardtii,* live in quite variable conditions of CO_2 availability. To survive in limiting CO_2 conditions, *C. reinhardtii* and other microalgae show adaptive changes, such as induction of a CCM (Badger et al., 1980; for review, see Spalding, 1998), changes in cell organization (Geraghty and Spalding, 1996), increased photorespiratory enzyme activity (Marek and Spalding, 1991), induction of pCA1 (*Cah1*) (Fujiwara et al., 1990; Fukuzawa et al., 1990; Ishida et al., 1993), mitochondrial CA (*Mca1* and *Mca2*) (Eriksson et al., 1996; Geraghty and Spalding, 1996), and Ccp (*Ccp1* and *Ccp2*) (Geraghty et al., 1990; Ramazanov et al., 1993; Chen et al., 1997), and transient down-regulation in the synthesis of Rubisco (Coleman and Grossman, 1984; Winder et al., 1992).

Of these the CCM is the most studied adaptive change (for review, see Spalding, 1998), because it affects photosynthetic characteristics through increasing intracellular CO_2 concentration. So far, at least C_i transport (Badger et al., 1980; Spalding et al., 1983b) and a thylakoid CA (*Cah3*) to supply CO_2 for Rubisco by dehydration of HCO_3^- (Spalding et al., 1983a; Funke et al., 1997; Karlsson et al., 1998) have been shown to be required for operation of the CCM. Characteristics resulting from the CCM include a high apparent affinity for CO_2, a low CO_2 compensation point, and reduced photorespiration due to a high ratio of CO_2 to O_2. It is not clear yet whether the induced genes (*Cah1*, *Mca1*, *Mca2*, *Ccp1*, and *Ccp2*) are required for function of the CCM.

The *Cah1* gene product, pCA1, is a Zn-containing metalloenzyme that catalyzes the interconversion of CO_2 and HCO_3^- in the cell wall space of *C. reinhardtii* (Badger and Price, 1994). There are two periplasmic CA isozymes: pCA1, which is induced by limiting CO_2, and pCA2, which is the *Cah2* gene product and is expressed only at very low abundance and only under elevated CO_2 (Fujiwara et al., 1990; Rawat and Moroney, 1991). pCA1 has been considered a candidate for involvement in the CCM because it is the most abundant gene product induced by limiting CO_2 in *C. reinhardtii*. However, evidence for any function of pCA1 in the CCM has been contradictory, with Moroney et al. (1985) arguing that pCA1 is required to supply CO_2 from HCO_3^- for rapid photosynthesis at low CO_2 concentrations and alkaline pH, and Williams and Turpin (1987) disagreeing. Among a collection of insertionally generated mutants, we found a mutant, named *cah1-1*, that showed no pCA1 protein and no *Cah1* mRNA. Here we report on the photosynthetic characteristics of this *Cah1* null mutant.

MATERIALS AND METHODS

Cell Strains, Culture Conditions, and Mating

All *Chlamydomonas reinhardtii* strains (Table I) were grown as previously described (Geraghty et al., 1990). Cells were cultured on an orbital shaker under aeration with 5% CO_2 in air (CO_2-enriched cells) or no aeration (air-adapted cells). Cell cultures were switched from elevated CO_2 to limiting CO_2 (no aeration) for 1 or 2 d of induction. All matings were performed by crossing PCA57-61 with CC1068 (Table I) according to the protocol of Harris (1989).

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Abbreviations: AZA, acetazolamide; CA, carbonic anhydrase; CCM, CO_2-concentrating mechanism; Ccp, chloroplast carrier protein; C_i, inorganic carbon; pCA1, periplasmic CA 1; pCA2, periplasmic CA 2.

Table I. *Strains*

Strain	Genotype	Description	Reference
CC125	mt^+	Wild type (137C)	Harris (1989)
CC400	*cw15* mt^+	Cell wall-less mutant	Harris (1989)
CC425	*arg2 cw15 sr-u-2-60* mt^+	Cell wall-less and Arg-requiring mutant	Harris (1989)
CC1068	*arg2 nr-u-2-1* mt^-	Arg-requiring and kanamycin resistant mutant	Harris (1989)
CC2702	*cia5* mt^+	No acclimation to limiting CO_2	Moroney et al. (1989) Spalding et al. (1991)
	ca1-1 mt^+	Defective in Cah3, thylakoid lumen CA	Spalding et al. (1983a) Karlsson et al. (1998) Funke et al. (1997)
CC1860	*pmp1-1* mt^+	Deficient in C_i transport	Spalding et al. (1983b)
CC2648	*pgp1-1* mt^+	Deficient in phosphoglycolate phosphatase	Suzuki et al. (1990)

Generation and Isolation of Mutants

Using glass bead transformation (Kindle, 1990; Davies et al., 1994), CC425 (Table I) was transformed with pArg7.8 (Debuchy et al., 1989) to generate a pool of insertional mutants on CO_2 minimal medium that lacked Arg. This plasmid has the structural gene (*Arg7*) for argininosuccinate lyase to complement the *arg2* mutation. Each of more than 7000 colonies was suspended with air minimal medium in a 1.5-mL microcentrifuge tube and grown with shaking but no aeration for 2 d. After centrifugation the supernatant of each culture was screened for a lack of pCA1 expression using a slot blot and immunodetection, as described for western blots. Mutants identified in this primary screen as having reduced pCA1 expression were screened again by western immunoblots, following SDS-PAGE of extracellular protein from larger scale cultures.

Protein Extracts, SDS-PAGE, and Western Immunoblots

Cell cultures exposed to limiting CO_2 (no aeration) for 2 d were harvested by centrifugation with a GSA rotor (5,000 rpm, 15 min, Sorvall). The supernatants were precipitated with $(NH_4)_2SO_4$ overnight, and the precipitated proteins were collected by centrifugation with a GSA rotor (10,000 rpm, 30 min), dissolved in 1× resolving gel buffer, and then desalted using spin columns (1-mL disposable syringe, Becton Dickinson) of Sephadex G-25. Protein samples were separated by SDS-PAGE with 12% polyacrylamide using the buffer system of Laemmli (1970). Separated proteins were electrophoretically transferred to nitrocellulose with a semi-dry blotting unit (Fisher Biotech, Itasca, IL) and immunodetected using affinity-purified anti-pCA1 polyclonal antiserum as the primary antibody (Roberts and Spalding, 1995), horseradish peroxidase-linked second antibody, and aminoethylcarbazole as the chromogenic substrate (Harlow and Lane, 1988).

Nucleic Acid Extractions and Analysis

Genomic DNA was isolated using Elu-Quik DNA purification kits (Schleicher & Schuell) and digested with appropriate restriction enzymes. Total RNA was prepared (Chomczynski and Sacchi, 1987) from induced cells exposed to limiting CO_2 for 1 or 2 d. Nucleic acids (10 μg) were separated in 0.8% agarose gels for Southern analysis or in 0.66 M formaldehyde-1.5% agarose gels for northern analysis, and then blotted onto nylon transfer membranes (Micron Separations Inc., Westborough, MA). After UV cross-linking, membranes were hybridized with appropriate ^{32}P-labeled probes (Random Primers DNA Labeling System, Life Technologies). Southern and northern analyses were performed according to the method of Ausubel et al. (1989).

Growth Tests and Photosynthetic O_2 Exchange

For spot growth tests, active growing cells were suspended to similar cell densities with minimal medium, spotted (10 μL) onto agar plates with different pHs, and kept at an air level of CO_2 for 10 d (Harris, 1989). Each medium was buffered with 25 mM Mes-KOH for pH 6.0, 25 mM Mops-KOH for pH 7.0, 25 mM Hepps (4–2 [-hydroxyethyl]-1-piperazine propane sulfonic acid)-KOH for pH 8.0 and 25 mM Ampso (3-(*N*-[1,1-dimethyl hydroxyethyl] amino)-2-hydroxypropane sulfonic acid)-KOH for pH 9.0.

For growth-curve tests, active, fully air-adapted cells were inoculated into different pH liquid minimal medium and buffered as described above. The cell density was determined by using a hemacytometer (Reichert Scientific Instruments, Buffalo, NY) (Harris, 1989).

For O_2-exchange measurements, 1-d-induced cells were harvested by centrifugation and resuspended in 25 mM Mops-KOH (pH 7.0) for analysis of the response to CO_2 concentration or in 25 mM of the appropriate buffer for analysis of the effects of pH. Suspended cells (1 mL) were added to an O_2 electrode (Rank Brothers, Bottisham, Cambridge, UK) at 25°C. The measurements were started by the addition of different concentrations of $NaHCO_3$ after confirmation by cessation of O_2 evolution of the depletion of C_i in medium under illumination (500 μmol photons m^{-2} s^{-1}) provided by a slide projector. O_2 concentration data were collected every second, averaged, recorded every 5 s by a 21× datalogger (Campbell Scientific, Logan, UT), and

transferred to an IBM computer. Smoothed data (Savitzky and Golay, 1964) were used for calculation of exchange rates. Maximum spontaneous CO_2 supply rates from uncatalyzed dehydration of bicarbonate were calculated as described by Miller and Colman (1980). Chlorophyll content was estimated after extraction with 96% (v/v) ethanol (Wintermans and De Mots, 1965).

RESULTS

Generation and Isolation of Mutants

A pool of more than 7000 transformants was generated by complementation transformation of an Arg-requiring mutant with the *Arg7* gene. In an attempt to identify mutants failing to adapt to limiting CO_2, this pool of potential insertional mutants was screened immunochemically for the absence of pCA1, which was being used as a reporter for induction of genes involved in adaptation to limiting CO_2. Sixty-eight putative mutants were identified in the primary screen, and 18 were selected for further characterization based on the absence of or reduction in pCA1 expression in western blots. Among this collection of insertionally generated mutants, we found a mutant (PCA57-61) that showed no detectable pCA1 protein (Fig. 1A) and no detectable *Cah1* mRNA (Fig. 1B). Since the *Cah1* cDNA probe used (Spalding et al., 1991) also detects the *Cah2* mRNA (Fujiwara et al., 1990) and the antibody used should cross react with pCA2, these blots also demonstrate that *Cah2* mRNA and pCA2 were undetectable as well. Expression of other proteins normally induced by limiting CO_2, mitochondrial CA (*Mca1* and *Mca2* genes) and Ccp (*Ccp1* and *Ccp2* genes), apparently was not affected by the mutation (data not shown).

Genomic Analysis of PCA57-61

PCA57-61 genomic DNA was compared with that of CC425 (Table I) by Southern analysis (Fig. 2). The presence of one insert in PCA57-61 was confirmed by probing with

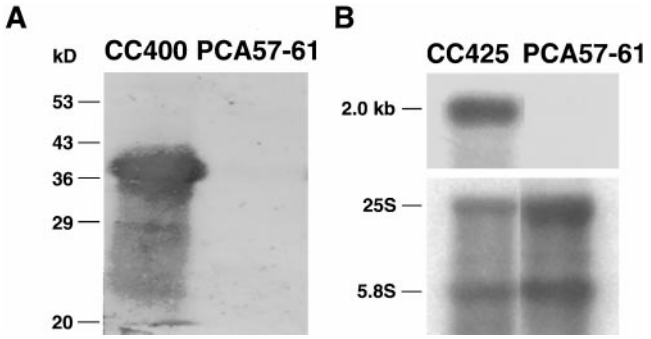

Figure 1. Western and northern analysis for wall-less "wild-type" *C. reinhardtii* (CC400 or CC425) and PCA57-61. Extracellular protein and total RNA (10 μg per lane) were isolated after adaptation of cells for 2 d in air. A, pCA1 protein detected with affinity-purified anti-pCA1 polyclonal antiserum. B, *Cah1* mRNA probed with 1.4-kb *Bgl*II and *Nco*I fragment of *Cah1* cDNA. The total RNA was probed with 25S and 5.8S rRNA. Northern analysis was performed on the same membrane, but CC425 and PCA57-61 are selectively shown.

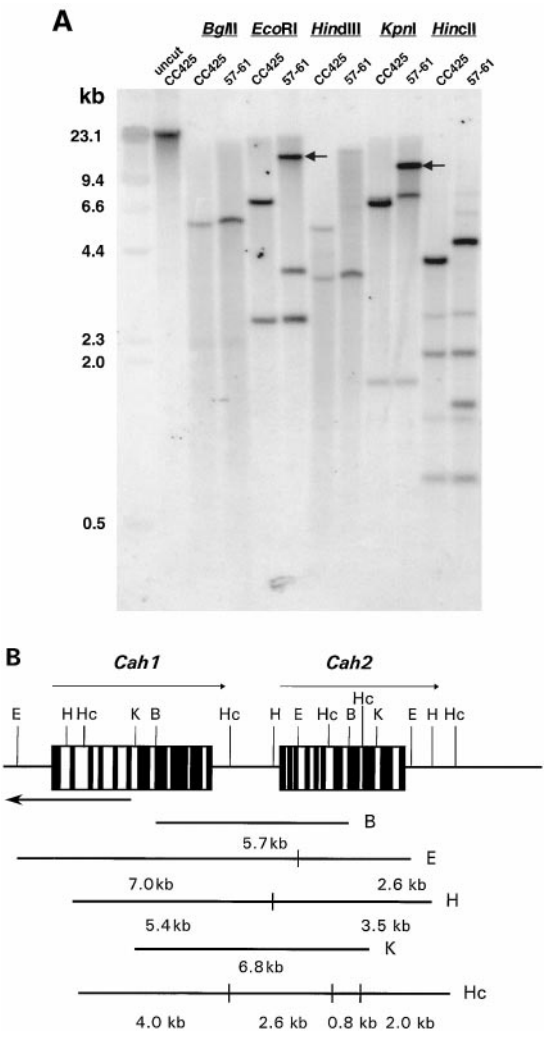

Figure 2. Southern analysis of wild-type CC425 and PCA57-61. A, Genomic DNA (10 μg per lane) was isolated, digested with different restriction enzymes, and hybridized with the full-length *Cah1* cDNA. Arrows indicate the band that hybridized both to the full-length *Cah1* cDNA and the 1.3-kb *Sal*I fragment of the *Arg7* probe. B, Restriction map of the *Cah1* and *Cah2* genomic region (adapted from Fujiwara et al., 1990). Restriction enzyme sites: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; K, *Kpn*I. The disrupted region is indicated by a bold arrow. Fragment sizes for each enzyme are indicated below the restriction map. Exons are indicated by filled boxes.

the 1.3-kb *Sal*I fragment of *Arg7* (no data shown). When the same blot was probed with the entire *Cah1* cDNA, different patterns were observed for CC425 and PCA57-61 (Fig. 2A), indicating that the *Cah1* structural gene had been disrupted. Some of the restriction fragments (indicated by arrows) apparently hybridized to both the *Cah1* and the *Arg7* probes (data not shown), indicating that the *Arg7* insert is located near the *Cah1* structural gene. *Cah1* and *Cah2* are arranged in tandem, separated by approximately 0.8 kb (Fig. 2B). The *Cah1* cDNA probe hybridizes with both genes (Fujiwara et al., 1990), so bands from both *Cah1* and *Cah2* can be seen on the Southern analysis in Figure 2A. The *Cah2* structural gene appeared undisturbed. The

Table II. *Genetic analysis of PCA57-61 progeny from a cross with CC1068*

Strain	Phenotype[a]	Cell Wall	*Arg7* Insert[b]	*Cah1* mRNA[c]	*Cah1* Polymorphism
PCA57-61 (*cah1-1*)	wt, Arg^+	−	1	−	+
CC1068	wt, Arg^-	+	0	+	−
Progeny					
6-1-1	wt, Arg^+	−	1	−	+
6-1-2	wt, Arg^+	+	1	−	+
6-1-3	wt, Arg^-	+	0	+	−
6-1-4	wt, Arg^-	−	0	+	−
10-1-1	wt, Arg^+	−	1	−	+
10-1-2	wt, Arg^-	+	0	+	−
10-1-3	wt, Arg^-	+	0	+	−
10-1-4	wt, Arg^+	−	1	−	+

[a] wt, Wild-type (5% CO_2 concentration not required for photoautotrophic growth); Arg^+, Arg not required for growth; Arg^-, Arg required for growth. [b] *Arg7* insert detected by Southern analysis using a 1.3-kb *Sal*I fragment of *Arg7* as the probe. [c] Total RNA was isolated from cells induced with limiting CO_2 for 1 d and probed with a 1.4-kb *Bg*lII/*Nco*I fragment of *Cah1* cDNA.

5.7-kb *Bgl*II fragment containing the 5′ end of the *Cah2* gene and the 3′ end of the *Cah1* gene appeared intact, but the 6.8-kb *Kpn*I fragment covering about the same area had been disrupted, along with all restriction fragments 5′ to the *Bgl*II site in *Cah1*. The simplest interpretation of these results is that the *Cah1* gene 5′ to the *Bgl*II site of *Cah1* had been disrupted by an insertion, a deletion, or a rearrangement. Since both the *Arg7* probe and the *Cah1* probe hybridized to the new *Eco*RI and *Kpn*I fragments of PCA57-61 (indicated by arrows in Fig. 2A), it appears that this disruption had been caused by insertion of the *Arg7* gene in or near the *Cah1* structural gene.

Phenotypes and Genetic Analysis of PCA57-61

The most interesting feature of PCA57-61 was its lack of any significant growth phenotype even though this mutant apparently lacked any *Cah1* expression in air. After PCA57-61 was crossed with CC1068 (Table I), progeny showed Mendalian 2:2 segregation of wild-type and Arg-requiring phenotypes (Table II). Both the absence of *Cah1* mRNA accumulation and the restriction polymorphisms of genomic DNA co-segregated with the *Arg7* insert, confirming that this insert was responsible for the absence of *Cah1* mRNA in PCA57-61 (Table II). Progeny 57-61-612 was chosen for further physiological analysis because it had the same biochemical phenotype as PCA57-61 but had a normal cell wall.

Effects of pH on Growth

Because Moroney et al. (1985) and Williams and Turpin (1987) reported contradictory results for photosynthesis at alkaline pH using either CA inhibitors (Moroney et al., 1985) or washed, wall-less cells to eliminate pCA1 activity (Williams and Turpin, 1987), a *Cah1* null mutant, such as PCA57-61, should help in resolving the function of pCA1 in the CCM. In spot tests growth of PCA57-61 and its walled progeny, PCA57-61-612, was similar to that of two wild types, one wall-less (CC400, Table I) and one walled

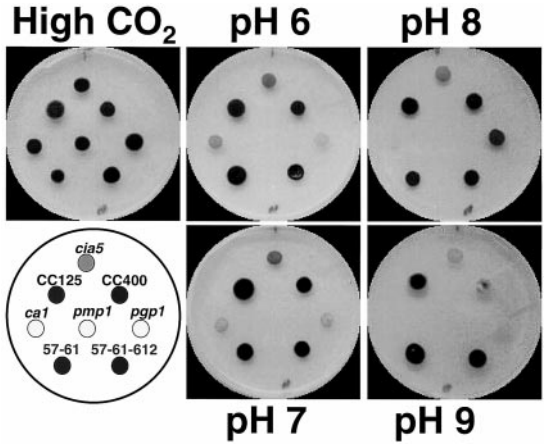

Figure 3. Spot test for growth response to different pH conditions of PCA57-61, wild-type strains (CC125 and CC400) and four high-CO_2-requiring mutants (*cia5, ca1-1, pmp1-1, and pgp1-1*). All plates (except the high CO_2 plate) were kept at air level of CO_2 for 10 d.

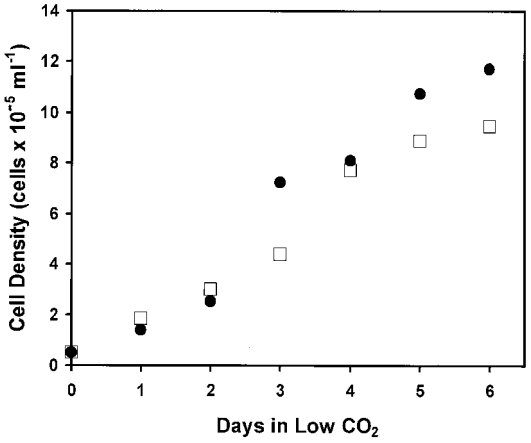

Figure 4. Cell growth curve of CC125 (□) and PCA57-61-612 (●) at pH 8.0 in air levels of CO_2. The growth curves shown are from a single experiment but are representative of three independent growth experiments.

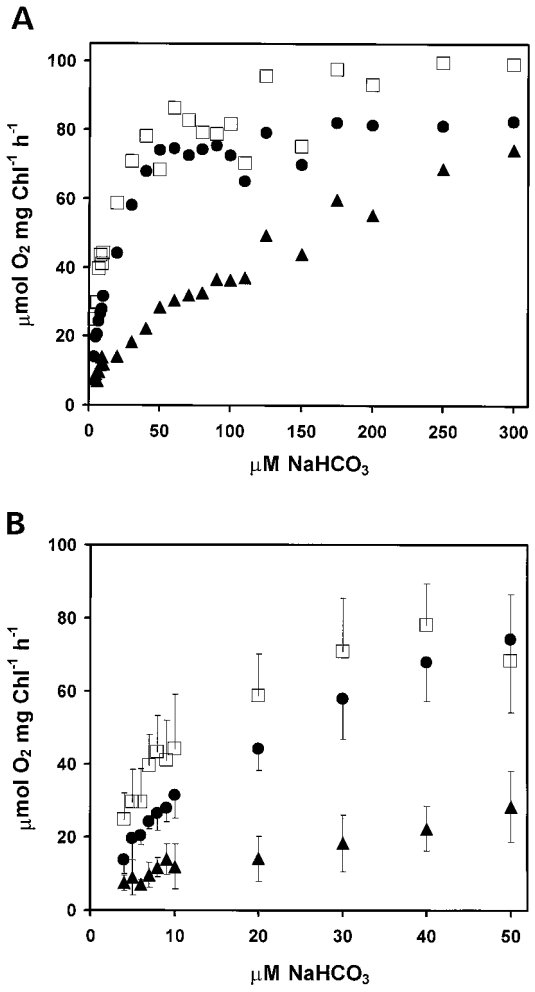

Figure 5. Photosynthetic response to $NaHCO_3$ concentration (pH 7.0) of wild-type CC125 (□), *cia5* (▲), and PCA57-61-612 (●). A, Air-adapted cells (1 d) were used for all measurements. Chlorophyll concentrations were: CC125, 11.09 μg mL^{-1}; cia5, 10.12 μg mL^{-1}; PCA57-61-612, 9.14 μg mL^{-1}. B, Expansion of the data in A at $NaHCO_3$ concentrations up to 50 μM. Three independent measurements were averaged from three different cultures. SD is indicated by error bars.

(CC125, Table I), at all pHs in air (Fig. 3). However, other known mutants with defects in the CCM and related pathways, *cia5*, *ca1-1*, *pmp1-1*, and *pgp1-1* (Table I), showed slow or no growth in air within the tested pH range. All cells grew poorly at pH 5.0 (data not shown). Both wild types, PCA57-61 and PCA57-61-612, showed variable growth at pH 9.0, as illustrated by the poor growth of CC400 at pH 9.0 in Figure 3. However, the slow growth usually caught up with that on the other pH plates. In liquid, the growth rate of PCA57-61-612 was similar to that of the CC125 wild type at pH 8.0 in air (Fig. 4) and at other pHs from 6.0 to 9.0 (data not shown). These growth tests demonstrated that there was no major difference in growth between the wild type and PCA57-61 over a pH range from 6.0 to 9.0, indicating that the mutant grows normally under low CO_2 in this pH range even without detectable pCA1.

Photosynthetic O_2 Exchange

Photosynthetic O_2 evolution of PCA57-61-612 was investigated at different concentrations of $NaHCO_3$ and at different pHs. PCA57-61-612 showed a similar pattern of photosynthetic response to $NaHCO_3$ concentration as the CC125 wild type (Fig. 5A), and there was no difference of photosynthesis rate between these strains at air levels of CO_2. At very low CO_2, however, PCA57-61-612 showed slightly lower photosynthesis rates than the wild type (Fig. 5B). The $K_{1/2}(CO_2)$ calculated from these data is higher for PCA57-61-612 (21 μM) than the wild type (11 μM). Since the O_2 evolution is measured in a closed system, these experiments used fairly low cell densities (9–11 μg chlorophyll mL^{-1}) to minimize problems with rapid C_i depletion at low C_i concentrations. At these relatively low cell densities, the rate of photosynthetic consumption in PCA57-61-612 was similar to the spontaneous CO_2 supply rate from un-

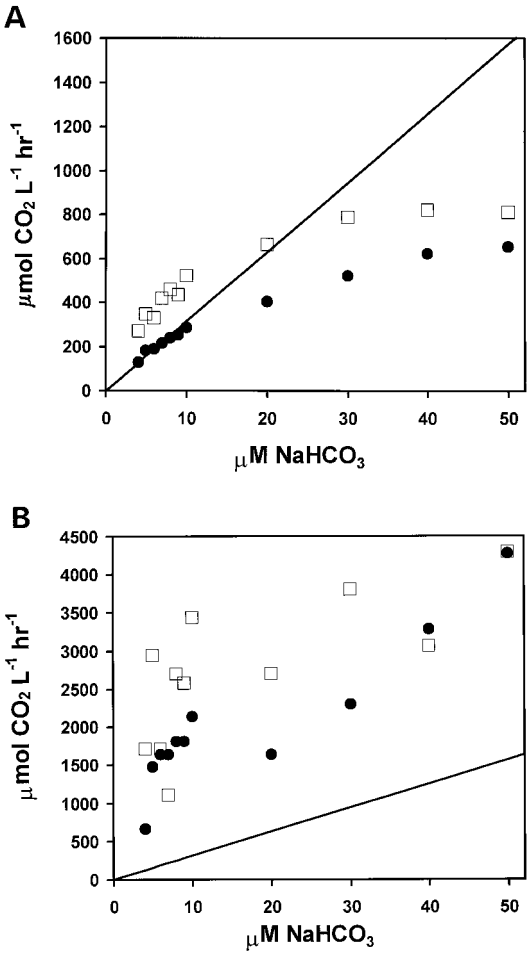

Figure 6. Photosynthetic CO_2 consumption rate of wild-type CC125 (□) and PCA57-61-612 (●) calculated from O_2 evolution assuming 1:1 O_2:CO_2. The calculated maximum spontaneous CO_2 supply rate is indicated by the diagonal line. A, Chlorophyll concentrations were: CC125, 11.09 μg mL^{-1}; PCA57-61-612, 9.14 μg mL^{-1}. Three independent measurements are averaged from three different cultures. B, Chlorophyll concentrations were: CC125, 37.53 μg mL^{-1}; PCA57-61-612, 35.11 μg mL^{-1}. One sample was measured.

Table III. *Effect of pH on the photosynthetic rate of wild-type CC125 and PCA57-61-612*

One-day air-adapted cells were resuspended in either 25 mM citrate-KOH buffer (pH 5.0), 25 mM Mes-KOH buffer (pH 6.0), 25 mM Mops-KOH buffer (pH 7.0), 25 mM HEPPS-KOH buffer (pH 8.0), or 25 mM AMPSO-KOH buffer (pH 9.0).

pH	10 μM $NaHCO_3$		75 μM $NaHCO_3$	
	CC125[a]	PCA57-61-612[a]	CC125	PCA57-61-612
	μmol O_2 mg^{-1} chlorophyll h^{-1}			
5.0	42.58 ± 9.24[b]	69.26 ± 34.16	88.62 ± 23.90	99.99 ± 29.19
6.0	56.88 ± 15.14	79.06 ± 32.08	104.31 ± 41.49	109.33 ± 37.07
7.0	50.78 ± 12.40	33.33 ± 16.28	82.04 ± 17.83	91.03 ± 27.52
8.0	16.82 ± 6.44	20.87 ± 17.66	49.44 ± 8.55	32.65 ± 9.59
9.0	8.21 ± 4.15	7.59 ± 7.01	7.83 ± 4.27	8.55 ± 6.65

[a] Chlorophyll concentrations were: CC125, 8 to 38 μg mL^{-1}; PCA57-61-612, 8 to 38 μg mL^{-1}. [b] Values are the means ± SD of four independent measurements from three different cultures.

catalyzed bicarbonate dehydration (Fig. 6A). However, this similarity is coincidental because at higher cell densities (35–38 μg chlorophyll mL^{-1}) CO_2 response curves for both strains were similar to those obtained at lower cell densities (data not shown), and the photosynthesis rates of both strains clearly exceeded the spontaneous CO_2 supply rate at all bicarbonate concentrations up to 100 μM (Fig. 6B). The apparently large difference between the calculated photosynthetic CO_2 consumption rates of PCA57-61-612 and CC125 in Figure 5A results only from the slightly lower cell density for the mutant because the rates are expressed as micromoles of CO_2 per liter per hour to match the units used for the spontaneous CO_2 supply rate. The measured rates of O_2 evolution were more variable at the higher cell density, but PCA57-61-612 maintained a rate similar to that of the wild type even though the calculated maximum spontaneous CO_2 supply rate was only one-half that of the photosynthetic rate.

Because it had been suggested that pCA1 was needed to supply CO_2 at alkaline pH (Moroney et al., 1985), photosynthetic O_2 evolution at different pHs was also investigated. Photosynthetic rates at two bicarbonate concentrations of wild type, CC125 and PCA57-61-612, decreased with increasing pH, but both showed approximately the same rate of photosynthetic O_2 evolution at the given concentration and pH (Table III). Although there was no significant difference between the wild type and PCA57-61-612 in photosynthetic O_2 evolution at any $NaHCO_3$ concentration or pH, small effects might be masked by the variation in the rate measurement. These results indicate that pCA1 is not required to supply CO_2 from HCO_3^- dehydration under the conditions used, but they do not exclude some minor benefit conferred by pCA1 at low C_i concentrations.

DISCUSSION

Like C_4 plants, *C. reinhardtii,* as well as many other microalgae and cyanobacteria, have an active CCM that allows cells to assimilate C_i efficiently when they grow under limiting CO_2 conditions. The CCM results in increased internal CO_2 concentration, which increases the substrate for Rubisco carboxylation and favors the carboxylation reaction of Rubisco over the oxygenation reaction (Badger et al., 1980; Aizawa and Miyachi, 1986; Spalding, 1998). This mechanism is inducible after transfer from high (5% CO_2 in air) to low CO_2 (0.03% CO_2 in air). As previously described, *C. reinhardtii* shows adaptive changes to limiting CO_2 conditions other than the CCM, including the induction of several major genes (*Cah1*, *Mca1*, *Mca2*, *Ccp1*, *Ccp2*) (for review, see Spalding, 1998). However, it is not clear yet whether any of these induced genes are required for function of the CCM.

pCA1 has been considered a potential candidate for an essential CCM component because large amounts of this polypeptide accumulate under low CO_2 conditions, and affinity for C_i is also increased, coincident with the induction of pCA1. For pCA1 to be essential, conversion between CO_2 and HCO_3^- would have to be a critical step for *C. reinhardtii* in terms of adapting to low CO_2, but many studies have shown that *C. reinhardtii* can use both CO_2 and HCO_3^- (Williams and Turpin, 1987; Sültemeyer et al., 1989; Palmqvist et al., 1994). On the other hand, the evidence does indicate that CO_2 is the preferred substrate (Moroney et al., 1985; Aizawa and Miyachi, 1986).

The function of pCA1 in the CCM has been controversial. Moroney et al. (1985) and Williams and Turpin (1987) arrived at contradictory conclusions regarding the need of pCA1 to supply CO_2 at alkaline pH. The photosynthetic rate of *C. reinhardtii* was significantly decreased in limiting CO_2 when pCA1 was inhibited by nominally nonpermeant CA inhibitors, but only at alkaline pH (Moroney et al., 1985). The authors interpreted this to mean pCA1 was required to supply CO_2 from HCO_3^- for rapid photosynthesis at low CO_2 concentrations and alkaline pH, because the HCO_3^- concentration is higher than that of CO_2 under these conditions. However, Williams and Turpin (1987) were unable to demonstrate decreased photosynthetic rates at alkaline pH using washed wall-less cells that lack pCA1 activity rather than using CA inhibitors. These authors concluded that the pCA1 activity is not absolutely required for utilizing C_i under alkaline pH and suggested that, in the work of Mononey et al. (1985), nominally nonpermeant CA inhibitors like AZA may have penetrated the cells and partially inhibited internal CA, which is essential for photosynthesis. Although Moroney and coworkers tried to

control for secondary effects of the CA inhibitors based on the work reported here and by Williams and Turpin, it appears that they did have effects other than inhibition of extracellular CA.

We have identified a mutant (PCA57-61) that shows no pCA1 protein and no *Cah1* mRNA (Fig. 1) but apparently normal *Mca1*, *Mca2*, *Ccp1*, and *Ccp2* mRNAs and normal levels of the corresponding proteins. This insertional mutant was isolated after transformation of strain CC425 with a plasmid containing the argininosuccinate lyase gene (*Arg7*). Southern and genetic analyses have established the presence of 1 insert in this mutant that co-segregates with the lack of *Cah1* mRNA and with polymorphisms in the 5′ region of *Cah1*. It is clear that insertion of this *Arg7* insert in the region of the *Cah1* and *Cah2* genes has disrupted the *Cah1* structural gene and that this disruption is responsible for lack of *Cah1* expression in this mutant. Thus, PCA57-61 is a *Cah1* structural gene mutant apparently null in pCA1 expression, which we have named *cah1-1*.

The most interesting feature of PCA57-61 is that it does not have a high CO_2-requiring phenotype even though it has no detectable pCA1. One might expect that the total absence of pCA1 would have a more significant effect on the growth rate of PCA57-61 at air level of CO_2, perhaps intermediate between wild type and *cia5*, a mutant that lacks *Cah1* expression along with all other induced genes (Moroney et al., 1989). Both in spot tests and in liquid culture, cell growth rates for PCA57-61 and PCA57-61-612 without detectable pCA1 were similar to those of the wild type over a pH range from 6.0 to 9.0, which argues against any essential role of pCA1 even at alkaline pH.

Consistent with the lack of any effect on growth, the measured photosynthetic rates of the mutant and the wild type were very similar over a wide range of pH values, as well (Table III). In addition, the C_i response curve of PCA57-61-612 for photosynthetic O_2 evolution at pH 7.0 was very similar to that of wild-type CC125, indicating that the lack of pCA1 had no major impact on photosynthetic rates over a range of C_i concentrations or a range of pH conditions. However, even though the photosynthetic rates of the two strains were indistinguishable at air levels of CO_2 (60 μM C_i), the mutant was found to have a slightly elevated $K_{1/2}(C_i)$ relative to wild-type (21 μM versus 11 μM) and slightly reduced photosynthetic O_2 evolution at C_i concentrations lower than 50 μM. Although not nearly as extreme as the differences reported by Moroney et al. (1985), it appears that the abundant pCA1 activity may be of some benefit under very low C_i concentrations.

The observations made here appear contradictory to the report by Moroney et al. (1985), based on inhibitor studies, that pCA1 was required to supply CO_2 from bicarbonate for rapid photosynthesis at low CO_2 concentrations, especially at alkaline pH. If pCA1 is essential to supply CO_2 through dehydration of bicarbonate, this should be most evident under conditions where the photosynthetic rate of the wild type clearly exceeds the calculated maximum spontaneous CO_2 supply rate. However, as demonstrated in Figure 5B, the *Cah1* null mutant showed a photosynthetic rate similar to that of wild-type CC125 even though the spontaneous CO_2 supply rate was only one-half the rate of photosynthesis. This could possibly be explained by residual CA activity from pCA2, but *Cah2* expression should be repressed under these low CO_2 growth conditions (Fujiwara et al., 1990; Rawat and Moroney, 1991), as confirmed by our inability to detect either the *Cah2* mRNA or the pCA2 protein. It seems more likely that, although *C. reinhardtii* apparently prefers CO_2 (Moroney et al., 1985; Aizawa and Miyachi, 1986), PCA57-61-612 must be using bicarbonate directly from the medium. These results confirm those of others (Sültemeyer et al., 1989; Palmqvist et al., 1994) including Williams and Turpin (1987), who reported similar findings using wall-less *C. reinhardtii* washed free of pCA1.

We conclude that, although some benefit may be derived from the presence of pCA1 at very low C_i concentrations, the benefit appears less substantial than that reported by Moroney et al. (1985), and this protein certainly does not appear to be essential either for function of the CCM or for growth of *C. reinhardtii* at limiting CO_2 concentrations. These conclusions beg the question, therefore, of why the expression level of pCA1 is so high if this protein provides only minimal benefit for photosynthesis and growth. It is possible that further work with this *Cah1* null mutant will allow the identification of conditions under which pCA1 provides more substantial benefits.

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