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Carbonic Anhydrase Activity Associated with the Cyanobacterium *Synechococcus* PCC7942[1]

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ABSTRACT

Intact cells and crude homogenates of high (1% CO_2) and low dissolved inorganic carbon (C_i) (30–50 microliters per liter of CO_2) grown *Synechococcus* PCC7942 have carbonic anhydrase (CA)-like activity, which enables them to catalyze the exchange of ^{18}O from CO_2 to H_2O. This activity was studied using a mass spectrometer coupled to a cuvette with a membrane inlet system. Intact high and low C_i cells were found to contain CA activity, separated from the medium by a membrane which is preferentially permeable to CO_2. This activity is most apparent in the light, where ^{18}O-labeled CO_2 species are being taken up by the cells but the effluxing CO_2 has lost most of its label to water. In the dark, low C_i cells catalyze the depletion of the ^{18}O enrichment of CO_2 and this activity is inhibited by both ethoxyzolamide and 2-(trifluoromethoxy)carbonyl cyanide. This may occur via a common inhibition of the C_i pump and the C_i pump is proposed as a potential site for the exchange of ^{18}O. CA activity was measurable in homogenates of both cell types but was 5- to 10-fold higher in low C_i cells. This was inhibited by ethoxyzolamide with an I_{50} of 50 to 100 micromolar in both low and high C_i cells. A large proportion of the internal CA activity appears to be pelletable in nature. This pelletability is increased by the presence of Mg^{2+} in a manner similar to that of ribulose bisphosphate carboxylase-oxygenase activity and chlorophyll (thylakoids) and may be the result of nonspecific aggregation. Separation of crude homogenates on sucrose gradients is consistent with the notion that CA and ribulose bisphosphate carboxylase-oxygenase activity may be associated with the same pelletable fraction. However, we cannot unequivocally establish that CA is located within the carboxysome. The sucrose gradients show the presence of separate soluble and pelletable CA activity. This may be due to the presence of separate forms of the enzyme or may arise from the same pelletable association which is unstable during extraction.

Both green microalgae and cyanobacteria have been shown to possess an inducible CO_2 concentrating mechanism which is expressed when the cells are grown on limiting levels of C_i[2] (5, 16). In green microalgae, it has become clear that various forms of CA are associated with the utilization of C_i from the external medium during photosynthesis. This is particularly so for cells which have been grown at limiting levels of C_i and possess a CO_2 concentrating mechanism based on the active transport of C_i either across the plasmamembrane or the chloroplast envelope (14, 18). This includes an external periplasmic CA activity, particularly in cells grown at limiting C_i, as well as internal CA activities which may be both soluble and membrane bound in nature, depending on the species examined (1, 6). The external CA activity appears to be functional in the conversion of HCO_3^- to CO_2, with CO_2 then entering the cell either by passive diffusion or active transport across the plasma membrane (14, 18). The internal CA activity is thought to be involved in the conversion of HCO_3^- to CO_2 which may then be used by the CO_2 fixing enzyme Rubisco. This role is particularly important in a cell which is involved in active C_i accumulation, as it is assumed that the C_i species which is delivered to the cell by the transporter is HCO_3^- (6).

The involvement of CA activity in the process of C_i accumulation and utilization in cyanobacteria is less clear. This is generally because of the lower and more variable CA activities associated with cyanobacterial species. There appears to be no periplasmically located CA activity associated with cyanobacterial cells examined to date (3, 28, 30). There is, however, low but variable CA activity associated with cell homogenates made from cyanobacteria grown at limiting levels of C_i. The nature of this CA activity appears to be both particulate and soluble, depending on the species examined. Ingle and Colman (10) concluded that with *Oscillatoria* sp. the CA activity in air grown cells was cytoplasmically located. Yagawa *et al.* (30) found that 50 to 70% of the CA activity in homogenates of two *Anabaena variabilis* strains was particulate but from a third strain appeared to be entirely soluble. In *Chlorogloeopsis fritschii*, Lanaras *et al.* (13) found that at least 90% of the CA activity was particulate in nature. The amount of activity found in homogenates of cells grown at limiting C_i also appears variable, ranging from 0.8 to 30 units per mg protein depending on species (4, 10, 11, 13, 30), and in certain strains of *A. variabilis* and *Anacystis nidulans* it has been reported to be undetectable (12).

The most likely role of internal CA in cyanobacteria is to catalyze the conversion of HCO_3^- to CO_2, as HCO_3^- appears to be the form of C_i accumulated by the cell during photosynthesis. Two high CO_2 requiring mutants of *A. nidulans* have been isolated which appear to accumulate internal C_i but are not able to utilize it for photosynthesis, suggesting that there

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[2] Abbreviations: C_i, dissolved inorganic carbon; CA, carbonic anhydrase; Rubisco, ribulose bisphosphate carboxylase-oxygenase; EZ, ethoxyzolamide; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; PMSF, phenyl methyl sulphonyl fluoride; FCCP, *p*-(trifluoromethoxy)carbonyl cyanide; I_{50}, 50% inhibition.

is a blockage in this conversion (15, 20). Unfortunately, conventional CA assays were unable to detect activity in either the mutant or wild-type strains and evidence for a lack of internal CA being responsible for the phenotype could not be established. This was the same *Anacystis* strain used in these experiments reported here. We could not detect CA in this strain using conventional assays but used mass spectrometry and isotope disequilibrium techniques to establish that this CA activity can be measured in cell homogenates. We used this assay technique to quantify and localize CA activity within the cell and to characterize the activity shown by intact cells in the light and the dark.

MATERIALS AND METHODS

Cell Culture

Cells of *Synechococcus* PCC 7942 were grown in BG11 medium (25) buffered with 10 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (pH 8.0). Cells were grown in batch culture in large test tubes (195 × 35 mm, 100 mL), illuminated from the side by a combination of Gro-Lux (Sylvania GTE) and white fluorescent tubes (60 μmol photons·m^{-2}·s^{-1}; 30°C).

Cultures were bubbled rapidly with gas dispersion tubes to ensure maximum equilibration between the gas and liquid phases and cells were harvested in log phase before a Chl density of 3 $\mu g \cdot mL^{-1}$ was reached. 'High C_i' cells were grown with 1% CO_2 in air in the gas phase, while 'low C_i' cells were produced with 30 to 50 $\mu L/L$ CO_2 in air. Culture pH remained constant at pH 8.0 when Chl densities were kept below 5 $\mu g \cdot mL^{-1}$.

Preparation of Cell Extracts

Cells were collected from culture by centrifugation (4000*g*, 10 min, 25°C) and resuspended in 50 mL of lysozyme buffer (0.6 M sucrose, 2 mM EDTA, 5 mM Tes-KOH, pH 7.0). Lysozyme was added (1–2 mg mL^{-1}) and the cells were incubated at 37°C for 1.5 to 2 h with occasional agitation. The cells were centrifuged from the suspension and washed once in the original lysozyme buffer, followed by a second centrifugation and suspension in breaking buffer (this varied from experiment to experiment and is detailed in the methods of individual experiments). Cells were broken by passage through a French pressure cell at 140 MPa (4°C) to produce a crude extract.

Carboxylase Assays

Assays were performed at 30°C in a total volume of 0.5 mL reaction mix (50 mM EPPS (pH 8.0), 10 mM $MgSO_4$, 25 mM $NaH^{14}CO_3$ [450 cpm/nmol]). Extract (5–10 μL) was preincubated in the assay mix for 5 min prior to the initiation of the reaction with ribulose bisphosphate (0.4 mM). Assays were run for 5 min and stopped by the addition of 0.4 mL 10% formic acid. Samples were dried and counted by liquid scintillation spectrometry.

CA Assays

CA activity was determined by two techniques:

1. A conventional assay based on measuring the rate of change in pH after the injection of a standard amount of CO_2 saturated water. Assays were performed in a stirred, water jacketed cuvette (2°C), with a pH electrode inserted into the assay solution. Assay buffer (2 mL, 5 mM EPPS, pH 8.0) was added to the cuvette followed by 0.2 to 0.5 mL of extract. After temperature equilibration had occurred, 0.5 mL of ice-cold CO_2 saturated water was injected into the assay and the time for a pH change between 8 and 7 was recorded. Control assays contained either boiled extracts or extracts inhibited with 200 μM EZ. Extracts for assays were prepared by breaking lysozyme digested cells into 20 mM Tes (pH 8.0), 10 mM $MgCl_2$, 2 mM PMSF, and ± 0.6 M sucrose, and assayed at 0.75 to 1.0 mg Chl·mL^{-1}.

2. A mass spectrometric MS technique was used based on the method previously described by Silverman (26) and used by Tu *et al.* (27) for *Chlorella vulgaris.* This procedure is based on measuring the loss of $^{18}O_2$ from labeled C_i to water, caused by the hydration and dehydration of CO_2 and HCO_3^-, which is catalyzed by CA.

The rate of ^{18}O loss was measured by monitoring the concentration of all CO_2 species in solution. This was achieved through the use of an aqueous membrane inlet vessel (9), which allows CO_2 and other gases to pass from the reaction solution to the source of the mass spectrometer (VG micromass 6, interfaced to a computer for peak switching, data collection and analysis). Measurements were done with ^{13}C-labeled C_i which had been enriched with ^{18}O. This enrichment was achieved by dissolving ^{13}C-labeled $NaHCO_3$ into $H_2{}^{18}O$ (99% enrichment) to a 1 M concentration and allowing the solution to reach isotopic equilibrium for at least 24 h. An aliquot of this solution was injected into the assay and the change in the enrichment of CO_2 species was measured after chemical equilibrium had been reached (3 to 4 min at 30°C). Crude extracts were assayed at a final Chl concentration of 20 to 100 $\mu g/mL$.

The height of the individual CO_2 peaks were measured (masses 45, 47, and 49) and the ^{18}O atom fraction in doubly labeled CO_2 (also termed atom % enrichment) was calculated as:

$$^{18}\text{O atom fraction} = \frac{(^{13}C^{18}O^{18}O)}{(^{13}CO_2)} = \frac{(49)}{(45 + 47 + 49)}.$$

For the calculation of carbonic anhydrase activities, the rate of decline of this atom fraction was calculated from the slope of the change in log (atom fraction) with time. This gives a measure of the first order rate constant. The rate in the presence of sample was compared to that in its absence and the percent increase calculated. In this assay system, one unit of enzyme activity is equivalent to a 100% stimulation in the first order rate constant for the loss of label from doubly labeled CO_2.

^{18}O Exchange Kinetics of Cells

The methods used here are modifications of the procedures developed by Silverman (26) and used by Tu *et al.* (28) with studies of a marine *Synechococcus* species. Cells were incubated with ^{13}C- and ^{18}O-labeled C_i in a cuvette connected to a mass spectrometer, as described for the CA assays outlined

above. The signals for the labeled species (masses 49, 47, 45) were monitored and the log (atom % enrichment) in doubly labeled CO_2 calculated as described above.

Sucrose Rate Gradients

Two 2.5 L cell cultures were grown in flat sided glass carboys (8 cm light path, 100 μmol photons $m^{-2}s^{-1}$ incident light), gassed with 30 μL/L CO_2 in air to a cell density of about 2.5 μg Chl mL^{-1}. Cells were harvested separately and incubated in lysozyme medium as described above, then isolated by centrifugation and resuspended in 2 mL of either 20 mM Tes (pH 7.0), 1 mM EDTA, 2 mM PMSF, 0.1 mg/mL DNAase or 100 mM Bicine (pH 7.9), 10 mM $MgSO_4$, 1 mM EDTA, 2 mM PMSF, 0.1 mg/mL DNAase and broken by passage through a French press as described above. A 1.5 mL sample of each homogenate was loaded onto a 35 mL 15 to 60% (w/w) linear sucrose gradient, 25 mM K-phosphate (pH 7.0) and spun for 2 h in an SW28 rotor at 27,000 rpm at 5°C. Fractions were collected from the bottom of the tube and assayed.

Sucrose Flotation Gradient

A flotation gradient technique has been developed for cyanobacteria that can achieve a separation of cell membrane, cell wall and thylakoid fractions from soluble materials (21). A modification of this technique was used to examine the association of CA activity with membranous cell fractions. Cells were grown in 2.5 L glass carboys, as described above. They were initially gassed with 1% CO_2 in air which was subsequently changed to air for the final 2 d of growth. Cells were harvested and incubated in lysozyme medium; as described above, then isolated by centrifugation and resuspended in 20 mM Tes (pH 7.0), 2 mM PMSF, 0.1 mg/mL DNAase, and broken by passage through a French press as described above. This sample was adjusted to 55% sucrose (w/v), 10 mM NaCl, 5 mM EDTA, 5 mM Tes (pH 7.0) by the addition of a concentrated stock sucrose solution. An aliquot of 9 mL of this sample was layered onto 9 mL of 65% sucrose solution in a SW-28 centrifuge tube. This sample was overlayed by 9 mL of 50% sucrose solution, 7 mL of 39% sucrose solution, and 3 mL of 10% sucrose solution. All added sucrose layers contained 5 mM Tes (pH 7.0), 5 mM EDTA, and 10 mM NaCl. The gradients were spun at 27,000 rpm for 18 h at 5°C and fractionated from the bottom of the tube.

Chl Determination

Chl was determined by the method of Wintermans and de Mots (29).

RESULTS

CA Activity of Intact Cells

Cells in the Dark

The experiments shown in Figure 1 were performed to assess the ability of both low and high C_i cells, under dark

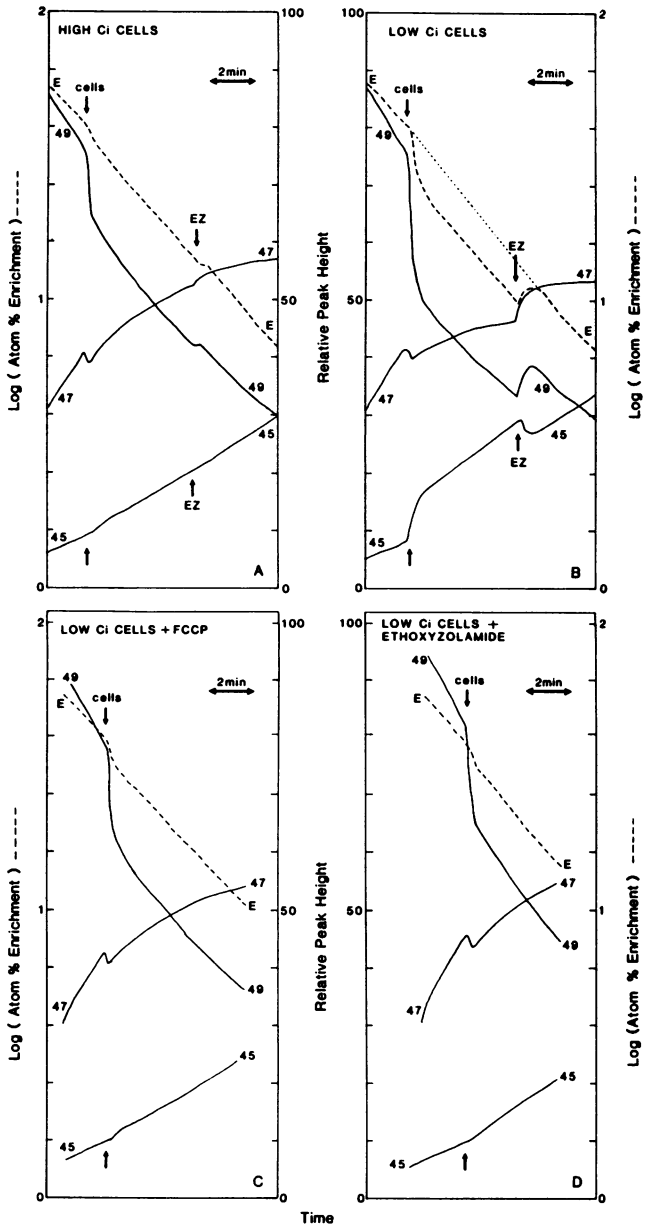


Figure 1. Effects of cells kept in the dark on the exchange of ^{18}O out of labeled CO_2 species. Assay buffer (2 mL BG11 growth medium buffered with 10 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane, pH 8.0, 30°C) plus $NaH^{13}C^{18}O_3$ (2.5 μL of 0.5 M to 625 μM final concentration) was allowed to equilibrate in the cuvette in the dark until chemical equilibrium had been reached between C_i species, *i.e.* a linear slope for the decrease in log (atom % enrichement) of doubly labeled CO_2 had been obtained (approximately 5 min). Cells (0.2 mL, resuspended in assay buffer) were then injected into the cuvette (8.0 μg Chl mL^{-1} final concentration) and the changes in the 45, 47, and 49 masses as well as the log (atom % enrichment) (E) were continuously monitored and are displayed on each figure. The responses of both (A) high C_i and (B) low C_i cells are shown in the top panels. In these two experiments, ethoxyzolamide was added (500 μM) about 5 min after the addition of the cells. The lower panels show the effect of preincubating low C_i cells for 2 min prior to injection, with either (C) FCCP (25 μM) or (D) EZ (500 μM). The inhibitor was also present in the assay at the same concentration as during the preincubation. Dotted lines show the change or enrichment which would have been expected if no cells or inhibitor had been added to the assay.

conditions, to catalyze the interconversion of C_i species in solution. When high C_i cells (Fig. 1A) were injected into the assay mixture there was a negligible effect on the rate of loss of ^{18}O label from doubly labeled CO_2, apart from a slight decline in the enrichment upon injection of the cells. Similarly, low C_i cells (Fig. 1B) did not alter the rate of decline of enrichment, but did cause a significant decline in the absolute level of enrichment immediately after they were injected into the assay. This effect on enrichment is similar to that seen by Tu *et al.* (27) with *Chlorella vulgaris* cells. It can be explained by assuming that there is carbonic anhydrase activity associated with the cells, but that it is internally located and that only CO_2 is then able to gain access to it via diffusion into the cell. The negligible effect of low C_i cells on the rate of decline of enrichment indicates that HCO_3^- is quite impermeable compared to CO_2.

The CA inhibitor, EZ, did not affect the rate of the decline in enrichment with either high or low C_i cells in the dark (Fig. 1), as expected if this rate is not influenced by the presence of cells. However, this inhibitor did considerably decrease the rapid decline in enrichment level seen when cells were first added to the assay. This can be seen from Figure 1B, when EZ was added to an assay where cells were already present. In this case, a rise in enrichment was seen which was approximately equivalent to the initial decline when cells were injected. This effect can also be seen in Figure 1D when cells were preincubated with the inhibitor prior to their addition to the assay. In this case, the initial decline in enrichment was much decreased.

The proton ionophore FCCP had a similar effect to EZ (Fig. 1C), in that it markedly decreased the initial decline of enrichment when low C_i cells were injected into the assay. This initial rapid decline in enrichment was assumed above to be due to the preferential access of CO_2 to internally located CA activity. Thus, these FCCP effects may be due to an indirect effect on internal CA activity, perhaps by internal pH modification, or by altering the access of CO_2 to the interior of the cell. It was initially assumed that access to internal CA was by means of passive diffusion across the cell membrane. However, as FCCP will inhibit residual activity of the C_i pump in the dark (19), it is possible that the C_i pump may be involved in either facilitating the transfer of CO_2 or more directly by catalyzing the exchange reaction itself as a consequence of the mechanism of the transport process.

Cells in the Light

The effect of light on the ability of both high and low C_i cells to promote the exchange of ^{18}O out of labeled inorganic carbon is shown in Figure 2. When the light was switched on, both high and low C_i cells showed similar patterns of exchange but they were more accentuated with low C_i cells. The results seen are similar to those previously reported for marine *Synechococcus* species (3, 27). Uptake of CO_2 species from the medium was rapid when the cells were illuminated. However, after 30 s to 1 min there was an increase in the unlabeled 45 mass while the labeled species continued to decline (Fig 2, A and B). This indicates that CO_2 which has lost its ^{18}O label to water was being released into the medium from the cells and that this could only be derived from labeled C_i species and not from an internal carbon source, as this would not be labeled with ^{13}C. This release of mass 45 leads to a decline in the enrichment of doubly labeled CO_2.

When the light was switched off an increase in CO_2 species was seen. This rise in CO_2 species could be due to two factors. First, it may arise from the efflux of CO_2 from the pool inside the cell during a period in which CO_2 uptake has ceased. An increase due to this effect would be expected to show a peak and then a decline to lower steady-state level as the efflux declined to zero. Second, it may be a result of a return to chemical equilibrium between CO_2 and HCO_3^- in the external solution after CO_2 uptake has stopped. An increase due to this would asymptotically approach a steady-state value and show no clear peak. The dark increase in CO_2 species was much more accentuated for the unlabeled 45 mass, and is consistent with a release of largely unlabeled CO_2 species from the inorganic carbon pool within the cell. It is hard to estimate to what extent the internal inorganic carbon pool retains any ^{18}O label, but the observation that only the 45 mass shows any appreciable overshoot would indicate that most of the internal C_i had undergone rapid exchange with water and lost most of its ^{18}O label. The rapid release of unlabeled CO_2 from the cell upon darkening leads to an initial decline in enrichment followed by a rise to an equilibrium level, presumably after efflux from the internal pool had ceased. In both high and low C_i cells, the rate of the decline of enrichment was the same both before and after the light period. However, with low C_i cells the decline in the absolute level of enrichment was greater than would have been expected by non-catalytic exchange reactions alone. Assuming that the exchange within the cells is equally rapid in both high and low C_i cells, this would be due to the greater C_i uptake rate in low C_i cells which would allow a higher proportion of the external C_i pool to gain access to the internal CA activity.

If the loss of ^{18}O label to water in the light is due to the action of carbonic anhydrase within the cell, then it might be possible to inhibit this by the use of ethoxyzolamide and by doing so produce a dark/light time-course (Fig. 2), which shows the release of ^{18}O labeled species from the internal C_i pool. However, we have recently shown that EZ also appears to inhibit the C_i transport process without necessarily inhibiting the internal CA activity of intact cells (22, 23). Thus, it might not be possible to inhibit the internal CA without first abolishing the transport of C_i. Experiments similar to those shown in Figure 2 have been performed with both low-C_i and high-C_i cells at EZ concentrations ranging from 40 to 200 μM (data not shown). There is no qualitative change in the time-course and all changes observed are consistent with a reduction in the C_i uptake process without a significant effect on the extent to which ^{18}O label is exchanged out of the internal C_i pool. These observations are consistent with our initial findings from silicon oil C_i accumulation studies (22), that ethoxyzolamide inhibits transport activity without significantly altering attainment of rapid equilibrium between C_i species within the cell.

CA Activity in Crude Homogenates

The foregoing assays of intact cells indicate carbonic anhydrase activity is associated with both high and low C_i cells

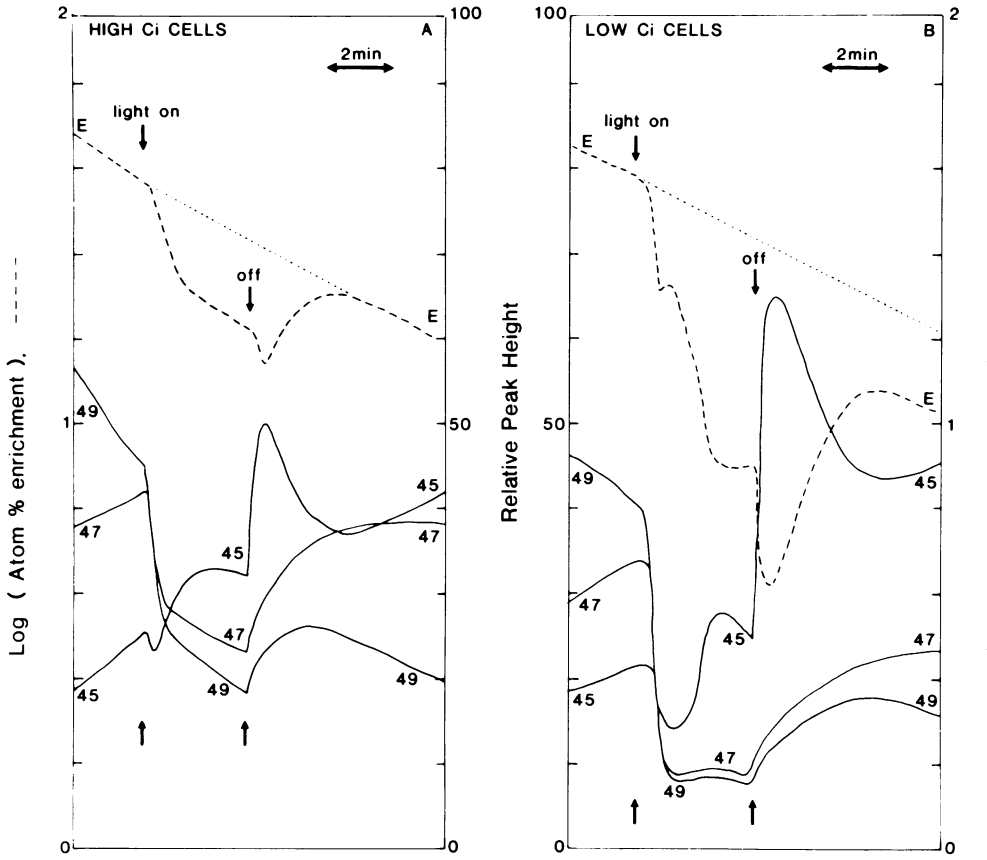


Figure 2. Effect of light on the ability of (A) high C_i and (B) low C_i cells to exchange ^{18}O out of labeled CO_2 species. Assay conditions were as for figure 1, except that cells were added to the cuvette (9.3 μg Chl mL^{-1}), in the dark, and time was allowed for chemical equilibrium between the C_i species to be reached and a linear slope for the decline in log (atom % enrichment) (E) obtained. At this point the light (200 μmol photons $m^{-2}s^{-1}$) was switched on and the cells were illuminated for about 3 min prior to darkening again.

and is located within the cell, separated by a permeability barrier from the external medium. Attempts were therefore made to measure this internal carbonic anhydrase activity in crude homogenates made from both cell types.

We were unable to reliably detect any appreciable CA activity using conventional CA assays, which are based on measuring the time taken to catalyze a pH change following the addition of a given amount of CO_2 saturated water. This was despite the use of the most concentrated cell suspensions that we could prepare (approximately 1 mg Chl/mL in final assay). However, using the MS CA assay we had no difficulty in detecting such activity in assays containing as little as 20 μg Chl/mL. This difference in detection level is due to two major features of the MS assay. First, the assay can be conducted at 30°C rather than 0°C, thus increasing any enzymic activity. Second, the rate of decline of the log (enrichment) is linear and may be measured over at least 5 to 10 min without the exchange reaction reaching equilibrium with water.

Table I shows the results of CA assays of crude extracts of both high and low C_i cells. Low C_i cells show activities varying from 50 to 100 units/mg Chl, while in high C_i cells a reduced level of 10 to 20 units/mg Chl is found. Given an average internal cell volume of 60 μL/mg Chl (22), then there would be sufficient internal CA in low C_i cells to increase the interconversion rate between C_i species by 800- to 1000-fold.

This CA activity is inhibited by EZ. An inhibition response curve of CA activity in both high and low C_i crude extracts is shown in Figure 3. The I_{50} for inhibition was similar for both homogenates, being in the region of 50 to 100 μM. As can be seen from the response, a small component of the apparent CA activity cannot be entirely inhibited by saturating levels of EZ. This background rate is probably due to components in the extract which catalyze the reaction in a nonspecific fashion.

Table I. *CA Activity in Crude Extracts of High and Low C_i Grown Cells of Synechococcus R2*

Cells were broken into 20 mM Tes (pH 7.0), 5 mM EDTA, 2 mM PMSF, 0.1 mg/mL DNAase, and the crude extracts were assayed using the mass spectrometer assay described in "Materials and Methods" at final Chl concentrations ranging from 20 μg/mL for low C_i cell to 100 μg/mL for highC_i cells.

Cell Type	CA Activity
	units/mg Chl
Low C_i cells[a]	
1	100.8
2	50.9
3	82.6
4	64.7
High C_i cells	
1	14.9
2	19.4
3	15.3
4	9.8

[a] Four separate measurements were made for each cell type.

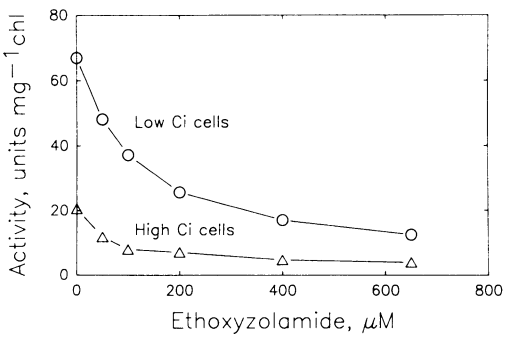


Figure 3. Inhibition of CA activity by EZ in crude extracts of high and low C_i cells. The crude extracts were prepared from high and low C_i cells grown as described in "Materials and Methods," and assays were conducted with the MS technique. Units of activity were derived as described in "Materials and Methods."

Localization of CA Activity

It has been proposed that CA within the cell plays a role in speeding up the conversion of HCO_3^- to CO_2 to aid in the elevation of the intracellular CO_2 partial pressure and thus allow the Rubisco reaction to operate near substrate saturation (4, 24). However, it has been proposed that rather than being located in the bulk cytoplasm of the cell, CA may be specifically localized within the Rubisco containing carboxysome (24). Given various assumptions about the properties of the carboxysome coat, this specialized localization could operate to elevate CO_2 preferentially within the carboxysome itself, rather than the whole cell (24). As some carbonic anhydrase in cyanobacteria has been reported to be particulate in nature (10, 13, 30), we examined the extent to which the CA activity in *Synechococcus* PCC7942 is associated with particulate fractions and, more importantly, whether there was any evidence for a specific association with carboxysomes.

Pelletability Studies

Table II shows a comparison of the pelletability of carbonic anhydrase, Rubisco and Chl under various experimental treatments. When a crude extract was prepared from high C_i cells at pH 7 with EDTA present (Experiment 1), the standard pelleting centrifugation was able to pellet 71% CA, 61% Rubisco, and 17% of the Chl. When 10 mM $MgSO_4$ was added to the supernatant and it was spun again, most of the remaining CA, Rubisco, and Chl could now be pelleted. When crude extracts of both high and low C_i cells were prepared at pH 8, in the absence of EDTA, then almost all the activities of CA and Rubisco and the Chl were pelletable (Experiments 2 and 3). The further addition of either EDTA or $MgSO_4$ caused no further change to this pelletability. These results (and others not shown) indicate that the ionic conditions of the breaking solution have significant effects on the association of CA and Rubisco activities with the pelletable fraction.

Coleman *et al.* (8) originally reported that the stability of carboxysomes was dramatically affected by the $[Mg^{2+}]$ of the solution. The results here suggest that there is a great potential for the thylakoid membranes and other cell components to form aggregates when exposed to Mg^{2+}, especially at pH 8. Such aggregates are apparent even at the light microscope level. It is difficult to know whether a 100% pelletability of both Rubisco and CA activity, when associated with similar pelleting patterns for chlorophyll, is due to the intrinsic association of these activities with a membrane or carboxysome fraction, or whether it is due to some increase of the association of soluble forms of these enzymes with the thylakoids by a general aggregation process. The pelleting of CA and Rubisco activity in experiment 1 was significantly higher than that of the Chl fraction. This may be evidence for a specific association of these activities with a fraction other than the thylakoids.

Sucrose Gradient Separation

To further resolve the association of CA with a specific particulate fraction, particulate fractions were separated on a sucrose density rate gradient. Figure 4 shows the distribution of Rubisco and CA activities and Chl, obtained when crude extracts of low C_i cells were separated on sucrose density rate gradients. Two crude homogenate samples were run, differing in whether the cells were broken in the presence or absence of added Mg^{2+}. In both instances, the majority of the CA activity ran toward the bottom of the tube, and is obviously associated with a readily pelletable fraction. The fractions containing CA activity are clearly associated with Rubisco activity and this is most obvious in the plus Mg^{2+} gradient. The absence of Mg^{2+} from the breaking medium caused Rubisco to be released from the pelletable fraction and thus appear with the soluble protein fraction at the top of the tube. The distribution of Chl is distinctly different to that of either CA or Rubisco activity and it can be concluded that CA is not associated with the thylakoid fraction of the cell.

Analysis of fractions from similar gradients by electron microscopy shows the presence of polyhedral bodies (carboxysomes) in the pelletable fractions which correlate with both Rubisco and CA activity at the bottom of the gradient (data not shown). While there is a correlation of the sedimentation of Rubisco with CA activity, the quantitative relationship between pelletable and soluble fractions as well as the effects of Mg^{2+} on this relationship, suggests that both activities may not be associated with the same fraction. There is obviously greater soluble Rubisco activity at the top of the gradient than is apparent for soluble CA activity and this is accentuated with the absence of Mg^{2+}. However, two arguments may be put forward to explain this observation. First, there may be both soluble and carboxysomal Rubisco in the cell prior to extraction, whereas the majority of the CA may be associated with the carboxysomal fraction. Second, the CA activity may be more intimately associated with the carboxysome, such that the release of Rubisco occurs much more readily than for CA activity. This reasoning could explain the relatively large effect of Mg^{2+} on Rubisco pelletability as compared to CA pelletability. In a number of sucrose gradients similar to those shown here, we have found some variability in the distribution of CA activity between pelletable and soluble fractions. In Figure 4, there appears to be less than 20% of the CA activity at the top of the gradient, but in other gradients we have found this to be as high as 60%. The reason for this variability cannot be readily attributed to either cell growth conditions or extraction procedures.

Table II. *Pelletability of CA and Rubisco Activities, and Chl*

Experiment 1. High C_i cells were incubated in lysozyme digestion solution (see methods) and subsequently broken into 20 mM Tes (pH 7.0), 5 mM EDTA, 2 mM PMSF, 0.1 mg/ml DNAase. This crude fraction was spun at 15,000 rpm in an Eppendorf centrifuge (1.5 mL tube; 15,000*g*) for 10 min at 4°C, and subsequently separated into pellet (EDTA) and supernatant (EDTA) fractions. The pellet was resuspended in breaking buffer with a volume equivalent to the supernant. The supernatant fraction was further treated by the addition of 10 mM $MgSO_4$ and allowed to sit on ice for 10 min prior to a further centrifugation. Pellet ($+Mg^{2+}$) and supernatant ($+Mg^{2+}$) fractions were prepared as above. *Experiment 2.* Low C_i cells were broken into 100 mM EPPS, (pH 8.0), 2 mM PMSF, 0.1 mg/mL DNAase. The crude homogenate was split into three equal fractions to which were added nothing, 10 mM $MgSO_4$, or 10 mM EDTA. These fractions were allowed to sit on ice for 10 min prior to centrifugation as described for experiment 1. Pellet and supernatant fractions were produced as described for experiment 1. *Experiment 3.* High C_i cells were broken into 100 mM EPPS (pH 8.0), 2 mM PMSF, 0.1 mg/mL DNAase. The crude extract was split into two and to one of these fractions 10 mM $MgSO_4$ was added. After 10 min on ice, the extracts were centrifuged as described for experiment 1. Supernatant and pellet fractions were produced as described for experiment 1.

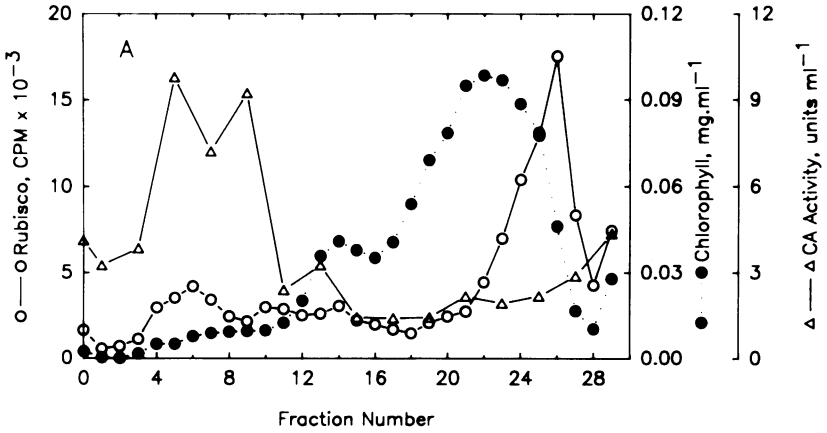
Treatment	CA	Rubisco	Chlorophyll
	units·mg⁻¹Chl	*mol·mg⁻¹Chl·min⁻¹*	*mg·ml⁻¹*
Experiment 1			
EDTA sup[a]	11.1 (29)[c]	1.73 (39)	0.342 (83)
EDTA pel[b]	28.5 (71)	2.80 (61)	0.068 (17)
$+Mg^{2+}$ sup	4.4 (26)	ND[d]	0.017 (6)
$+Mg^{2+}$ pel	12.7 (74)	1.37 (100)	0.283 (94)
Experiment 2			
$-Mg^{2+}$ sup	6.6 (19)	0.02	0.004 (2)
$-Mg^{2+}$ pel	28.2 (81)	2.80 (>99)	0.216 (98)
+EDTAsup	9.7 (27)	ND	0.003 (1)
+EDTA pel	26.6 (73)	3.14 (100)	0.219 (99)
$+Mg^{2+}$ sup	7.0 (16)	0.02	0.003 (1)
$+Mg^{2+}$ pel	36.2 (84)	2.91 (>99)	0.212 (99)
Experiment 3			
$-Mg^{2+}$ sup	1.8 (5)	ND	0.001
$-Mg^{2+}$ pel	37.1 (95)	2.60 (100)	0.207 (>99)
$+Mg^{2+}$ sup	2.9 (11)	ND	0.011 (5)
$+Mg^{2+}$ pel	25.7 (89)	2.61 (100)	0.193 (95)

[a] Supernatant fraction. [b] Pellet fraction. [c] Percentage of total activity in parentheses. [d] Not detectable.

A flotation gradient technique has been developed for cyanobacteria that can achieve a separation of cell membrane, cell wall and thylakoid fractions from soluble material (21). This technique was used to further examine the association of CA activity with other cell fractions (Fig. 5). There is clearly no CA activity associated with the light cytoplasmic membrane fraction which floats to the top of the gradient and is found at the interface of the 10 and 39% sucrose steps. A small amount of activity was associated with the thylakoid fraction which is found at the interface between the 50 and 39% steps but given the results of the rate gradient (Fig. 4), where the pelletable CA may be clearly separated from the Chl, this is probably a nonspecific association. The majority of the CA activity is associated with the soluble fraction of the 55% sample and in a pellet at the bottom of the tube as is the case for Rubisco activity. These results support the observations from the sucrose rate gradient (Fig. 4) that CA and Rubisco activity are associated with a similar pelletable fraction. In several experiments of this type, the distribution of both CA and Rubisco activity between the pelletable and soluble fractions was variable, as was observed for the sucrose rate gradients.

DISCUSSION

Intact cells of both high and low C_i grown *Synechococcus* PCC7942 clearly contain CA activity which appears to be internally located. In low C_i cells in the dark, this activity is preferentially accessible to external CO_2 and may be inhibited by both ethoxyzolamide and FCCP (Fig. 1). The effects of these two inhibitors may be either independent or they could perhaps act on a common step. The effects of EZ can be explained if it is assumed to inhibit internal CA activity as is the case for *in vitro* activity (Fig. 3). FCCP on the other hand will not have a direct effect on CA, other than perhaps by internal pH modification, but it will decrease the activity of the C_i pump in the dark, due to the reduction in ATP supply (19). We have recently shown (22, 23) that EZ also appears to inhibit the C_i transport process without necessarily inhibiting the internal CA activity in intact cells. If this is the case, then the common mode of inhibition may be through the inhibition of the C_i pump. This would indicate that the access of CO_2 to the internal CA is largely via the C_i pump and not by passive diffusion. This would be consistent with a large



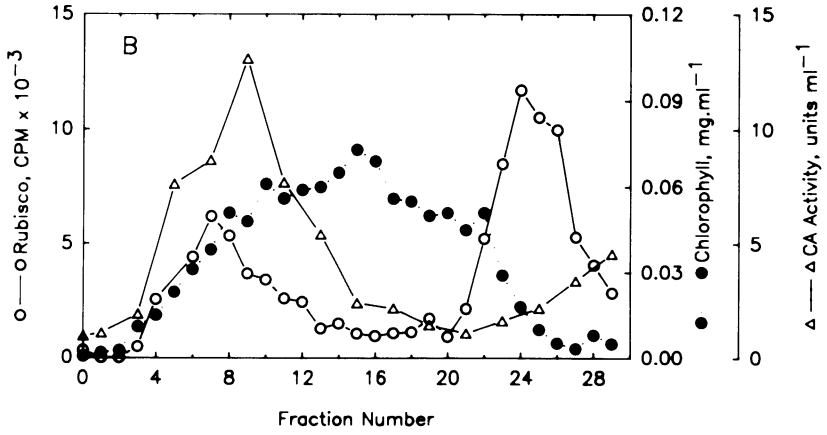


Figure 4. Two sucrose density rate gradients (15–60%, w/w), fractionating Rubisco and CA activities as well as Chl. See "Materials and Methods" for experimental details. A, Plus $MgSO_4$ (10 mM) in the breaking buffer; B, minus Mg^{2+} in breaking buffer.

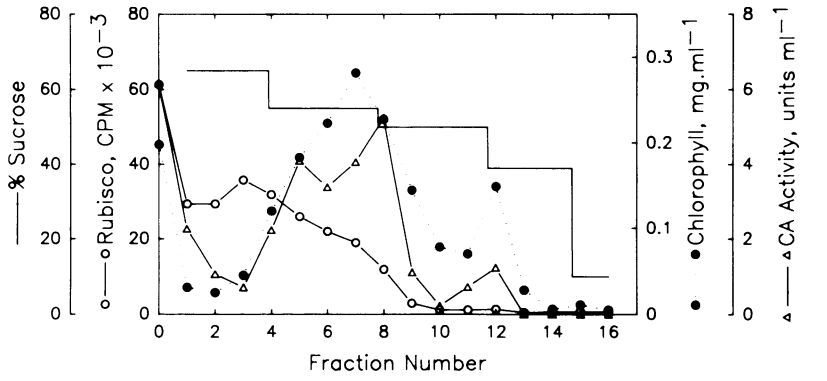


Figure 5. Sucrose flotation gradient showing the distribution of CA and Rubisco activities and Chl. See "Materials and Methods" for experimental details.

resistance to CO_2 diffusion calculated from CO_2 efflux studies with cyanobacteria (4) (our unpublished data). In addition, it would mean that, in the dark, the pump is transporting CO_2 preferentially, even though the levels of HCO_3^- are about two orders of magnitude higher.

In the light, operation of the C_i pump allows external C_i species to gain access to an internal CA where rapid exchange between C_i species occurs (Fig. 2). This is particularly evident for low C_i cells. The results obtained here are very similar to those seen with two marine *Synechococcus* species (3, 28) and establish without doubt that during the C_i transport process the inorganic carbon comes into contact with a region which promotes the exchange of C_i species with water. This could be most readily interpreted as being due to the internal CA activity which has been found in these cells (Table I) but there is an additional possibility. The C_i pump itself may catalyze a reaction which causes the exchange of the CO_2 oxygen with water. This could occur via the following scenario: It has been shown that the C_i pump in both low and high C_i cells preferentially takes up CO_2 from the external medium (5). However, for various reasons, it seems most likely that HCO_3^- is delivered to the interior of the cell (6). If this is the case, then the transport process must lead to the combination of OH^- from water with CO_2. This would introduce an oxygen from water into HCO_3^- and would thus speed up the exchange

rate. If the pump also operated in the reverse direction, then the pump would operate as a light stimulated CA activity. This reaction would have many similarities with the carbonic anhydrase reaction and it is significant that EZ appears to be an inhibitor of the pump (22, 23).

There is clearly internal CA activity measurable in homogenates of both high and low C_i cells (Table I). The levels of activity appear to be some 5- to 10-fold higher in low C_i cells and this is consistent with reports for other cyanobacteria (10, 11, 30). While the absolute levels of activity are different in the two cell types, there is evidence that the internal CA in both cell types is present in an amount sufficient to bring C_i species close to rapid equilibrium. Badger and Gallagher (5) have shown the response of photosynthesis to internal C_i is similar in both high and low C_i cells of *Synechococcus* PCC6301 and in both cases is similar to that of purified cyanobacterial Rubisco. Two mechanisms have been proposed for the manner in which this internal CA may be involved in elevating the CO_2 at the site of Rubisco. The first involves the localization of CA in the cytoplasm, where it participates in speeding up the conversion of HCO_3^- to CO_2 which is used by Rubisco (4). The second proposes that CA may be specifically located within the Rubisco containing carboxysome (24). This model proposes that HCO_3^- is preferentially permeable into the carboxysome and that the presence of CA in this environment would be essential to cause a local elevation of CO_2. The level of CA measured here is sufficient to meet the functional requirements of either model (4, 24).

The internal CA is inhibited by EZ (Fig. 3) and the I_{50} for inhibition is similar to that found for the marine *Synechococcus* sp. (UTEX 2380) (28). This is a very high value compared to that measured for green algae (2, 17) and the cyanobacterium *Anabaena variabilis* (30). Recent studies by us have shown that the $K_{0.5}$ (C_i) for photosynthesis in both high and low C_i cells is dramatically affected by levels of EZ in the 100 to 600 μM concentration range (23). This would be consistent with the inhibition characteristics of internal CA activity. However, in these same studies, it appears as though the intracellular relationship between CO_2 and HCO_3^- may not be disturbed during the inhibition. This was also seen when examining the effect of EZ on the ability of cells to promote the loss of ^{18}O label from C_i (see discussion with Fig. 2). This would not be consistent with the notion that CA is rate limiting to internal CO_2 generation, and it appears as though there is a direct effect of EZ on the C_i pump, which may impose a much more severe limitation on photosynthesis than does the inhibition of internal CA. Given that both the C_i pump and *in vitro* CA activity appear to have similar sensitivities to EZ, then two explanations for the preferential inhibition of C_i transport may be advanced. First, it may be due to a problem of penetration of EZ to the site of internal CA. However secondly, it may simply be the result of both activities being inhibited to a similar extent with the result that sufficient CA activity is always present to promote rapid equilibrium at the reduced levels of C_i flux that are produced when the pump is inhibited.

The studies on the pelletability and localization of CA activity (Table II and Fig. 4) tend to support the theory that CA activity may be associated with the Rubisco containing carboxysome fraction. However, this cannot be fully established from these results and the association with another particulate fraction cannot be ruled out. These results are different to those obtained by Lanaras *et al.* (13), with *Chlorogloeopsis*, where rate gradient separation showed that although the CA activity was particulate, it was associated with a lighter fraction than either the carboxysome or the thylakoid fraction. This casts some doubts on the possibility that CA and Rubisco may be universally associated with carboxysomes as part of the CO_2 concentrating mechanism.

There is evidence for some of the CA activity being of a soluble nature (Table II, Fig. 4). The proportion which appears to be soluble may be regulated by similar factors which affect carboxysome stability. Certainly Mg^{2+} addition increases the pelletability, but as discussed before, this may be via a nonspecific aggregation process. In direct contrast, the results of Fig. 4 show that the absence of Mg^{2+} may cause Rubisco to be released more readily from the pelletable fraction than CA activity. It has been suggested that Rubisco may exist in both carboxysomal and soluble forms, and that the distribution may depend on growth conditions (7). A similar situation may exist for CA activity. If the CA were to be located in carboxysomes, then its distribution between particulate and soluble forms may be similar to Rubisco. Whether the soluble and particulate activities are a result of the same protein remains to be answered. If there were different forms of CA involved then speculation about the roles of these forms could be made. Both particulate and soluble forms of CA have been reported for other cyanobacteria (10, 13, 30), using criteria for pelletability similar to those shown here. In studies of *A. variabilis*, Yagawa *et al.* (30) found that one particular strain appeared to contain only soluble CA activity and that addition of Mg^{2+} to extracts did not cause precipitation of this activity. Thus, there is precedent for more than one form of CA activity being found in cyanobacteria. There was clear evidence that no CA activity is associated with isolated cytoplasmic membrane fractions. If this is where the C_i pump is located then there is no accessible intrinsic CA activity that can be assayed readily.

Recently, it has been reported that two high CO_2 requiring mutants of *Synechococcus* R2 appear blocked in the utilization of internal inorganic carbon for photosynthesis (15, 20). A logical explanation for this phenotype may be absence of internal carbonic anhydrase. Unfortunately, Marcus *et al.* (15) were unable to detect CA in either the wild type or the mutant and could not test this hypothesis. It should be possible using the MS assay described here to establish whether these mutants are deficient in internal CA, and thus establish a primary role for CA activity in the operation of the CO_2 concentrating mechanism.

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