Identification of a New Chloroplast Carbonic Anhydrase in *Chlamydomonas reinhardtii*[1]

Mautusi Mitra, Scott M. Lato, Ruby A. Ynalvez, Ying Xiao, and James V. Moroney*

Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803

Carbonic anhydrases (CA) are zinc-containing metalloenzymes that catalyze the reversible hydration of CO_2. The three evolutionarily unrelated families of CAs are designated α-, β-, and γ-CA. Aquatic photosynthetic organisms have evolved different forms of CO_2 concentrating mechanisms (CCMs) to aid Rubisco in capturing CO_2 from the surrounding environment. One aspect of all CCMs is the critical roles played by various specially localized extracellular and intracellular CAs. Five CAs have previously been identified in *Chlamydomonas reinhardtii*, a green alga with a well-studied CCM. Here we identify a sixth gene encoding a β-type CA. This new β-CA, designated Cah6, is distinct from the two mitochondrial β-CAs in *C. reinhardtii*. Nucleotide sequence data show that the *Cah6* cDNA contains an open reading frame encoding a polypeptide of 264 amino acids with a leader sequence likely targeting the protein to the chloroplast stroma. We have fused the *Cah6* open reading frame to the coding sequence of maltose-binding protein in a pMal expression vector. The purified recombinant fusion protein is active and was used to partially characterize the Cah6 protein. The purified recombinant fusion protein was cleaved with protease Factor Xa to separate Cah6 from the maltose-binding protein and the purified Cah6 protein was used to raise an antibody. Western blots, immunolocalization studies, and northern blots collectively indicated that Cah6 is constitutively expressed in the stroma of chloroplasts. A possible role for Cah6 in the CCM of *C. reinhardtii* is proposed.

Carbonic anhydrase (CA) is a zinc-containing metalloenzyme that catalyzes the reversible interconversion of CO_2 and HCO_3^-. The enzyme was first discovered in human erythrocytes (Meldrum and Roughton, 1933) but has since been found in many organisms, including animals, plants, archaebacteria, and eubacteria (Hewett-Emmett and Tashian, 1996). CA plays an important role in many physiological functions that involve decarboxylation or carboxylation reactions, including both photosynthesis and respiration. It also participates in the transport of inorganic carbon (C_i) to actively photosynthesizing cells or away from actively respiring cells (Henry, 1996). The known CAs can be grouped broadly into three independent families (Hewett-Emmett and Tashian, 1996) called α-CA, β-CA, and γ-CA. These three families have no significant sequence identities and are an example of convergent evolution of catalytic function (Hewett-Emmett and Tashian, 1996). Most α-CAs are active as monomers of about 30 kD with three His coordinating the zinc atom and are generally highly susceptible to inhibition by sulfonamide compounds (Moroney et al., 1985, 2001). All active β-CAs are either monomers, or homo/heteromers with a His and two Cys residues that serve as the zinc coordinating residues (Bracey

et al., 1994; Rowlett et al., 1994). The structure of the γ-CA is remarkably different from that of α-CA or β-CA (Alber and Ferry, 1994). The γ-CA functions as a trimer of identical subunits. As in α-CAs, three His coordinate the zinc atom but the His residues are provided by two subunits. Like the α-CAs, γ-CAs are highly sensitive to sulfonamide compounds (Moroney et al., 1985, 2001). Although the primary sequences of these CA families are different, the active sites of these three types of CAs contain Zn^{2+} and all of them employ a two-step catalytic mechanism (Lindskog, 1997).

Five CAs have previously been identified in the green alga *Chlamydomonas reinhardtii* (Moroney et al., 2001). These known CAs include three α-CAs and two β-CAs. Two of these α-CAs, Cah1 and Cah2, are localized to the periplasmic space (Fujiwara et al., 1990; Fukuzawa et al., 1990). Cah1 and Cah2 are very similar proteins although they are differentially regulated. *Cah1* is expressed under low CO_2 conditions (0.035% CO_2 [v/v]) but not under high CO_2 (5% CO_2 [v/v] in air) conditions. The role of Cah1, the most abundant CA in *C. reinhardtii,* is to facilitate the movement of CO_2 across the plasma membrane, particularly when the pH in the medium is alkaline (Moroney et al., 1985). Cah2 is present in much lower amounts. *Cah2* is poorly expressed under low CO_2 and only slightly up-regulated under high CO_2 conditions. The third α-CA, Cah3, is localized to the thylakoid lumen (Karlsson et al., 1998). Mutants defective in Cah3 require elevated CO_2 levels for growth compared to wild-type cells. The primary function of Cah3 is to provide CO_2 to Rubisco (Hanson et al., 2003), which is localized to the pyrenoid of the chloroplast (Borkhsenious et al., 1998).

Figure 1. The genomic map of *Cah6*. *Cah6* is 2,886 bp in length. The arrows represent the four exons while the horizontal line represents the three introns. The start and stop codons are labeled by black vertical lines.

Two β-CAs have also been identified in *C. reinhardtii* (Eriksson et al., 1996). Both of these β-CAs are localized to the mitochondria. Expression of the β-CAs in *C. reinhardtii* is strongly influenced by the CO_2 concentration during growth (Eriksson et al., 1998) as these CAs are induced strongly under low CO_2 conditions but not under high CO_2 conditions. This report describes the identification of a newly discovered β-CA gene (*Cah6*) in *C. reinhardtii* and the partial biochemical characterization of the recombinant Cah6 protein. Sequence and immunolocalization studies suggest that the Cah6 protein is targeted to the chloroplast stroma. The expression of *Cah6* is constitutive and is slightly up-regulated under low CO_2 (air) conditions.

RESULTS

Screening of the Cosmid and cDNA Library for Genomic and cDNA Clones of *Cah6*

To find new β-CA genes, the mitochondrial β-CA protein (Ca1) sequence of *C. reinhardtii* was used to BLAST the expressed sequence tag (EST) database of *C. reinhardtii*. The search yielded many ESTs that were the gene products of the mitochondrial β-CA genes. However, several other ESTs also were found that, while encoding a β-CA, did not belong to either of the two known mitochondrial β-CA genes. These ESTs were from the same gene and were analyzed by contig assembly program (CAP; http://www.infobiogen.fr/services/analyseq/cgi-bin/cap_in.pl) to form a consensus *Cah6* sequence. In order to amplify *Cah6* and screen for the Cah6 cDNA, several PCR primers were designed based on this *Cah6* contig.

The PCR primers F4 and R5 were used on an indexed cosmid library to isolate a cosmid carrying the *Cah6* gene (Colombo et al., 2002). After several rounds of screening by PCR, two cosmid clones, designated 72-E-6 and 29-D-12, were isolated. Positive cosmid clones were verified as *Cah6* clones after each round of screening by PCR, followed by sequencing of the PCR product using different *Cah6* primers. PCR using primers F4 and R5 yielded a product of 2.8 kb when used on the isolated cosmid clones. The same 2 primers used on the cDNA core library yielded a PCR product of 2.4 kb which is 6 bp short from being a full-length Cah6 cDNA clone (excluding the poly A tail).

Sequencing and Homology Search

The cosmid clones 72-E-6 and 29-D-12, and the 2.4-kb cDNA PCR product mentioned above were se-

quenced in both directions. The sequencing results were confirmed by the EST and genomic Chlamydomonas databases (http://www.biology.duke.edu/chlamy_genome/). The *Cah6* gene is 2,886-bp long with 4 exons and 3 introns (Fig. 1). The exons range in size from 93 bp to 1,652 bp while the introns range from 75 bp to 189 bp long. The genomic and cDNA sequence of Cah6 can be obtained from the GenBank (accession nos. AY463238 and AY463239).

The *Cah6* cDNA is 2,452-bp long and encodes a putative protein of 264 amino acids. It contains a translation start site at nucleotide 299 and a stop site at nucleotide 1,091. It has an extremely long 3′ untranslated region (UTR) of more than 1,300 bp. Predictions based on various protein prediction programs (SORT P, CHLOR P, AND TARGET P) listed under ExPasy tools (http://ca.expasy.org/tools/#translate) indicate that the Cah6 protein is likely to be targeted to the chloroplast, as the protein contains a putative chloroplastic transit peptide of 39 amino acids. A protein database search using the Cah6 protein sequence showed that it is similar to β-CAs from *Escherichia coli*, green algae, and higher plants. A multiple sequence alignment of the Cah6 protein with that of β-CAs from other green algae and higher plants shows that it contains the characteristic one His and two Cys residues as zinc coordination residues, characteristic of enzymatically active β-CAs (Fig. 2).

Cloning of the *Cah6* in an Overexpression Vector

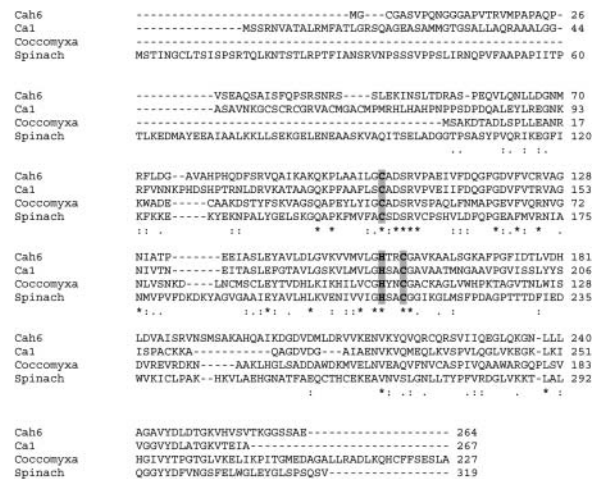*Cah6* was cloned into the expression vector pMal to study the properties of Cah6 protein and to raise an

Figure 2. Alignment of Cah6 protein sequence with those of other well-characterized β-CAs. Ca1 represents the *C. reinhardtii* mitochondrial β-CA. Mitochondrial β-Ca1 and β-Ca2 (sequence not shown in the alignment) are almost identical in amino acid sequence and have only one amino acid difference in their sequences. Coccomyxa represents the cytosolic β-CA from the symbiotic alga Coccomyxa. Active site residues are in bold and highlighted. Asterisks represent a completely conserved amino acid; colons represent conserved amino acid substitutions; and periods represent semiconserved amino acid substitutions.
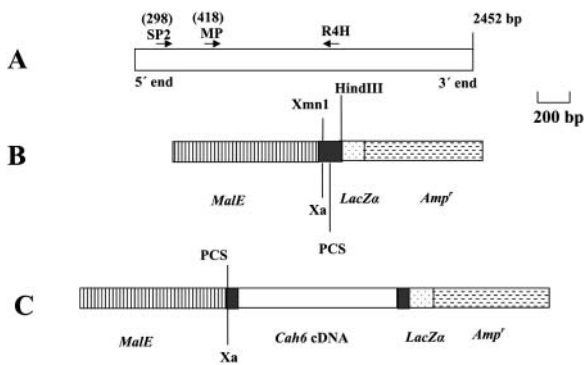
Figure 3. Generation of the recombinant pMal-Cah6 expression construct. A, A schematic figure showing the alignment of primers used to amplify *Cah6* cDNA. PCR primers are denoted by black arrows. SP2 and R4H primers were used to amplify a cDNA PCR product that codes for the full-length protein while MP and R4H primers were used to amplify a cDNA product that codes for the putative mature protein. R4H primer has a *Hind*III site incorporated at the 5′ end. The numbers on the top of the primers denote the primer position in bp. B, A part of the pMal vector showing the polylinker cloning site (PCS), β-galactosidase (*LacZα*), and β-lactamase (*Amp^r*) genes, *Xmn*I and *Hind*III recognition sites. Xa denotes Factor Xa cleavage site. *MalE* codes for MBP. C, A schematic figure of the pMal-Cah6 recombinant construct.

antibody against it. Two different *MalE-Cah6* recombinant overexpression constructs were generated. One contained the cDNA sequence coding for the full-length open reading frame (ORF) of the Cah6 protein and the other contained the cDNA sequence that would code for the mature Cah6 protein predicted by the web protein prediction programs. The SP2 + R4H primers and the MP + R4H primers were used to amplify the cDNA coding for the full-length and putative mature Cah6 proteins, respectively (Fig. 3A).

Amplified cDNAs were purified from the gel and cloned into the overexpression vector pMal-c2x (Fig. 3B) to generate two different types of recombinant constructs. An in-frame insertion of *Cah6* with the sequence of MBP in the recombinant clone was verified by restriction enzyme digestion analyses and DNA sequencing. Two clones were selected out of the 25 recombinant clones of each type. Clone B48 had a cDNA insert coding for the full-length Cah6 protein while clone B3 had one coding for the mature Cah6 protein.

Overexpression of MBP-Cah6

E. coli cells harboring the recombinant B48 and B3 recombinant constructs were induced with 1 mM isopropylthio-β-galactoside for 2 h at 37°C to overexpress the MBP-Cah6 fusion protein. Equal amounts of proteins from induced and uninduced cells were loaded on a 12% SDS-polyacrylamide gel and subjected to electrophoresis (Fig. 4). The overexpressed recombinant Cah6 fusion protein was 15% of the total *E. coli* cell protein.

Purification and Activity Assays of the Recombinant Cah6

Crude cell extracts from the B48 and B3 clones were used for CA activity assays. CA activity was detected in cell extracts of the induced B48 clone but not in that of the B3 clone. The B48 clone was selected for further study. This clone had the entire ORF of Cah6. The overexpressed recombinant Cah6 protein was purified by affinity chromatography using amylose resin following a protocol in the New England Biolabs technical catalog. Purified recombinant Cah6 was further concentrated by using a 100-kD cut-off centricon column.

The CA activity in the sample at each step of purification was assayed to check the purity of the Cah6 sample (Table I). The recombinant Cah6 protein was found to have a specific activity of 400 Wilbur-Anderson units (WAU)/mg. This calculation of specific activity was based on the total amount of recombinant fusion protein in the sample. CA activity assays were done using the method of Wilbur and Anderson (1948). CA activity was not detected in the extracts from uninduced cells containing the B48 clone or *E. coli* cells containing only the pMal vector.

The fusion protein purified from the B48 clone was cleaved by the protease Factor Xa for 4 h at 23°C to separate Cah6 from the MBP. Factor Xa cleaved the fusion protein to yield 42 kD MBP and 31 kD Cah6 protein. It also nonspecifically cleaved the 31-kD Cah6 protein band to yield a 28-kD fragment. Factor Xa sometimes cleaves nonspecifically at other basic residues depending on the conformation of the protein substrate. The mature Cah6 protein starts with an Arg residue. This can explain the generation of the 28-kD fragment (similar in molecular mass to that of the mature Cah6 protein) by the nonspecific cleavage of 31 kD Cah6 protein. Use of common protease inhibitors
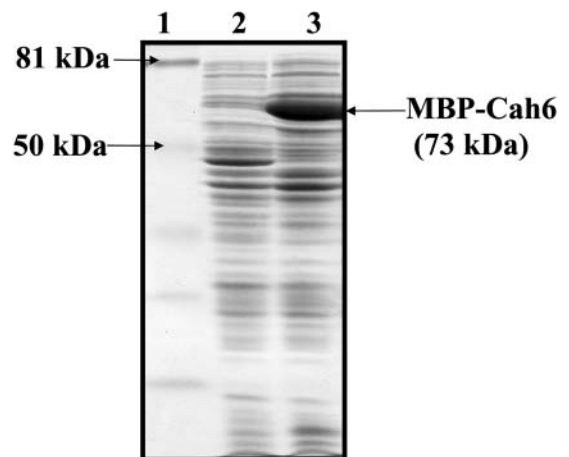

Figure 4. A 12% SDS-polyacrylamide gel showing overexpression of recombinant MBP-Cah6. Lane 1 represents prestained low molecular mass markers. Lanes 2 and 3 represent 30 μg of proteins from uninduced and induced *E. coli* cells, respectively.

Table I. *Purification of the chimeric MBP-Cah6*

Step	Total Activity	Protein	Specific Activity	Recovery *100%*	Purification Fold
	WAU[a]	mg[b]	WAU/mg		
Sonicated cells	1,200	8,000	0.15	100	1
Amylose column	800	3	300	67	2,000
Centricon column	400	1	400	33	2,567

[a]One WAU $= (t_0-t)/t$ where t_0 is the time for uncatalyzed reaction and t is the time for the enzyme catalyzed reaction. [b]Determined by Lowry's protein assay (Lowry et al., 1951).

like leupeptine did not prevent this nonspecific cleavage of Cah6. Purification and cleavage of the fusion protein was confirmed by performing SDS-PAGE (Fig. 5). There was no significant difference between the CA activities of the Factor Xa cleaved MBP-Cah6 fusion protein and undigested fusion protein. The 31-kD Cah6 protein band was excised from the gel to be used as an antigen for production of polyclonal Cah6 primary antibodies.

Western-Blotting and Northern-Blotting Analyses of Cah6 Expression

To test the specificity of the Cah6 antibody, Factor Xa (protease) cleaved MBP-Cah6 and purified MBP-Cah6 fusion proteins were separated by 12% SDS-PAGE and probed with the Cah6 antibody (Fig. 6). The antibody did not react with the MBP with induced *E. coli* cells containing only the pMal-c2x vector (data not shown). Proteins extracted from high CO_2 and air acclimated D66 cells were separated by electrophoresis and probed with the Cah6 and mitochondrial β-CA primary antibodies (Fig. 7). The mitochondrial CAs are expressed only under low CO_2 conditions but not under high CO_2 conditions. Hence, the mitochondrial β-CA primary antibodies were used as a control to confirm that the cells used for the experiment were high and low CO_2 adapted. The Cah6 antibody detected the Cah6 protein (28.5-kD band) in both the high CO_2 and air acclimated cells. The air acclimated cells showed slight up-regulation of the Cah6 protein compared to that of the high CO_2 acclimated cells (Fig. 7A). The mitochondrial β-CA antibody recognized the 22-kD mitochondrial β-CA protein in the air acclimated cells but not in the high CO_2 grown cells (Fig. 7B), in agreement with earlier observations (Eriksson et al., 1998).

The primers X-9 and R5 were used to amplify an 826-bp PCR product from the cDNA core library (Fig. 8A). This 826-bp PCR product is contained within the 3′ UTR of *Cah6* and was used as a probe for northern-blot analyses using mRNA extracted from high and low CO_2 acclimated *C. reinhardtii* (strain D66) cells grown in minimal medium. Twenty micrograms of RNA from both cell types were loaded on the RNA gel (Fig. 8B). Northern blotting shows that Cah6 is expressed under both low and high CO_2 conditions, but is slightly up-regulated in cells acclimated to air levels of CO_2 (Fig. 8C).

Immunolocalization of Cah6

Air acclimated D66 and CC-124 cells grown in minimal medium were used for immunolocalization of Cah6. *C. reinhardtii* cell sections were probed with the Cah6 antibody or the preimmune serum and observed under a transmission electron microscope (Fig. 9). Immunogold densities in different cell compartments are given in Table II. The immunogold densities in different cell compartments in sections were calculated by dividing the number of immunogold particles in a particular cell organelle with the area of that cell organelle. Immunolocalization results demonstrated that Cah6 is located in the stroma of the chloroplast (Fig. 9A) and is 4-fold more abundant in the area around the pyrenoid (Fig. 9B) compared to the other areas of the stroma. *C. reinhardtii* cell sections probed with the preimmune serum served as negative controls for the immunolocalization study (Fig. 9C).

Partial Characterization of the *Cah6* Activity

The effects of sulfonamide and anion inhibitors on the CA activity of recombinant Cah6 were studied. Table III shows the inhibition of recombinant Cah6 by sulfonamides and anions. Cah6 was comparatively less inhibited by the sulfonamides and more inhibited
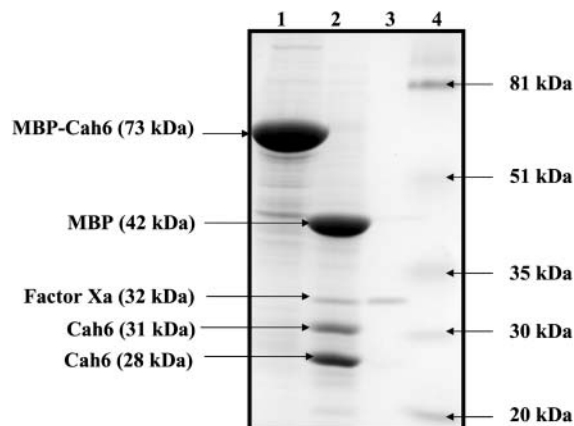

Figure 5. A 12% SDS-polyacrylamide gel showing undigested and Factor Xa digested purified MBP-Cah6 protein. Lane 1, 55 μg of undigested purified recombinant protein; lane 2, 55 μg of purified recombinant protein digested by 1 μg of Factor Xa; lane 3, 1 μg of Factor Xa; lane 4, prestained low molecular mass markers.
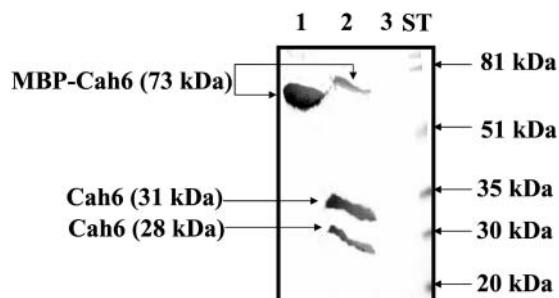
Figure 6. A western blot probed by the Cah6 antibody using purified overexpressed MBP-Cah6 protein. Lane 1, 20 μg of purified undigested fusion protein; lane 2, 20 μg of fusion protein cleaved by 1 μg of Factor Xa; lane 3, 1 μg of Factor Xa. ST represents prestained low molecular mass markers.

by the anions azide and cyanide than bovine CAII, which is an α-CA. Generally, all β-CAs are less sensitive to sulfonamide inhibition than are α-CAs and have sulfonamide I_{50} ranging from 2 μM to 10 μM (Johansson and Forsman, 1993). The I_{50} of Cah6 falls within this range.

DISCUSSION

β-CAs were first recognized in photosynthetic organisms (Burnell et al., 1990; Fawcett et al., 1990) but later have been identified in eubacteria, cyanobacteria, yeast, micro-algae, and higher plants. They play important physiological roles in these organisms. For example, a carboxysomal β-CA in cyanobacteria, coded by *icfA* gene, converts HCO_3^- to CO_2 (Badger and Price, 1992). This increases the concentration of CO_2 at the site of Rubisco, ensuring efficient CO_2 fixation. In *Saccharomyces cerevisiae*, deletion of the β-CA-like gene *NCE103* causes an oxygen-sensitive growth defect (Götz et al., 1999). It has also been found that the tobacco salicylic acid-binding protein 3 (SABP3) is a chloroplast β-CA that exhibits antioxidant activity and plays a role in the hypersensitive defense response (Slaymaker et al., 2002). In *E. coli*, the *cynT* gene that codes for a β-CA is a part of the *cyn* operon. CO_2 is produced in the reaction of cyanate with HCO_3^-, and this β-CA recycles the CO_2 back to HCO_3^-, so that it does not diffuse out of the cell (Guilloton et al., 1993). Finally, a β-CA gene identified in *Corynebacterium glutamicum* has been shown to be essential for achieving normal growth under atmospheric conditions (Mitsuhashi et al., 2003). These researchers have shown that the effect of this β-CA is most likely due to its ability to maintain favorable intracellular HCO_3^- levels, particularly during exponential growth phases and during L-lysine overproduction, both of which are conditions of higher HCO_3^- demand (Mitsuhashi et al., 2003).

Two nearly identical β-CAs were identified in 1996 in *C. reinhardtii* (Eriksson et al., 1996). Both the β-CAs (Ca1 and Ca2) are located in the mitochondria. It has been suggested that the mitochondrial CAs of *C.*
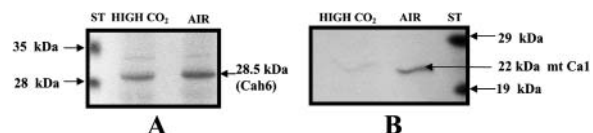

Figure 7. Western blots probed by the Cah6 and mitochondrial β-CA antibodies using wild-type Chlamydomonas cells. A, A western blot probed by Cah6 antibody using high (5% CO_2 in air [v/v]) and air (0.035% CO_2 [v/v]) acclimated wild-type Chlamydomonas cells grown in minimal medium. B, A western blot probed by mitochondrial β-CA antibody using high and air acclimated wild-type Chlamydomonas cells grown in minimal medium.

reinhardtii may play roles in recycling both respiratory and photorespiratory CO_2 by converting it to HCO_3^- in the mitochondrial matrix (Raven, 2001). This HCO_3^- then would leak back into the cytosol where it would be available for transport into the chloroplast stroma. Recently, it has been shown that the expression of mitochondrial CAs (Ca1 and Ca2) decreases when the external NH_4^+ concentration decreases, to the point of being undetectable when the supply of NH_4^+ restricts the rate of photoautotrophic growth (Giordano et al., 2003). The expression of these CAs was induced at 0.2% CO_2 condition by increasing the NH_4^+ concentration in the growth medium. These researchers have proposed that the mitochondrial CAs are involved in supplying HCO_3^- for anaplerotic assimilation catalyzed by phosphoenolpyruvate carboxylase, which in turn provides carbon skeletons for nitrogen assimilation.

Researchers in several laboratories have tried to assay CA activity in *C. reinhardtii* chloroplasts. Using mass-spectrometric measurements of ^{18}O exchange, Sültemeyer et al. (1995) have characterized two chloroplastic CA activities in *C. reinhardtii* cells. One CA activity is an insoluble form associated with the thylakoid fraction and is less sensitive to ethoxyzolamide (EZ) while the other is a soluble form and sensitive to EZ (Amoroso et al., 1996). Villarejo et al.
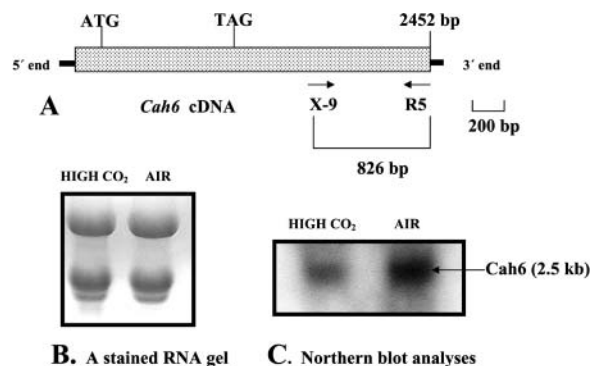

Figure 8. Northern-blot analyses of Cah6 expression. A, A schematic figure showing the alignment of primers used for making the probe for northern-blot analyses of *Cah6* expression. B, A stained RNA gel showing RNA extracted from high and low CO_2 acclimated wild-type cells grown in minimal medium. Each lane contains 20 μg of total RNA. C, Northern-blot result using 826-bp PCR product as a probe.
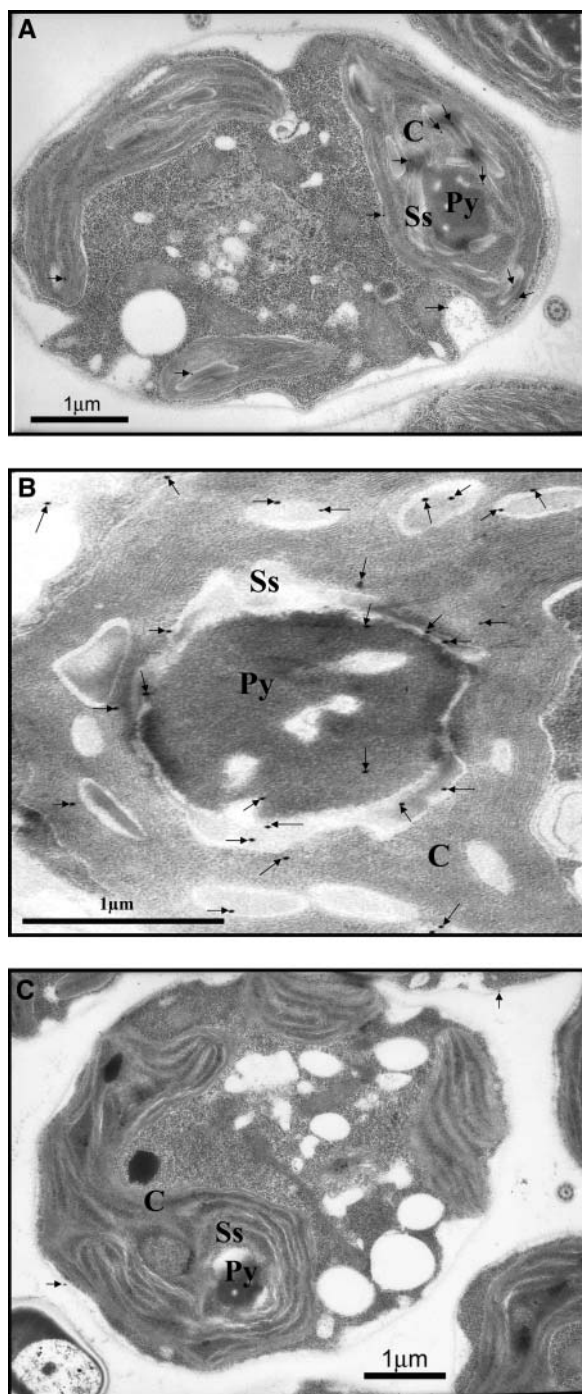
Figure 9. A, Transmission electron micrograph showing the immuno-gold labeling of *C. reinhardtii* CC-124 cells probed with the Cah6 antibody. Cells grown under low CO_2 (0.035%) conditions in minimal medium were probed with the Cah6 antibody. B, Transmission electron micrograph showing the immunogold density around the pyrenoid in *C. reinhardtii* cells probed with the Cah6 antibody. Cells grown under low CO_2 (0.035%) conditions in minimal medium were probed with the Cah6 antibody. C, Transmission electron micrograph showing the immunogold labeling of *C. reinhardtii* cells probed with the preimmune serum. Low CO_2 (0.035%) acclimated CC-124 cells grown in minimal medium were used. For all micrographs, Ss, Py, and C denote starch sheath, pyrenoid, and chloroplast, respectively. Immunogold labelings are shown by small black arrows.

(2001) have found a new chloroplast envelope CA activity that is sensitive to EZ and is constitutively expressed. Support for the presence of the insoluble chloroplastic CA activity is provided by the identification of the 29-kD Cah3, which is located on the lumenal side of thylakoid membrane (Karlsson et al., 1998).

Here we report the identification of a nuclear gene encoding a chloroplastic β-CA, *Cah6*, in *C. reinhardtii*. Cah6, the sixth carbonic anhydrase gene and the third β-CA gene to be identified in *C. reinhardtii*, is located in the stroma of the chloroplast and likely represents the soluble chloroplastic CA activity detected earlier. The Cah6 protein has two Cys residues and one His residue that coordinate zinc, similar to all known enzymatically active β-CAs (Moroney et al., 2001). Furthermore, like all other β-CAs, Cah6 is 100- to 1,000-fold less sensitive to sulfonamide compounds like acetazolamide and EZ than the bovine CAII, an α-CA (Table III). Cah6 was 100-fold and 10-fold more sensitive to azide and cyanide, respectively, than the bovine alpha CAII. The full-length protein has a calculated molecular mass of 28 kD and a pI of 7.0. It has an apparent weight of 31 kD on a SDS-polyacrylamide gel. The mature Cah6 protein (beginning from amino acid residue 40) has a calculated molecular mass of 26 kD and a pI of 6.58. It has an apparent molecular mass of 28.5 kD on SDS-poly-acrylamide gels (Fig. 7A). Cah6 is similar to β-CAs from *E. coli*, green algae, and higher plants, with an amino acid identity of 23% to 34%. Cah6 most closely resembles the green alga Coccomyxa β-CA with a 34% identity.

CA activity was detected in cell extracts of the induced B48 clone but not in that of the B3 clone of *E. coli* expressing the Cah6 gene. The B48 clone contains the entire ORF of Cah6, whereas the B3 clone had only the cDNA coding for the putative mature Cah6 protein. One explanation for the lack of activity is that Cah6 is cleaved at a point different than that predicted by the different target prediction programs. A second possible explanation for the lack of activity of the protein from B3 clone is that the first amino acid of the mature Cah6 was eliminated to facilitate cloning. During the cloning of the putative mature Cah6, the codon for Arg was substituted as Factor Xa does not cleave any protein that starts with an Arg after the Factor Xa recognition sequence (Ile-[Glu-Asp]-Gly-Arg). Thus the mature Cah6 protein in the B3 clone started with a Ser instead of an Arg residue. It is therefore possible that this change caused the recombinant fusion protein to become insoluble or misfold. It is clear however that Cah6 is an active CA as the full-length protein had a specific activity well within the normal range found for β-CAs.

Western- and northern-blotting analyses show that Cah6 is constitutively expressed and slightly up-regulated under low CO_2 conditions. Immunolocalization shows that Cah6 is localized in the stroma of the chloroplast and is not present in the pyrenoid.

Table II. *Intracellular localization of Cah6 using wild type C. reinhardtii cells*

| Location | Area | Immunogold Density | | |
		Immune	Preimmune	Difference
	μm^2	*No. of Immunogold Particles/μm^2*		
Outside	7.80 ± 0.38	1.67 ± 0.10	1.54 ± 0.11	0.13 ± 0.19
Cytoplasm	6.84 ± 1.2	0.44 ± 0.02	0.30 ± 0.02	0.14 ± 0.03
Nucleus	0.36 ± 0.05	0.47 ± 0.02	0.47 ± 0.02	0.00 ± 0.01
Stroma	5.93 ± 1.07	3.0 ± 0.14	0.85 ± 0.04	2.15 ± 0.13
Pyrenoid	1.85 ± 0.30	0.99 ± 0.08	0.97 ± 0.7	0.02 ± 0.03
Starch sheath	0.86 ± 0.11	10.9 ± 1.9	2.3 ± 0.14	8.6 ± 1.95

Immunogold densities in different cell compartments in sections were calculated by dividing the number of immunogold particles in a particular cell organelle with the area of that cell organelle. The data presented in the table is the average ± SD of 15 cell sections. The stroma includes the thylakoid area. The cytoplasmic area was calculated by subtracting the total area of chloroplast and the nucleus from the cell area. The Cah6 antibody dilution used for immunolocalization is 1:10. The cells used for immunolocalization are air acclimated D66 cells grown in minimal medium.

In Chlamydomonas, 90% of the Rubisco is localized inside the pyrenoid under low CO_2 conditions (Borkhsenious et al., 1998). Western blots and the lack of immunogold labeling of the pyrenoid indicated that the Cah6 antibody is not cross reacting with Rubisco. Interestingly, in *C. reinhardtii* immunogold density is 4-fold higher in the area around the pyrenoid, particularly around the starch sheath that surrounds the pyrenoid, compared to that in the other areas in the stroma of the chloroplast (Table III). Based on these observations, we hypothesize that Cah6 indirectly plays a role in the CCM by trapping CO_2 diffusing out from the pyrenoid, the site of localization of Rubisco in *C. reinhardtii*, and converting it to HCO_3^- (Fig. 10). This conversion of CO_2 to HCO_3^- would increase the HCO_3^- pool in the stroma, thereby retaining inorganic carbon within the chloroplast.

A mutant of *C. reinhardtii, cia5,* which requires elevated levels of CO_2 for growth, fails to induce CCM and does not synthesize any of the low CO_2 inducible mitochondrial and periplasmic CAs along with other low CO_2 inducible polypeptides (Manuel and Moroney, 1988). It is deficient in a putative transcription factor coded by the *Cia5* gene (Fukuzawa et al., 2001; Xiang, et al., 2001). Katzman et al. (1994) used a ^{14}C assay to measure the CA activity in the chloroplasts of *cia5* and wild-type cells of Chlamydomonas and found that the CA activity of *cia5* is almost identical to that in wild-type cells. Cah3 is an α-CA that is localized to the lumenal side of thylakoids in the chloroplast (Karlsson et al., 1998). The Cah3 and the Cah6 antibodies specifically detect the Cah3 and Cah6

protein in the *cia5* cells (data not shown). So it appears that both Cah3 and Cah6 contributed to the total CA activity measured by Katzman et al. (1994) in the chloroplasts of *cia5*. This also suggests that Cah6 expression is probably not under the regulation of the *Cia5* gene. Immunoblotting and northern-blotting analysis (using Cah3 and Cah6 specific probes) of *cia5* and wild-type cells of *C. reinhardtii* could show the expression pattern of *Cah6* gene in this mutant and could confirm if these genes are under the regulation of the *Cia5* gene. Generation of mutants of Cah6 could help to confirm the physiological role of Cah6 in photosynthesis and CCM.

There has been a surge of interest in CAs from plants and algae over the past decade. This interest began with the discovery of the β-CA in plants in 1990 (Fawcett et al., 1990) and has continued with the

Table III. *The inhibition constants of the bovine CAII and heterologously produced Cah6*

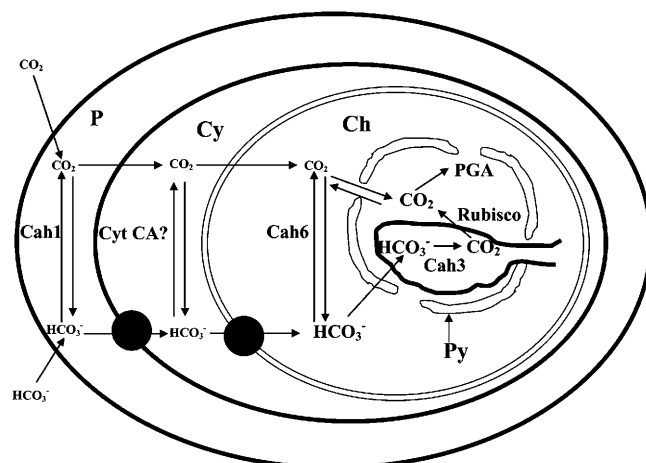| Inhibitor	I_{50} of Bovine CA	I_{50} of Cah6
	M	*M*
Acetazolamide	1.4×10^{-8}	2×10^{-6}
Ethoxyzolamide	1.2×10^{-9}	9×10^{-6}
Azide	1.1×10^{-3}	1.5×10^{-5}
Cyanide	4.9×10^{-5}	5×10^{-6}

Figure 10. A model showing the potential role of Cah6 and other known CAs in the operation of CCM in *C. reinhardtii*. The font sizes of CO_2 and HCO_3^- indicate the relative concentrations of these Ci species. Cyt CA? represents a putative cytoplasmic carbonic anhydrase. P, Cy, Ch, and Py represent periplasm, cytoplasm, chloroplast, and pyrenoid, respectively. Cah1 and Cah3 represent the periplasmic and thylakoid CAs, respectively. Putative HCO_3^- transporters are denoted by small black circles.

finding of multiple α- and β-CAs in *C. reinhardtii* and Arabidopsis and the determination of the critical physiological roles CAs have in cyanobacteria and macro-algae. Analysis of the Arabidopsis genomic database reveals at least 14 genes potentially encoding CAs that have homologies with ESTs and are expressed in cells (Moroney et al., 2001). Clearly the number of CAs in plants is much greater than previously thought. *C. reinhardtii*, a unicellular green alga, is not far behind with six CAs already characterized. The availability of Arabidopsis and Chlamydomonas genome sequences and EST databases can be used to find out the exact number of expressed CA isoforms in these organisms. As there appears to be a large number of isoforms in plants and algae, the challenge for future researchers will be to determine the expression patterns, localization, and physiological roles for each of these isoforms.

MATERIALS AND METHODS

Strains and Media

Chlamydomonas reinhardtii strain D66 ($nit2^-$, $cw15$, mt^+) was obtained from Rogene Schnell, University of Arkansas (Little Rock, AR; Schnell and LeFebvre, 1993). The wild-type strain CC-124 was obtained from the Duke culture collection. To start cultures, cells from yeast acetate medium plates were inoculated into 100 mL of Tris-acetate phosphate (TAP) medium (Sueoka, 1960) and grown with continuous shaking and light (300 μmol photons $m^{-2} s^{-1}$) for 2 d. An aliquot of the culture was then transferred to 1.5 L of minimal medium (Sueoka, 1960) and bubbled with high CO_2 (5% CO_2 [v/v] in air) until it reached a cell density of about 2×10^6 cells mL^{-1}. The culture was diluted with an equal volume of fresh medium and split into two flasks. One was bubbled with high CO_2 (5% CO_2 [v/v] in air) and the other with air (0.035% [v/v] CO_2). The cells were low CO_2 acclimated for 12 h. The high and low CO_2 acclimated D66 cells were used for RNA isolation, measurement of chlorophyll content, and western blotting. Only low CO_2 acclimated cells were used for immunolocalization studies.

Total RNA Isolation and Northern-Blot Analysis

Extraction of total RNA from low CO_2 and high CO_2 acclimated D66 cells and RNA gel-blot analyses were performed by standard procedures (Sambrook et al., 1989). RNAs from high and low CO_2 acclimated cells were transferred to a BA-S 85 nitrocellulose membrane (Schleicher and Schuell Bioscience, Keene, NH). When *Cah6* 5'end primer X-9 (5' AAACTCAACTC-CTTCATAATAGGC-3') and 3'end primer R5 (5'-TGCGGTACAGATTA-CAGTCA-3') were used to perform PCR on the cDNA core library, an 826-bp PCR product was generated. This product was used to make a radioactive probe to study the expression pattern of *Cah6* under low and high CO_2 conditions. The PCR product corresponded to a unique 826 bp region in the 3' UTR of *Cah6*. ^{32}P-dCTP labeled probes were prepared using a random primer procedure (Sambrook et al., 1989).

DNA Preparation, Sequencing, and Homology Analysis

Plasmid and cosmid DNA was purified using a combination of the standard ethanol precipitation method (Sambrook et al., 1989) followed by the purification using the spin columns from a commercial kit (Qiagen, Chatsworth, CA). cDNA and genomic PCR products were purified from the 0.8% [w/v] agarose gels. Amplified bands were digested from the gel and were treated with 6 M NaI at 55°C to melt the gel piece. DNA was purified from the liquefied gel using the mini spin columns from the commercial kit mentioned above. DNA was sequenced using ABI dye terminators (Perkin Elmer Applied Biosystems, Foster City, CA; for some PCR fragments and cosmids enriched in GC content, the use of dGTP-BigDye generated better sequences than dITP-BigDye).

Homology searches (against Chlamydomonas EST and the full database) were performed using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST; Altschul et al., 1997) Exon/intron splice sites and ORFs were identified manually (Silflow, 1998) as well as by using GreenGenie (Kulp et al., 1996; http://www.cse.ucsc.edu/%7Edkulp/cgi-bin/greenGenie). Signal peptide analysis, molecular mass, and pI calculations were determined with different protein prediction programs like CHLOR P, TARGET P, and SORT P, which had hyperlinks in the ExPasy server (http://ca.expasy.org/tools/#translate).

cDNA Library Preparation and Amplification of *Cah6* cDNA from the Prepared Library

A cDNA core library (in bacteriophage λ) was obtained from the Chlamydomonas Genetics Center at Duke University (Durham, NC). This core library was made from cDNAs prepared from CC-1690 cells grown to mid-log phase in TAP (acetate-containing) medium in the light, TAP medium in the dark, high salt (HS; minimal) medium in ambient levels of CO_2, and HS medium bubbled with 5% CO_2. cDNAs were cloned into the lambda Zap II (Stratagene, La Jolla, CA) in the *Eco*RI (5') and *Xho*I (3') sites. This cDNA library was amplified following the protocol given in the Stratagene manual. *Cah6* primers F4 (5'-GCACGAGGCAACATTAAACA-3') and R5 when used on the cDNA core library yielded a PCR product of 2446 bp. This product codes for the full-length Cah6 protein.

Screening of the Cosmid Library

To obtain wild-type genomic clones of the *Cah6* gene, a PCR-based screen of an indexed cosmid library was used (Pollock et al., 2003). An indexed cosmid library was constructed using a cosmid library from Saul Purton, University of London (Purton and Rochaix, 1994). Briefly, 7,680 different *Escherichia coli* lines carrying single cosmids were grown in Luria-Bertani media on 80 different 96-well microtiter plates. Using this indexed library, 80 pools of cells, each containing 96 single cosmids, were generated. DNA from each pool, obtained by common alkaline lysis procedures (Sambrook et al., 1989), was used to create 10 superpools (each containing about 768 individual cosmids) that were suitable for PCR.

The mitochondrial β-CA protein (Ca1) sequence of *C. reinhardtii* was used to BLAST the EST database of *C. reinhardtii*. The search yielded many ESTs that were not the gene products of the mitochondrial β-CA genes although they encoded a β-CA. These ESTs were all from the same gene and were analyzed by contig assemblage program to form a consensus *Cah6* sequence. Based on this *Cah6* contig, primer sets were designed and used to screen the superpools. *Cah6* primers F4 and R4 (5'-TTGCGCCATGAAGTCCCTAA-3') were used for PCR. These primers amplified a *Cah6* product of 1.8 kb. Once a plate carrying the correct cosmid was identified, a new set of pools was generated (12 pools, each containing 8 single cosmids). Finally, a new PCR reaction was performed with the single cosmid from the positive pool described above. Using this protocol, after four rounds of PCR, two cosmids, 72-E-6 and 29-D-12, containing the *Cah6* gene were isolated from the cosmid library. PCR using primers F4 and R5 yielded a product of 2,880 bp when used on the isolated cosmid clones. This PCR product is just short of 6 bp from being the full-length genomic sequence of *Cah6*.

Production of Overexpression Constructs

Cah6 was cloned into the pMal-c2x overexpression vector (NEB, Beverly, MA) and fused to the *MalE* gene that encodes MBP. Two different pMal-*Cah6* recombinant overexpression vectors were constructed. One contained the cDNA sequence coding for the ORF of Cah6 protein and the other contained the cDNA sequence that would code for the putative mature Cah6 protein. SP2 (5' A TGGGATGCGGTGCCAGCGTG 3') + R4H (5'-ATATAA-GCTTTTGCGCCATGAAGTCCCT AA-3') primers and MP (5'- AGCAACCG-CAGCAGCTT-3') + R4H primers were used to amplify the cDNA coding for the full-length and mature Cah6 protein, respectively. SP2 + M1RH and MP + M1RH primers yielded a PCR product of 1.1 kb and 979 bp, respectively. A high fidelity DNA polymerase (Platinum Pfx DNA polymerase from Invitrogen, Carlsbad, California) was used for PCRs. The vector was double digested with the *Xmn*I and *Hin*dIII. Amplified cDNAs were purified from the DNA gel using Qiagen spin columns and were digested with *Hin*dIII. Ligation of the insert to the overexpression vector pMal-c2x vector was performed

following the protocol in the NEB technical manual. Transformations of DH5α cells were performed following the protocol in Sambrook et al. (1989). In-frame insertion of *Cah6* with the sequence of MBP in the recombinant clone was verified by double restriction enzyme digestion analyses with *Sac*I and *Hin*dIII and DNA sequencing.

Overexpression and Purification of MBP Fusion Proteins

Selected clones of *Cah6* were grown at 37°C in 2 L Luria-Bertani medium + Glc (0.2%) + Amp (100 μg mL^{-1}) cultures on a rotary shaker. Glc was added to the growth medium to repress the maltose genes on the chromosome of the *E. coli* host, one of which codes for amylase that can degrade the amylose on the affinity resin that is used for purification. The cells were induced for 2 h with 1 mM isopropylthio-β-galactoside at 37°C when the culture OD_{600} was between 0.6 and 0.7. Both induced and uninduced cells were harvested and resuspended in column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, and 1 mM EDTA with or without 10 mM 2-mercaptoethanol) and ruptured in a prechilled French pressure cell. Equal amounts of protein samples of ruptured induced and uninduced cells were loaded on a 12% SDS-polyacrylamide gel and subjected to electrophoresis to verify the over-expression of the recombinant protein. The fusion protein was purified by one-step affinity chromatography using amylose resin. Amylose resin (1 mL of amylose resin binds 3 mg of the recombinant protein) was mixed with the crude ruptured cell extract on a shaker at room temperature for 2 h and poured into a 2.5-cm × 10-cm column to perform batch purification. The column was washed with 6 L of column buffer to remove other proteins. At the final step, fusion proteins were eluted from the column by column buffer containing 10 mM maltose. Purified recombinant fusion proteins were further concentrated by passing the extract through the 100-kD centricon columns (Amicon, Billerica, MA).

Recombinant proteins were cleaved from the MBP by digestion with Factor Xa. Fifty micrograms of the recombinant protein was digested by 1 μg of Factor Xa enzyme in the Factor Xa digestion buffer (20 mM Tris-HCl, 100 mM NaCl, and 2 mM CaCl$_2$ [pH 8.0]) at 23°C for 4 to 6 h. Purification and Factor Xa digestion of the recombinant protein were verified using SDS-PAGE.

Generation of Polyclonal Cah6 Primary Antibodies

Factor Xa digested purified recombinant proteins were separated on a 12% gel by SDS-PAGE at 15 mA for 18 to 20 h. The 31-kD Cah6 protein band was excised carefully from the polyacrylamide gel. The gel pieces were shipped to Strategic BioSolutions (Ramona, CA) for production of antibody. Antibodies were raised against Cah6 proteins by a standard 70-d protocol using two pathogen-free rabbits. Approximately 1.6 mg of the Cah6 protein was used to raise the antibody.

Carbonic Anhydrase Assays and CA Inhibition Studies

CA activity was assayed electrometrically using a modification of the Wilbur-Anderson method (1948). The samples were assayed at 4°C by adding 50 to 200 μL of the test sample to 3.5 mL of 20 mM 4-(2-hydroxyethyl)-1-piperazine propane sulfonic acid (EPPS, pH 8.0). The reaction was initiated by addition of 1.5 mL of ice-cold CO_2 saturated water. The time required for the pH drop from 7.7 to 6.3 was measured. The activity of the test sample was calculated using the equation: WAU = $t_0/t - 1$ where t is the time required for the pH change when the test sample is present and t_0 is the time required for the pH change when the buffer is substituted for the test sample. Bovine CAII (Sigma, St. Louis) was used as a positive control. Both Factor Xa digested and undigested purified Cah6 fusion proteins were used for activity assays.

The effects of the sulfonamide inhibitors ethoxyzolamide and acetazol-amide and the anions azide and cyanide on CA activities of recombinant Cah6 were studied. I_{50} was determined by plotting the percentage of inhibition versus the concentration of the inhibitor. Sodium salts of azide and potassium salts of cyanide were used. All the inhibitors were purchased from Sigma.

Electrophoresis and Immunoblotting

For protein analyses and western blots, cells were harvested, washed twice with fresh medium, and resuspended in 10 mM Tris-HCl, 10 mM EDTA, and 150 mM NaCl, pH 7.5. Proteins were separated on 12% and 15% poly-acrylamide gels (0.8% bis-acrylamide) as described previously (Laemmli,

1970). SDS-PAGE was performed using prestained low molecular mass markers as protein standards (Bio-Rad, Hercules, CA).

Immunoblotting was performed as described in the protocol from Bio-Rad. Cah6 primary antibody was used to probe proteins from high and low CO_2 acclimated D66 as well as the Factor Xa digested and undigested purified MBP-Cah6 fusion proteins. The Cah6 antibody was diluted with the antibody buffer (Tris-buffered saline + 0.005% Tween 20 + 1% bovine serum albumin, pH 7.4) in the ratio of 1:1,000, before being used as a probe. The secondary antibody used for western blotting was conjugated to the enzyme horseradish peroxidase (Bio-Rad) and was diluted at a ratio of 1:3,000 with the antibody buffer. Western blots were developed following the protocol from Bio-Rad using a mixture of the horse radish peroxidase color development reagent (Bio-Rad) in ice-cold 100% methanol (20 mL), Tris-buffered saline (80 mL, pH 7.4, and 30% H_2O_2, 60 μL).

Immunolocalization Studies Using Electron Microscopy

Air acclimated D66 or CC-124 cells were fixed in a mixture of 1% OsO_4, 2% formaldehyde, and 0.5% glutaraldehyde in a 1:1 ratio for 15 min. The sample was then fixed for an additional 15 min in 1% OsO_4, 2% formaldehyde, 0.5% glutaraldehyde, and 0.1 mM sodium cacodylate buffer. Materials were rinsed with distilled water and stained with 0.5% uranyl acetate for 30 min. After this, excess stain was rinsed and the samples were dehydrated in ethyl alcohol series. Samples were then infiltrated and embedded in LR White resin (EMS, Fort Washington, PA). Embedded tissues were sectioned with a DuPont Sorvall microtome (Wilmington, DE). The sections were 70-μm thick.

The immunocytochemical procedure was similar to the method of Borkhsenious et al. (1998) with some modifications. The sections were incubated for 90 min with diluted primary antibody (1:10 dilutions of the Cah6 primary antibody) or with the preimmune serum diluted similarly (used as a negative control). The grids were transferred to 1:50 dilution of Protein A (Sigma) conjugated to 20 nm colloidal gold particles for 1 h. Protein A was diluted with 1% bovine serum albumin and 0.1% Tween 20 in phosphate buffered saline (Sigma). Finally the sections were rinsed with distilled water and photographed under transmission electron microscopy.

Other Methods

The CO_2 concentration in the growth chambers was measured using an infrared gas analyzer (Analytical Development, Hoddesdon, UK), which reads at an accuracy of ±2%. The CO_2 concentration was checked at least once every day, while the alga was growing in the high and low CO_2 growth chambers. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Chlorophyll concentration was determined spectrophotometrically (Arnon, 1949) using the equations of Holden (1976). The solvent used for extraction of chlorophyll was 100% methanol. Cell density values were determined by direct counting in a hemacytometer chamber.

LITERATURE CITED

Alber BE, Ferry JG (1994) A carbonic anhydrase from the archaeon *Methanosarcina thermophila*. Proc Natl Acad Sci USA **91:** 6909–6913

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:** 3389–3402

Amoroso G, Weber C, Sültemeyer DF, Fock HP (1996) Intracellular

carbonic anhydrase activities in *Dunaliella tertiolecta* (Butcher) and *Chlamydomonas reinhardtii* (Dangeard) in relation to inorganic carbon concentration during growth: further evidence for the existence of two distinct carbonic anhydrases associated with the chloroplast. Planta **199:** 177–184

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris.* Plant Physiol **24:** 1–15

Badger MR, Price GD (1992) The CO_2 concentrating mechanism in cyanobacteria and microalgae. Physiol Plant **84:** 606–615

Borkhsenious ON, Mason CB, Moroney JV (1998) The intracellular localization of ribulose-1, 5-bisphosphate carboxylase/oxygenase in *Chlamydomonas reinhardtii.* Plant Physiol **116:** 1585–1591

Bracey MH, Christiansen J, Tovar P, Cramer SP, Bartlett SG (1994) Spinach carbonic anhydrase: investigation of the zinc-binding ligands by site-directed mutagenesis, elemental analysis and EXAFS. Biochemistry **33:** 13126–13131

Burnell JN, Gibbs MJ, Mason JG (1990) Spinach chloroplastic carbonic anhydrase-nucleotide sequence analysis of cDNA. Plant Physiol **92:** 37–40

Colombo SL, Pollock SV, Eger KA, Godfrey AC, Adams JE, Mason CB, Moroney JV (2002) Use of the bleomycin resistance gene to generate tagged insertional mutants of *Chlamydomonas reinhardtii* that require elevated CO_2 for optimal growth. Funct Plant Biol **29:** 231–241

Eriksson M, Karlsson J, Ramazanov Z, Gardeström P, Samuelsson G (1996) Discovery of an algal mitochondrial carbonic anhydrase: molecular cloning and characterization of a low-CO_2-induced polypeptide in *Chlamydomonas reinhardtii.* Proc Natl Acad Sci USA **93:** 12031–12034

Eriksson M, Villand P, Gardeström P, Samuelsson G (1998) Induction and regulation of expression of a low-CO_2-induced mitochondrial carbonic anhydrase in *Chlamydomonas reinhardtii.* Plant Physiol **116:** 637–641

Fawcett TW, Browse JA, Volokita M, Bartlett SG (1990) Spinach carbonic-anhydrase primary structure deduced from the sequence of a cDNA clone. J Biol Chem **265:** 5414–5417

Fujiwara S, Fukuzawa H, Tachiki A, Miyachi S (1990) Structure and differential expression of two genes encoding carbonic anhydrase in *Chlamydomonas reinhardtii.* Proc Natl Acad Sci USA **87:** 9779–9783

Fukuzawa H, Fujiwara S, Yamamoto Y, Dionisio-Sese ML, Miyachi S (1990) cDNA cloning, sequence, and expression of carbonic anhydrase in *Chlamydomonas reinhardtii*- regulation by environmental CO_2 concentration. Proc Natl Acad Sci USA **87:** 4383–4387

Fukuzawa H, Miura K, Ishizaki K, Kucho K, Saito T, Kohinata T, Ohyama K (2001) Ccm1, a regulatory gene controlling the induction of a carbon-concentrating mechanism in *Chlamydomonas reinhardtii* by sensing CO_2 availability. Proc Natl Acad Sci USA **98:** 5347–5352

Giordano M, Norici A, Forssen M, Eriksson M, Raven J (2003) An anaplerotic role for mitochondrial carbonic anhydrase in *Chlamydomonas reinhardtii.* Plant Physiol **132:** 2126–2134

Götz R, Gnann A, Zimmermann FK (1999) Deletion of the carbonic anhydrase-like gene NCE103 of the yeast *Saccharomyces cerevisiae* causes an oxygen-sensitive growth defect. Yeast **15:** 855–864

Guilloton MB, Lamblin AF, Kozliak EI, Gerami-Nejad M, Tu C, Silverman D, Anderson PM, Fuchs JA (1993) A physiological role for cyanate-induced carbonic anhydrase in *Escherichia coli.* J Bacteriol **175:** 1443–1451

Hanson DT, Franklin LA, Samuelsson G, Badger MR (2003) The *Chlamydomonas reinhardtii* cia3 mutant lacking a thylakoid lumen-localized carbonic anhydrase is limited by CO_2 utilization by Rubisco and not PSII function *in vivo.* Plant Physiol **132:** 2267–2275

Henry RP (1996) Multiple roles of carbonic anhydrase in cellular transport and metabolism. Annu Rev Physiol **58:** 523–538

Hewett-Emmett D, Tashian RE (1996) Functional diversity, conservation, and convergence in the evolution of the α-, β-, and γ-carbonic anhydrase gene families. Mol Phylogenet Evol **5:** 50–77

Holden M (1976) Chlorophylls. *In* TW Goodwin, ed, Chemistry and Biochemistry of Plant Pigments. Academic Press, London, pp 2–37

Johansson IM, Forsman C (1993) Kinetic-studies of pea carbonic-anhydrase. Eur J Biochem **218:** 439–446

Karlsson J, Clarke AK, Chen ZY, Hugghins SY, Park YI, Husic HD, Moroney JV, Samuelsson G (1998) A novel alpha-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required for growth at ambient CO_2. EMBO J **17:** 1208–1216

Katzman GL, Carlson SJ, Marcus Y, Moroney JV, Togasaki RK (1994) Carbonic anhydrase activity in isolated chloroplasts of wild-type and high-CO_2-dependent mutants of *Chlamydomonas reinhardtii* as studied by a new assay. Plant Physiol **105:** 1197–1202

Kulp D, Haussler D, Reese MG, Eeckman FH (1996) A generalized hidden Markov model for the recognition of human genes in DNA. Proc Int Conf Intell Systems Mol Biol **4:** 134–142

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lindskog S (1997) Structure and mechanism of carbonic anhydrase. Pharmacol Ther **74:** 1–20

Lowry OH, Rosebrough NJ, Fart AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem **193:** 265–275

Manuel LJ, Moroney JV (1988) Inorganic carbon accumulation by *Chlamydomonas reinhardtii*: new proteins are made during adaptation to low CO_2. Plant Physiol **88:** 491–496

Meldrum NU, Roughton FJW (1933) Carbonic anhydrase. Its preparation and properties. J Physiol **80:** 113–142

Mitsuhashi S, Ohnishi J, Hayashi M, Ikeda M (2003) A gene homologous to beta-type carbonic anhydrase is essential for the growth of *Corynebacterium glutamicum* under atmospheric conditions. Appl Microbiol Biotechnol (in press)

Moroney JV, Bartlett SG, Samuelsson G (2001) Carbonic anhydrases in plants and algae. Plant Cell Environ **24:** 141–153

Moroney JV, Husic HD, Tolbert NE (1985) Effect of carbonic anhydrase inhibitors on inorganic carbon accumulation by *Chlamydomonas reinhardtii.* Plant Physiol **79:** 177–183

Pollock SV, Colombo SL, Prout DL Jr, Godfrey AC, Moroney JV (2003) Rubisco activase is required for optimal photosynthesis in the green alga *Chlamydomonas reinhardtii* in a low CO_2 atmosphere. Plant Physiol **133:** 1854–1861

Purton S, Rochaix JP (1994) Complementation of a *Chlamydomonas reinhardtii* mutant using a genomic cosmid library. Plant Mol Biol **24:** 533–537

Raven JA (2001) A role for mitochondrial carbonic anhydrase in limiting CO_2 leakage from low CO_2-grown cells of *Chlamydomonas reinhardtii.* Plant Cell Environ **24:** 261–265

Rowlett RS, Chance MR, Wirt MD, Sidelinger DE, Royal JR, Woodroffe M, Wang YFA, Saha RP, Lam MG (1994) Kinetic and structural characterization of spinach carbonic-anhydrase. Biochemistry **33:** 13967–13976

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schnell RA, Lefebvre PA (1993) Isolation of the *Chlamydomonas reinhardtii* regulatory gene NIT2 by transposon tagging. Genetics **134:** 737–747

Silflow CD (1998) Organization of the nuclear genome. *In* JD Rochaix, M Goldschmidt-Clermont, S Merchant, eds, The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas.* Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 25–40

Slaymaker DH, Navarre DA, Clark D, del Pozo O, Martin GB, Klessig D (2002) The tobacco salicylic acid binding protein 3 (SABP3) is the chloroplast carbonic anhydrase which exhibits antioxidant activity and plays a role in the hypersensitive defense response. Proc Natl Acad Sci USA **99:** 11640–11645

Sueoka N (1960) Mitotic replication of deoxyribonucleic acids in *Chlamydomonas reinhardtii.* Proc Natl Acad Sci USA **46:** 83–91

Sültemeyer DF, Price GD, Yu JW, Badger MR (1995) Characterization of carbon dioxide and bicarbonate transport during steady state photosynthesis in the marine cyanobacterium *Synechococcus strain* PCC 7002. Planta **197:** 597–607

Villarejo A, Rolland N, Martinez F, Sültemeyer D (2001) A new chloroplast envelope carbonic anhydrase activity is induced during acclimation to low inorganic carbon concentrations in *Chlamydomonas reinhardtii.* Planta **213:** 286–295

Wilbur KM, Anderson NG (1948) Electrometric and colorimetric determination of carbonic anhydrase. J Biol Chem **176:** 147–154

Xiang YB, Zhang J, Weeks DP (2001) The Cia5 gene controls formation of the carbon concentrating mechanism in *Chlamydomonas reinhardtii.* Proc Natl Acad Sci USA **98:** 5341–5346