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Identification of Distinct Internal and External Isozymes of Carbonic Anhydrase in *Chlorella saccharophila*[1]

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External carbonic anhydrase (CA) was detected in whole cells of alkaline-grown *Chlorella saccharophila* but was suppressed by growth at acid pH or growth on elevated levels of CO_2. Internal CA activity was measured potentiometrically as an increase in activity in cell extracts over that of intact cells. Cells grown under all conditions had equal levels of internal CA activity. Two isozymes were identified after electrophoretic separation of soluble proteins on cellulose acetate plates. The fast isozyme was found in cells grown under all conditions, whereas the slow isozyme was found only in cells grown at alkaline pH. Western blot analysis following sodium dodecyl sulfate-polyacrylamide gel electrophoresis using antibodies produced against the periplasmic form of CA from *Chlamydomonas reinhardtii* revealed a single band at 39 kD, which did not change in intensity between growth conditions and was associated only with proteins eluted from the fast band. The slow isozyme was inactivated by incubation of cell extract at 30°C and by incubation in 10 mM dithiothreitol, whereas the internal form was unaffected. These results indicate that external and internal forms of CA differ in structure and their activities respond differently to environmental conditions.

CA catalyzes the reversible hydration of CO_2 in aqueous solution. Numerous forms of CA have been implicated in many physiological processes in bacteria (Guilloton et al., 1992), animals (Tashian, 1989), and plants (Graham et al., 1984). In higher plants CA has been shown to be required for maximum carbon fixation in both C_3 (Everson, 1970) and C_4 (Hatch and Burnell, 1990) species, since it increases the supply of inorganic carbon to the initial fixation step. Burnell and Hatch (1988) also emphasized that the distribution of CA within C_4 plants is essential to optimize photosynthesis. In unicellular microalgae and cyanobacteria internal CA is also essential to optimize photosynthesis: a mutant of *Chlamydomonas reinhardtii* that lacks internal CA activity (Spalding et al., 1983) had a high CO_2-requiring phenotype; and in a cyanobacterium, disruption of a gene homologous to that coding for CA in *Escherichia coli* and higher plants also created a high CO_2-requiring mutant (Fukuzawa et al., 1992). The importance of the intracellular distribution of CA has also been emphasized in cyanobacteria (Price et al., 1992) and *C. reinhardtii* (Kuchitsu et al., 1991).

In addition to internal CA many eukaryotic microalgae exhibit external CA activity that is repressed by growth at elevated levels (1–5%) of CO_2 (Tsuzuki et al., 1984). Since the DIC-concentrating mechanism in these algae is also repressed by CO_2, it has been suggested that external CA is required at alkaline pH to facilitate the supply of CO_2 to a transporter from a large HCO_3^- pool at alkaline pH (Aizawa and Miyachi, 1986). Other evidence suggests that, under some conditions, the ability to transport inorganic carbon is not affected by the removal of external CA (Williams and Turpin, 1987; Gehl et al., 1990).

The sequences of some forms of CA from spinach (Fawcett et al., 1990), pea (Majeau and Coleman, 1991), and *C. reinhardtii* (Fukuzawa et al., 1990) as well as *E. coli* (Guilloton et al., 1992) have been established. However, there are often three or more isozymes of CA in higher plants (Atkins et al., 1972) that can be immunologically disparate (Burnell, 1990). Three isozymes of CA have been described in *C. reinhardtii* that are distinct in their immunological response, inhibitor sensitivities, and control by CO_2 (Moroney et al., 1987; Husic et al., 1989).

Preliminary analysis of algal CAs in species other than *C. reinhardtii* suggests a diversity of CAs within the algae. The presence of external CA varies between species in some genera of algae. This fact has been used to compare internal and external CA in *Dunaliella* and *Chlorella*. Internal and external CA activity in *Dunaliella* showed different sensitivities to NaCl, whereas only a single immunoreactive band at 30 kD was observed (Goyal et al., 1992) when extracts of all species were probed with the antibody produced against the external form of CA from *C. reinhardtii*. Using similar antibodies, bands at 38 and 36 kD have been shown in *Chlorella saccharophila* that have been associated with internal and external CA, respectively (Coleman et al., 1991), whereas one strain of *Chlorella vulgaris* showed only weak reactivity at 27, 29, and 205 kD, and another showed no reactivity (Goyal et al., 1992).

Here we report on experiments that clearly identify isozymes of CA within a single clone of *C. saccharophila* that differ in immunoreactivity, stability at 30°C, and response to DTT.

MATERIALS AND METHODS

Chlorella saccharophila (UTCC 91, UTEX 2469) was grown axenically in medium as previously described (Gehl et al.,

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Abbreviations: CA, carbonic anhydrase; DIC, dissolved inorganic carbon.

1990) with a molar equivalent of NH_4Cl replacing $NaNO_3$. Culture pH was controlled by buffering with 10 mM Bicine, pH 8.5, and 10 mM Mes, pH 5.5. High-CO_2 cells were obtained by bubbling cultures with 2% CO_2 and buffering with 50 mM Bicine (final pH 8.0) for at least eight doublings. Cells were harvested at mid-log phase (A_{730} 0.40–0.60).

Cell extracts were obtained by resuspending 1 mL of packed cell volume in 1 mL of ice-cold buffer containing 50 mM Bicine (pH 8.5), 1 mM EDTA, and 10 mM NaCl. The suspension was added to 2 mL each of 425 to 600 μm and 150 to 212 μm of acid-washed, ice-cold glass beads in a 15-mL test tube. The sample was then homogenized using a Super-Mixer (Lab-Line Instruments, Inc., Melrose Park, IL) in five 1-min intervals with a minimum of 1 min between sessions to prevent any warming. The extract was collected and the beads were washed with 2 mL of buffer. The combined extract and wash was centrifuged at 30,000*g* for 1 h. Subsequent centrifugation at 100,000*g* for 2 h had no effect on the results obtained.

CA assays were carried out using a modification of the technique of Wilbur and Anderson (1948). An aliquot of extract (usually 50 μL) was added to 1.45 mL of ice-cold 20 mM veronal buffer, pH 8.3, in a water-jacketed chamber (2.0–4.0°C) containing a pencil-type pH electrode. Ice-cold CO_2-saturated water (0.5 mL) was injected rapidly into the sample with a syringe and the time taken for the pH to drop from pH 8.3 to 8.0 was measured. Units of activity were calculated using the formula $(T_c/T) - 1$, where T_c and T are the time taken in the absence or presence of sample, respectively. Activity was standardized using the total amount of Chl in the assay.

Chl was extracted by centrifuging 200 μL of cell suspension at 15,000*g* for 1 min and resuspending the pellet in 100 μL of DMSO. After a minimum 1-min incubation, 1.0 mL of methanol was added and the sample was vortexed briefly. Total Chl content was calculated using the method of Holden (1965).

Electrophoresis of extract was carried out using Titan III Zip Zone cellulose acetate membrane plates (Helena Laboratories, Mississauga, Ontario, Canada) at 200 mV for 1 h at 4°C. The running buffer contained 15.74 g of Tris, 0.82 g of EDTA, 0.24 g of NaCl, and 4.41 g of boric acid dissolved in 1.0 L of double-distilled water and adjusted to pH 8.9. Approximately 0.3 μL of extract containing about 10 mg protein/mL was loaded into each lane.

CA activity was visualized using a modification of the method of Atkins et al. (1972). Cellulose acetate plates were soaked in ice-cold 20 mM veronal buffer containing 0.1% bromcresol purple dye for 1 min. The plate was then placed on an aluminum plate on ice, blotted with Whatman paper, and exposed to a stream of CO_2 through an inverted filter funnel. CA appeared as yellow bands on a purple background within 2 min. Photographs were taken using TMAX 400 ASA film (Kodak).

Analysis of proteins associated with CA visualized on the cellulose acetate plates was performed by loading approximately 1.0 mg of protein onto the cellulose acetate plates by spotting 12 lanes 10 times with sample. The portions of the plates associated with the CA bands were scraped into 2 mL of extraction buffer and eluted by shaking overnight at 4°C.

The effects of DTT were examined by incubating cell-free extract of alkaline-grown cells in varying DTT concentrations at room temperature for 30 min. Extracts were then subjected to cellulose acetate membrane electrophoresis and observed as described above. The effects of temperature were examined after incubating a similar extract at room temperature (22°C) and at 30°C.

Protein samples for SDS-PAGE were prepared using TCA precipitation as described by Piccioni et al. (1982) and resuspended in sample buffer to a concentration of 1 mg/mL. SDS-PAGE was carried out as described by Piccioni et al. (1982) in a 10% running gel with a 4% stacking gel at 7 mA overnight. Gels were stained using the diamine silver method as described by Merril (1990). Molecular mass was estimated using prestained mol wt markers (Amersham).

Transfers of gels to nitrocellulose was accomplished in a Trans-Blot cell (Bio-Rad) containing 25 mM Tris, 192 mM Gly, 20% (v/v) methanol, and 0.05% (w/v) SDS, which was necessary for full transfer.

Proteins thus transferred were probed with a purified immunoglobulin G fraction containing the polyclonal antibody raised against the glycosylated form of the 37-kD monomer of the extracellular CA isolated from *C. reinhardtii* (kindly donated by Dr. John Coleman, University of Toronto). Specifically bound antibody was visualized using the Protoblot western blot alkaline phosphatase system (Promega Corp.).

RESULTS

Intact cells of *C. saccharophila* grown at alkaline pH under air levels of CO_2 or high CO_2 or grown at acid pH were assayed for CA activity using the potentiometric technique. CA activity in whole alkaline-grown cells was reduced to undetectable levels by growth at acid pH or on elevated CO_2 (Table I). Cell extracts of alkaline-grown cells had more activity than whole cells. Similarly, CA activity in cell extracts of high CO_2-grown cells and acid-grown cells was detected (Table I). The difference between activity in extracts and whole cells in all growth conditions remained relatively constant. Assuming that the increase in activity seen in cell extracts is due to the presence of internal CA, it would then seem that there is an internal CA that is expressed constitutively in *C. saccharophila*.

Further evidence for the existence of isozymes of CA in *C. saccharophila* was obtained. Extracts of *C. saccharophila* grown under the three conditions were electrophoretically separated on nondenaturing cellulose acetate plates. CA activity is readily visualized on these plates with the use of a pH-

Table I. *CA activity[a] of whole cells and whole cell extracts of cells grown at alkaline pH (8.5), acid pH (5.5), and at high CO_2 (2%)*

Growth Conditions	Whole Cells	Extract	Difference
Alkaline pH	18.1 ± 2.6 (5)	30.2 ± 1.0 (5)	12.1
Acid pH	1.1 ± 0.5 (2)	12.1 ± 0.6 (2)	11.1
High CO_2	0.4 ± 1.0 (4)	15.1 ± 2.7 (2)	14.7

[a] $(T_c/T - 1)$/mg Chl ± SE (no. of replicates in one experiment).

indicating dye. Extracts from alkaline-grown cells have two bands of activity (Fig. 1, lane A). The CA that moves slowly appeared more rapidly than the fast-moving CA, indicating that a greater amount of activity is associated with this band. Extracts from acid-grown cells and high CO_2-grown cells had only the fast-moving CA (Fig. 1, lanes B and C, respectively) and, although precise quantification of CA on the cellulose acetate plates is not possible, the rapidity of pH alteration between treatments suggests that this CA was present in similar quantities in cells grown under all conditions. This evidence supports the hypothesis that the fast-running band is associated with the internal CA activity and the slow band is associated with the external CA. The lack of CA seen at the slow band correlates closely with the effect of acid pH and high CO_2 on external CA activity seen using the potentiometric assay (Table I), strongly suggesting that this band is associated with external CA.

Transfer of protein to nitrocellulose from the cellulose acetate plates followed by western blot analysis using the antibody produced against the external form of CA from *C. reinhardtii* revealed only a weak reaction in all three extracts associated with the fast band (data not shown).

Western blot techniques were also carried out after SDS-PAGE of cell-free extracts of cells grown under the three conditions. A single band was visible at 39 kD in all extracts that did not vary between treatment (Fig. 2, lanes D–F). This suggests that the antibody recognizes only the internal form of CA from *C. saccharophila*. The possibility that the gels were overloaded (20 μg protein/lane) was examined by loading one-fourth of the amount of protein, and it was found that the 39-kD band was present at greatly reduced intensities that did not vary between growth conditions, which suggests that the reaction was not being saturated (data not shown).

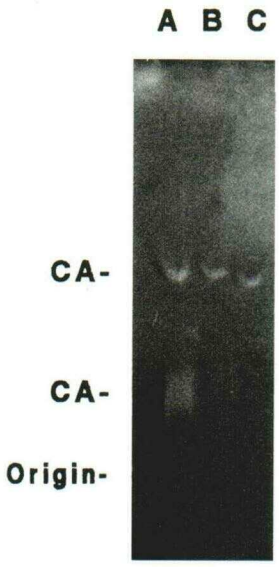

Figure 1. CA activity after cellulose acetate electrophoresis of cell-free extracts of *C. saccharophila* grown at alkaline pH (lane A), acid pH (lane B), and at 2% CO_2 (lane C). CA activity was visualized by soaking the plates in the pH-indicating dye bromcresol purple and exposing the plate to CO_2.

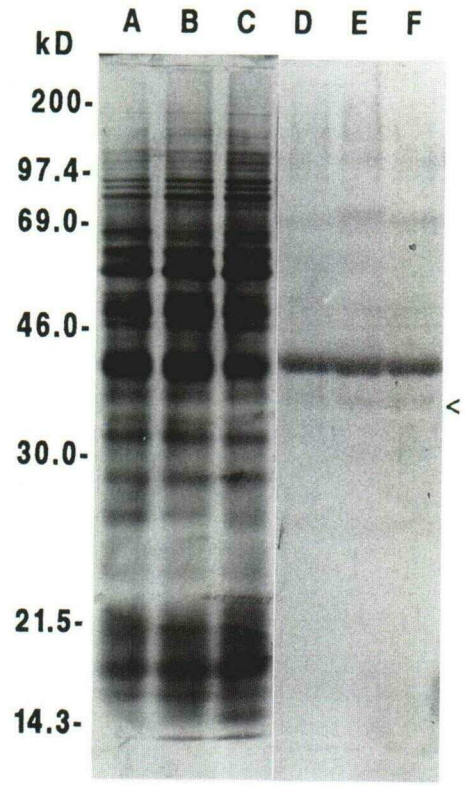

Figure 2. Silver stain (lanes A-C) and western blot analysis (lanes D–F) of cell-free extract of *C. saccharophila* grown at alkaline pH (lanes A and D), acid pH (lanes B and E), and 2% CO_2 (lanes C and F).

Proteins associated with the two bands were extracted from cellulose acetate plates and subjected to SDS-PAGE followed by western blot analysis. The single band at 39 kD was present at equal levels in all treatments but it was associated only with proteins extracted from cellulose acetate associated with the fast isozyme (Fig. 3, lanes A–C). Proteins eluted from cellulose acetate scraped at the position of the slow isozyme showed no reaction with the antibody in any of the treatments (Fig. 3, lanes D–F).

Previous studies have reported the existence of a second band at 36 kD associated with immunoreactivity to the antibody produced against CA from *C. reinhardtii* (Coleman et al., 1991). Occasionally, we have seen a second band (Fig. 2, lanes D–F), but it has been weak and has never been observed with proteins eluted from the slow band.

The external CA was inactivated partially by incubation of extract at room temperature and was inactivated entirely when incubated at 30°C for 1 h (Fig. 4). This inactivation was not alleviated by addition of protease inhibitors or 500 μM DTT (data not shown). The internal CA was unaffected by this treatment. The external CA was inactivated by 10 mM DTT and the internal CA was unaffected by DTT treatment (Fig. 5). Although most activity associated with the slow band was inactivated by DTT, another band became evident that was less susceptible to DTT (Fig. 5). This activity may be due to dissociated CA monomers as described by Husic et al. (1991), or by a third isozyme of CA. A doublet is seen

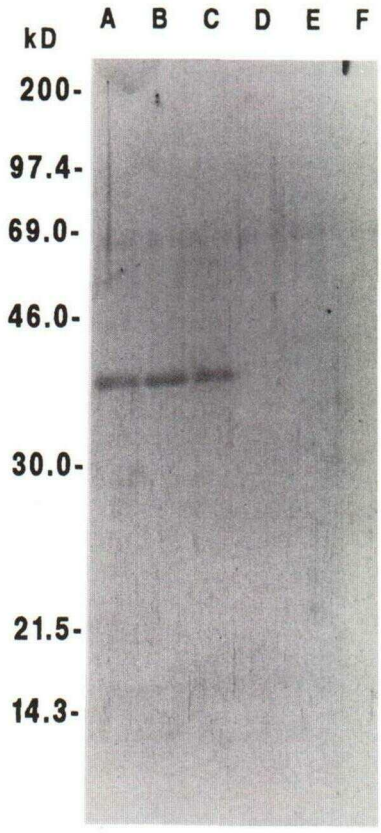

Figure 3. Western blot analysis of proteins eluted from cellulose acetate associated with the fast CA (lanes A–C) and the slow CA isozymes (lanes D–F). Extracts from alkaline-grown cells (lanes A and D), acid-grown cells (lanes B and E), and high CO_2-grown cells (lanes C and F) were separated electrophoretically on cellulose acetate plates, the CA isozymes were visualized as described in the text, the cellulose acetate was scraped from the plastic backing into buffer, and the proteins were eluted overnight.

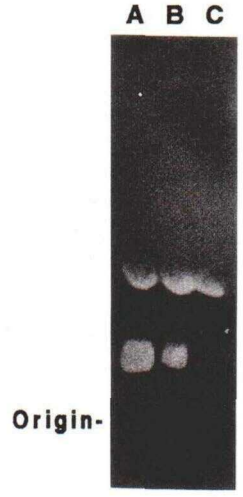

Figure 4. CA activity after electrophoresis on cellulose acetate plates of extracts of alkaline-grown cells of *C. saccharophila* incubated at 4°C (lane A), 22°C (lane B), and 30°C (lane C) for 1 h.

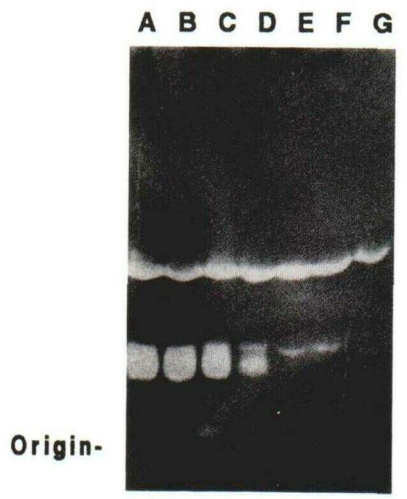

Figure 5. CA activity after electrophoresis on cellulose acetate plates of extracts of alkaline-grown cells of *C. saccharophila* incubated in 0, 0.1, 1.0, 5.0, 10.0, 50.0, and 100.0 mM DTT for 30 min at room temperature (lanes A–G, respectively).

occasionally at this location, which suggests that it is a third isozyme that is difficult to resolve from the major slow band.

DISCUSSION

Evidence has been presented that supports the hypothesis that *C. saccharophila* has at least two distinct isozymes of CA. Growth at acid pH or high CO_2 suppresses external CA activity, whereas cell extracts exhibit a consistent increase in activity over whole cells (Table I). Other workers have concluded that *C. reinhardtii* also has an internal CA that is under different regulatory control than the external form (Moroney et al., 1987), and others have described two periplasmic CA genes that are under inverse control by CO_2 (Fujiwara et al., 1990).

Two isozymes of CA were separated electrophoretically on cellulose acetate plates at pH 8.9 (Fig. 1). The fast-moving isozyme was present in cells grown at alkaline and acid pH as well as in cells grown at high CO_2. The slow-moving enzyme was present only in cells grown at alkaline pH and seemed to be present in greater quantities in these cells than the fast-moving isozyme. When taken in conjunction with the potentiometric results, these facts lead to the conclusion that the slow isozyme is an external CA and the fast isozyme is an internal CA. The fact that the two forms are readily separable on cellulose acetate plates indicates that the two forms have quite different primary structures, which is manifest in differing electrical charge at pH 8.9.

Further evidence to support the fact that the two forms are distinct comes from analysis of the peptides associated with the two bands using the antibody produced against the external form of CA from *C. reinhardtii*. Diffusion blotting of the cellulose acetate plates onto nitrocellulose followed by western blot analysis showed the presence of a single band in cells grown under all conditions that coincided with the location of the putative internal CA, but the reaction was very weak, which made photographic reproduction difficult.

No reaction was seen in extracts of alkaline-grown cells in the region of the slow-moving isozyme.

Western blot analysis of cell-free extracts following SDS-PAGE revealed a strong reaction in all growth conditions at 39 kD. A very weak band was also seen at 36 kD. The strong unvarying reaction at 39 kD in all treatments suggests that the internal CA has a monomeric molecular mass of 39 kD and that it is immunologically similar to the external form of CA from *C. reinhardtii*. Analysis of protein eluted from the cellulose acetate plates is consistent with the hypothesis that the 39-kD peptide is the internal form of CA. The 39-kD immunoreactive peptide was eluted from the cellulose acetate associated with the internal activity only and was present in extracts of cells from all growth conditions (Fig. 3). The proteins associated with the external CA exhibited no immunoreaction with the antibody.

The thermal stability of the two forms of the enzyme in cell extracts was also tested and it was shown that the external CA activity was extremely sensitive to a 1-h treatment at 30°C, whereas the internal CA was unaffected. Whole cells incubated at 30°C do not lose activity. The instability of the external enzyme has also been seen in attempts to purify the external CA. Given the instability of the external CA, it may be that some of its activity is lost during the homogenization process. If this is true, then estimates of internal activity based on the difference between whole-cell activity and cell extracts may underestimate internal CA activity in *Chlorella* species that exhibit external CA activity.

The external CA was shown to be susceptible to 10 mM DTT, but the internal form was not. In *C. reinhardtii* a similar effect of DTT has been seen on periplasmic CA that has been attributed to subunit dissociation (Husic et al., 1991). Our results also suggest that the external form of CA in *C. saccharophila* has subunits dependent on disulfide bonds in a manner similar to those of the periplasmic CA of *C. reinhardtii*. The internal CA was unaffected by DTT, suggesting that its quarternary structure is not dependent on disulfide linkages in the manner of the external CA from *C. reinhardtii*. A third band appeared in proximity to the slow band in extract incubated with concentrations of DTT greater than 5 mM, suggesting that there may be a third isozyme of CA that is difficult to resolve from the major slow band using cellulose acetate membrane electrophoresis. It may be that this band is a result of remnant activity of dissociated subunits as seen by Husic et al. (1991), but transient doublets have been seen during CO_2 incubation of the cellulose acetate plates, which suggests that the large slow band may be due to the presence of more than one isozyme.

Recent cloning of homologous CA genes from higher plants (Fawcett et al., 1990; Majeau and Coleman, 1991) and bacteria (Guilloton et al., 1992), as well as the discovery of a cyanobacterial gene homologous to higher plant CA (Fukuzawa et al., 1992), has led to the hypothesis that higher plant chloroplastic CA is of an evolutionary line that stems from bacteria. The external CA from *C. reinhardtii*, being homologous with animal CAs (Fukuzawa et al., 1990), suggests that the external CA in *C. reinhardtii* is of a different origin than higher plant CAs. Evidence presented elsewhere (Coleman et al., 1991) suggests that a 38-kD protein associated with isolated chloroplasts of *Chlorella ellipsopidea* is immunologically similar to the external CA of *C. reinhardtii*. Our evidence also suggests that the internal form of CA from *C. saccharophila* is similar to the external form of CA from *C. reinhardtii*. If this CA is chloroplastic and proves to be homologous with the periplasmic CA of *C. reinhardtii*, then *Chlorella* species would be in an evolutionary position unique from higher plants. Evidence from *Dunalliela* (Goyal et al., 1992) also suggests that there may be a great diversity in the structure, function, and location of CAs in the microalgae.

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LITERATURE CITED

Aizawa K, Miyachi S (1986) Carbonic anhydrase and CO_2 concentrating mechanisms in microalgae and cyanobacteria. FEMS Microbiol Rev **39:** 215–233

Atkins CA, Patterson BD, Graham D (1972) Plant carbonic anhydrases I: Distribution of types among species. Plant Physiol **50:** 214–217

Burnell JN (1990) Immunological study of carbonic anhydrase in C_3 and C_4 plants using antibiodies to maize cytosolic and spinach chloroplastic carbonic anhydrase. Plant Cell Physiol **31:** 423–427

Burnell JN, Hatch MD (1988) Low bundle sheath carbonic anhydrase is apparently essential for effective C_4 pathway operation. Plant Physiol **86:** 1252–1256

Coleman JR, Rotatore C, Williams TG, Colman B (1991) Identification and localization of carbonic anhydrase in two *Chlorella* species. Plant Physiol **95:** 331–334

Everson RG (1970) Carbonic anhydrase and CO_2 fixation in isolated chloroplasts. Phytochemistry **9:** 25–32

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, Suzuki E, Komukai Y, Miyachi S (1992) A gene homologous to chloroplastic carbonic anhydrase (icfA) is essential to photosynthetic carbon dioxide fixation by *Synechococcus* PCC7942. Proc Natl Acad Sci USA **89:** 4437–4441

Gehl KA, Colman B, Sposato L (1990) Mechanism of inorganic carbon-uptake in *Chlorella saccharophila:* the lack of involvement of carbonic anhydrase. J Exp Bot **41:** 1385–1391

Goyal A, Shiraiwa S, Husic HD, Tolbert NE (1992) External and internal carbonic anhydrases in *Dunaliella* species. Mar Biol **113:** 349–355

Graham D, Reed ML, Patterson BD, Hockley DG (1984) Chemical properties, distribution and physiology of plant and algal carbonic anhydrases. Ann NY Acad Sci **429:** 222–237

Guilloton MB, Korte JJ, Lamblin AF, Fuchs JA, Anderson PM (1992) Carbonic anhydrase in *Escherichia coli:* A product of the cyn operon. J Biol Chem **267:** 3731–3734

Hatch MD, Burnell JN (1990) Carbonic anhydrase activity in leaves and its role in the first step of C_4 photosynthesis. Plant Physiol **93:** 825–828

Holden M (1965) Chlorophylls. *In* TW Goodwin, ed, Chemistry and Biochemistry of Plant Pigments. Academic Press, New York, pp 461–488

Husic HD, Hsieh S, Berrier AL (1991) Effect of dithiothreitol on the catalytic activity, quarternary structure and sulfonamide-binding properties of an extracellular carbonic anhydrase from *Chlamydo-*

monas reinhardtii. Biochim Biophys Acta **1078:** 35–42

Husic HD, Kitayama M, Togasaki RK, Moroney JV, Morris KL, Tolbert NE (1989) Identification of intracellular carbonic anhydrase in *Chlamydomonas reinhardtii* which is distinct from the periplasmic form of the enzyme. Plant Physiol **89:** 904–909

Kuchitsu K, Tsuzuki M, Miyachi S (1991) Polypeptide composition and enzyme activities of the pyrenoid and its regulation by CO_2 concentration in unicellular green algae. Can J Bot **69:** 1062–1069

Majeau N, Coleman JR (1991) Isolation and characterization of a cDNA coding for pea chloroplastic carbonic anhydrase. Plant Physiol **95:** 264–268

Merril CR (1990) Gel-staining techniques. Methods Enzymol **182:** 477–487

Moroney JV, Togasaki RK, Husic HD, Tolbert NE (1987) Evidence that an internal carbonic anhydrase is present in 5% CO_2-grown and air-grown *Chlamydomonas.* Plant Physiol **84:** 757–761

Piccioni R, Bellemare G, Chua N (1982) Methods of polyacrylamide gel electrophoresis in the analysis and preparation of plant polypeptides. *In* M Edelman, RB Hallick, N Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, Amsterdam, pp 985–1014

Price GD, Coleman JR, Badger MR (1992) Association of carbonic anhydrase activity with carboxysomes isolated from the cyanobacterium *Synechococcus* PCC7942. Plant Physiol **100:** 784–793

Spalding MH, Spreitzer RJ, Ogren WL (1983) Carbonic anhydrase-deficient mutant of *Chlamydomonas reinhardtii* requires elevated carbon dioxide concentration for photoautotrophic growth. Plant Physiol **73:** 268–272

Tashian RE (1989) The carbonic anhydrases: widening perspectives on their evolution, expression and function. BioEssays **10:** 186–192

Tsuzuki M, Shimamoto T, Yang SY, Miyachi S (1984) Diversity in intracellular locality, nature, and function of carbonic anhydrase in various plants. Ann NY Acad Sci **429:** 238–240

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

Williams TG, Turpin DH (1987) The role of external carbonic anhydrase in inorganic carbon acquisition by *Chlamydomonas reinhardtii* at alkaline pH. Plant Physiol **83:** 92–96