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Ethoxyzolamide Inhibition of CO_2-Dependent Photosynthesis in the Cyanobacterium Synechococcus PCC7942[1]

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ABSTRACT

Cells of the cyanobacterium, *Synechococcus* PCC7942, grown under high inorganic carbon (C_i) conditions (1% CO_2; pH 8) were found to be photosynthetically dependent on exogenous CO_2. This was judged by the fact that they had a similar photosynthetic affinity for CO_2 ($K_{0.5}[CO_2]$ of 3.4–5.4 micromolar) over the pH range 7 to 9 and that the low photosynthetic affinity for C_i measured in dense cell suspensions was improved by the addition of exogenous carbonic anhydrase (CA). The CA inhibitor, ethoxyzolamide (EZ), was shown to reduce photosynthetic affinity for CO_2 in high C_i cells. The addition of 200 micromolar EZ to high C_i cells increased $K_{0.5}(CO_2)$ from 4.6 micromolar to more than 155 micromolar at pH 8.0, whereas low C_i cells (grown at 30 microliters CO_2 per liter of air) were less sensitive to EZ. EZ inhibition in high and low C_i cells was largely relieved by increasing exogenous C_i up to 100 millimolar. Lipid soluble CA inhibitors such as EZ and chlorazolamide were shown to be the most effective inhibitors of CO_2 usage, whereas water soluble CA inhibitors such as methazolamide and acetazolamide had little or no effect. EZ was found to cause a small drop in photosystem II activity, but this level of inhibition was not sufficient to explain the large effect that EZ had on CO_2 usage. High C_i cells of *Anabaena variabilis* M3 and *Synechocystis* PCC6803 were also found to be sensitive to 200 micromolar EZ. We discuss the possibility that the inhibitory effect of EZ on CO_2 usage in high C_i cells of *Synechococcus* PCC7942 may be due to inhibition of a 'CA-like' function associated with the CO_2 utilizing C_i pump or due to inhibition of an internal CA activity, thus affecting CO_2 supply to ribulose bisphosphate carboxylase-oxygenase.

It is now well documented that cyanobacteria possess an inducible CO_2 concentrating mechanism (CCM[2]) which functions to elevate internal CO_2 levels around Rubisco (5, 6, 10, 13, 17). Both CO_2 and HCO_3^- can be transported and/or utilized by the CCM (6, 7, 24), but the ability to utilize HCO_3^- is most apparent when cells are grown at very low levels of C_i (7, 15). Although most research has centered upon characterizing the high affinity HCO_3^- uptake system, it has been known for some time that CO_2 is often a more efficient substrate for the CCM than HCO_3^- (6,7,24) Nevertheless, the most recent model for the CCM postulates a pivotal role for a HCO_3^- transporter on the plasma membrane (24). In this proposal the ability to utilize CO_2 is accommodated for by the addition of a 'CA-like' moiety that converts CO_2 to HCO_3^- in close proximity to the HCO_3^- pump. It was assumed that CO_2 acts as a better substrate due to a higher diffusion rate into the periplasmic space than the charged HCO_3^- ion. The best evidence for this model is that in low CO_2 grown cells of *Anabaena variabilis* M3, a low concentration (10 μM) of the CA inhibitor, EZ, inhibits CO_2 uptake more than HCO_3^- uptake (24). Unfortunately this type of evidence is not generally applicable to other cyanobacteria, such as *Synechococcus* sp., which are virtually unaffected by 10 to 100 μM EZ (9).

Recent studies have verified that low CO_2 grown cyanobacteria possess the ability to transport or utilize both CO_2 and HCO_3^- (1, 7). More important, it has been shown that cells of *Synechococcus* PCC6301 grown at high CO_2 retain the ability to take up CO_2 while losing the capacity for HCO_3^- uptake (7). This CO_2 uptake activity predominates in cells grown in media (pH 8.2) equilibrated with CO_2 levels above normal air levels (>350 μL/L), whereas maximum induction of HCO_3^- uptake capacity requires CO_2 levels of less than 50 μL/L in the gas phase (7). They suggest, in contrast to Volokita *et al.* (24), that a CO_2 utilizing C_i pump may be the primary component of the CCM and that HCO_3^- utilization would require 'front end'conversion of HCO_3^- to CO_2 in the vicinity of the C_i pump.

The studies described in this paper were aimed at extending the characterization of the ecologically important CO_2 utilization system in *Synechococcus* PC7942. We show that CO_2 utilization can be severely inhibited by high concentrations of the CA inhibitor, EZ, with high CO_2 grown cells being more sensitive to EZ than low CO_2 grown cells. We also show that EZ inhibits CO_2 utilization in other cyanobacterial strains, namely *Anabaena variabilis* M3 and *Synechocystis* PCC6803.

MATERIAL AND METHODS

Growth Conditions

Cells of *Synechococcus* PCC7942 (*Anacystis nidulans* R2), *Anabaena variabilis* M3, and *Synechocystis* PCC6803 were

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[2] Abbreviations: CCM, CO_2 concentrating mechanism; CA, carbonic anhydrase; C_i, dissolved inorganic carbon; DMQ, 2,5-dimethyl-*p*-benzoquinone; EZ, ethoxyzolamide; Rubisco, ribulose bisphosphate carboxylase-oxygenase; $K_{0.5}$, concentration required for half maximal response.

grown in BG11 media (21) 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 by a combination of Gro-Lux and white fluorescent tubes (60 $\mu E \cdot m^{-2} \cdot s^{-1}$; 30°C). Equilibrium between CO_2 in the gas phase and total C_i in the media was assured by vigorous aeration, using gas dispersion tubes, and by harvesting cells (in log phase) before a Chl density of 3 μg/mL was reached (7). 'High C_i' cells were grown with 1% CO_2 (v/v of air) in the gas phase and 'low C_i' cells with 30 to 50 ppm CO_2 in the gas phase. Culture pH remained constant at pH 8.0 when Chl densities were kept below 5 μg/mL.

Photosynthetic Measurements

The photosynthetic response of cyanobacteria to added inorganic carbon (as $NaHCO_3$) was measured using a Clarke-type O_2 electrode (Hansatech, Kings Lynn, Norfok, UK) maintained at 30°C and illuminated with a quartz halogen projector lamp at 250 $\mu E \cdot m^{-2} \cdot s^{-1}$. Cells were collected by centrifugation (5 min; 4000*g*; 25°C) and washed twice and resuspended in low C_i containing (<30 μM C_i) BG11 media buffered with 20 mM 1,3-bis[tris(hydroxymethyl) methylamino]propane-HCl at pH 7.0, 8.0, or 9.0. Most experiments were performed at pH 8.0 and with a Chl density in the O_2 electrode cuvette of 1.5 to 2.5 μg/mL. Low C_i cells were allowed to run out of C_i in the cuvette before additions of $NaHCO_3$ were made. This was not usually necessary with high C_i cells. In calculation of $K_{0.5}$ (C_i) values from the photosynthetic response to C_i, rates of O_2 evolution at zero C_i were assumed to be due to respiratory CO_2 efflux (23). In such cases, rates were extrapolated to zero and the small C_i value was added in determining $K_{0.5}$ (C_i).

Chl *a* was determined by the method of Wintermans and de Mots (25). EZ (obtained from Sigma; 1978 catalog) additions were made from 100 mM stock solutions dissolved in DMSO. When used at a final concentration of 0.2% (v/v), DMSO had no apparent effect on the photosynthetic response to C_i (results not shown). Concentrations above 0.2% had a small effect on photosynthesis and PSII electron transport (see text). PSII electron transport in whole cells was measured as O_2 evolution in the presence 1 mM DMQ as described by Ogawa *et al.* (18).

RESULTS

CO_2 Dependent Photosynthesis in High C_i Grown Cells.

Some of the photosynthetic characteristics of high C_i and low C_i cells of *Synechococcus* PCC7942, assayed at different pH are shown in Table I. Over the pH range 7 to 9, high C_i cells had virtually the same photosynthetic affinity for CO_2 (*i.e.* $K_{0.5}$ (CO_2) of 3.4–5.4 μM). This indicates that high C_i cells have a strong dependence on CO_2 species for photosynthesis.

Low C_i cells displayed a constant and high photosynthetic affinity for C_i ($K_{0.5}$ (C_i) of 10–11 μM) over the pH range 7 to 9 (Table I), indicating the ability to utilize both HCO_3^- and CO_2. This is consistent with the ability of low C_i cells to utilize HCO_3^- and CO_2 which is now well established (5–7, 10, 24).

Table I. *Photosynthetic Parameters of High C_i and Low C_i Grown Cells*

High C_i and low C_i grown cells of *Synechococcus* PCC7942 were incubated in buffered BG11 media of the desired pH and $K_{0.5}(C_i)$, $K_{0.5}(CO_2)$ and P_{max} were determined from the photosynthetic response to C_i.

Cells	$K_{0.5}(C_i)\mu M$	$K_{0.5}(CO_2)\mu M$	P_{max}
			$\mu mol\ O_2 \cdot mg\ Chl^{-1} \cdot h^{-1}$
High C_i grown cells			
pH 7.0	24	4.2	298.0
pH 8.0	262	5.4	310.6
pH 9.0	1700	3.4	272.7
Low C_i grown cells			
pH 7.0	9.5	1.65	297.3
pH 8.0	9.8	0.20	295.6
pH 9.0	11.1	0.02	280.9

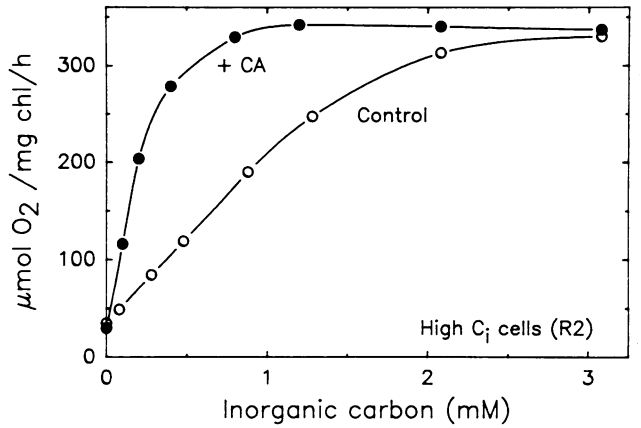

Figure 1. Effect of exogenous CA (0.17 mg/ml) on the photosynthetic response of high C_i cells of *Synechococcus* PCC7942 to C_i (pH 8.0). Cells were suspended at a Chl density of 18 μg/ml.

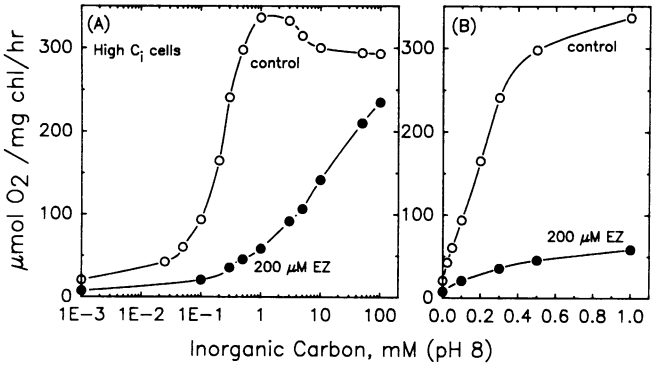

Figure 2. Effect of 200 μM EZ on the photosynthetic response of high C_i cells of *Synechococcus* PCC7942 to exogenous C_i (pH 8.0). Responses are shown on an extended scale (A, log plot) and over a 1 mM C_i range (B, linear plot).

Further evidence that high C_i cells are dependent on CO_2 species for photosynthesis comes from examining the photosynthetic affinity for C_i under conditions where the rate of CO_2 supply is limiting—such as when cell densities are high. In the absence of CA and at a Chl density of 18 μg/mL, high

C_i cells exhibited a lower than normal affinity for C_i, with a $K_{0.5}$ (C_i) of 960 μM (Fig. 1). Addition of CA increased the photosynthetic affinity for C_i more than 4-fold to a $K_{0.5}$ (C_i) of 212 μM (Fig. 1). P_{max} (maximum photosynthetic rate) was unchanged. Since the addition of CA at this pH (pH 8) would have caused a large increase in the rate of CO_2 supply but little change in HCO_3^- supply, it must be concluded that photosynthesis in high C_i cells is strongly CO_2 dependent.

Effect of EZ on the Photosynthetic Affinity for C_i

The photosynthetic response of high C_i grown cells to C_i (pH 8) was dramatically inhibited in the presence of 200 μM EZ (Fig. 2). The addition of EZ caused the rate at 1 mM C_i to drop by 83% (Fig. 2B), even though this C_i concentration was normally saturating for photosynthesis in high C_i cells (Fig. 2B). EZ also increased the $K_{0.5}$ (CO_2) by over 33-fold, from 4.6 to 155 μM (Fig. 2A; assuming P_{max} in the presence of EZ was attained at 100 mM C_i). Photosynthesis in EZ-inhibited cells could be restored to better than 85% of the control level by progressively elevating exogenous C_i up to 100 mM (Fig. 2A), presumably in response to free diffusion of CO_2 into the cell. Alleviation of EZ inhibition by high levels of C_i indicates that photosynthesis, *per se,* was largely unaffected by EZ. This is consistent with the response expected if a CO_2 accumulating mechanism were largely inoperative such that photosynthetic affinity reflected the affinity of Rubisco for CO_2. Cyanobacterial Rubisco has a K_m (CO_2) of 150 to 200 μM (3, 4).

EZ was less effective in inhibiting the photosynthetic response to C_i in low C_i grown cells (Fig. 3). The addition of 200 μM EZ caused a 44% reduction in the photosynthetic rate at 100 μM C_i (Fig. 3B), a level of C_i that was normally saturating for low C_i cells assayed at pH 8 (Fig. 3A). The addition of 400 μM EZ had a larger inhibitory effect than 200 μM EZ. With either concentration the level of inhibition at 100 μM C_i was similar to that at 1 mM C_i. Again, in the presence of EZ it was possible to completely restore photosynthesis by progessively increasing C_i up to 100 mM (Fig. 3A). Curiously, much of the response of low C_i cells to limiting C_i concentrations (0–100 μM C_i range) was insensitive to EZ (Fig. 3B).

Effect of EZ at Different pH

Figure 4 displays the photosynthetic response of high C_i cells to C_i at pH 7, 8, and 9 in the presence and absence of 200 μM EZ. As described above, photosynthesis in high C_i cells is CO_2-dependent (Table I) such that $K_{0.5}$ (C_i) rises approximately 10-fold for each 1 unit rise in pH over the pH range 7 to 9. It is apparent that 200 μM EZ had a greater inhibitory effect on high C_i cells at pH 7 than at pH 8 or 9, with inhibition decreasing with increasing pH (Fig. 4). This can be seen by comparing degrees of inhibition at the $K_{0.5}$ (C_i) value in each case. At pH 7, 8, and 9 the degree of inhibition due to 200 μM EZ was 87%, 71%, and 39%, respectively. This may indicate that the inhibitory effect of EZ is dependent on the uncharged (unprotonated) form of the inhibitor. EZ is a lipid soluble CA inhibitor with a pK_a of 8.1 (14). This pH dependence of EZ inhibition suggests that the inhibitor may need access to the cytoplasm or the interior of the plasma membrane. At pH 8 and 9, higher concentrations of EZ were capable of producing greater levels of inhibition (Fig. 4, B and C).

Concentration Dependence of EZ Inhibition

The effect of various concentrations of EZ on photosynthesis in 1% CO_2 grown and air grown cells (high C_i) and 30 μL/L grown cells (low C_i) is shown in Figure 5. Photosynthetic rate was measured in the presence of 1 mM C_i (pH 8), a C_i concentration that was saturating for all three cell types in the absence of EZ. Inhibition by EZ in high C_i cells (1% CO_2 or air grown) was close to maximal at 200 to 300 μM EZ (Fig. 5). As noted above (Fig. 3), low C_i cells were less sensitive to EZ than high C_i cells with the effect in low C_i cells being maximal at 400 to 500 μM EZ (Fig. 5). A component of the photosynthetic response of low C_i cells was unaffected by up to 600 μM EZ. The addition of less than 50 μM EZ had little effect on either high C_i or low C_i cells. In experiments similar to those in Figure 4, it was found that the concentration dependence of EZ inhibition at limiting C_i levels (25 μM C_i) was similar to that at saturating C_i levels (data not shown).

Levels of DMSO above 0.2% had a small effect on photosynthetic rate (Fig. 5, control). Although EZ dissolved in DMSO has the advantage of not introducing C_i into the O_2

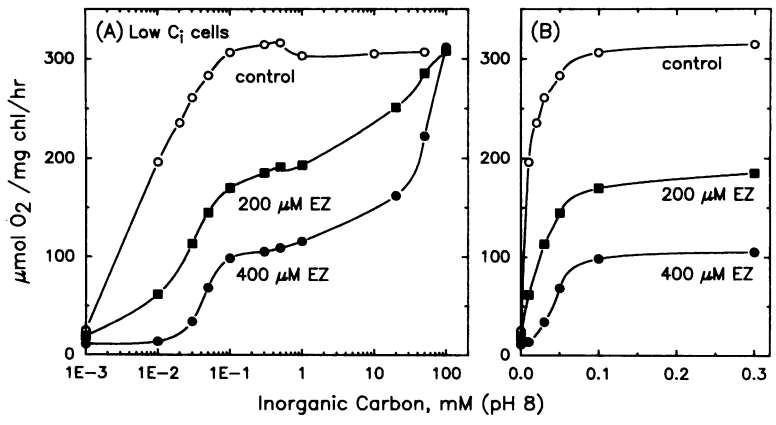

Figure 3. Effect of 200 and 400 μM EZ on the photosynthetic response of low C_i grown *Synechococcus* PCC7942 cells to exogenous C_i. Data are presented over an extended C_i range (A) and a 0 to 1 mM C_i range (B).

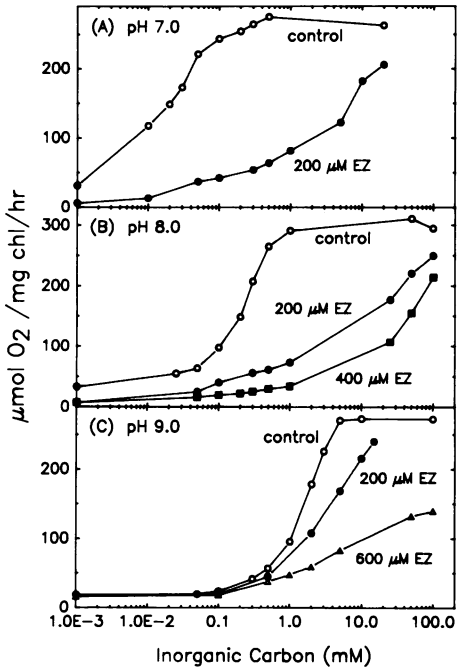

Figure 4. Effect of various concentrations of EZ on the photosynthetic responses of high C_i grown *Synechococcus* PCC7942 cells to C_i at pH 7.0 (A), pH 8.0 (B), and pH 9.0 (C). Data presented over an extended C_i range (log plot).

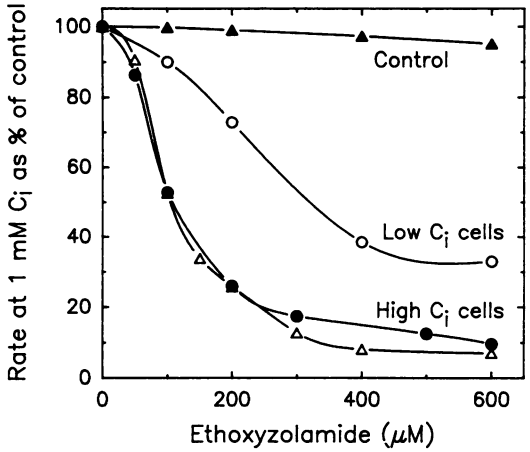

Figure 5. Concentration dependence for EZ inhibition of photosynthetic rate at 1 mM C_i (pH 8.0) in high and low C_i grown cells of *Synechococcus* PCC7942. Data represented as percentage of control rate. Controls (▲; 1% CO_2 grown cells) contained DMSO only at the rate of 0.1% DMSO per 100 μM change in EZ concentration. Each cell type had the following control rate at 1 mM C_i (μmol·mg $Chl^{-1}\cdot h^{-1}$):-1% CO_2 grown, 349.4 (△); air CO_2 grown, 352.2 (●) and 30 ppm CO_2 grown cells, 330.5 (○).

electrode, as do hydroxide dissolved stocks, suitable controls need to be included when using more than 0.2% DMSO.

Effect of Other Carbonic Anhydrase Inhibitors

In addition to EZ, three other CA inhibitors, namely acetazolamide (Diamox), methazolamide, and chlorazolamide (Cl

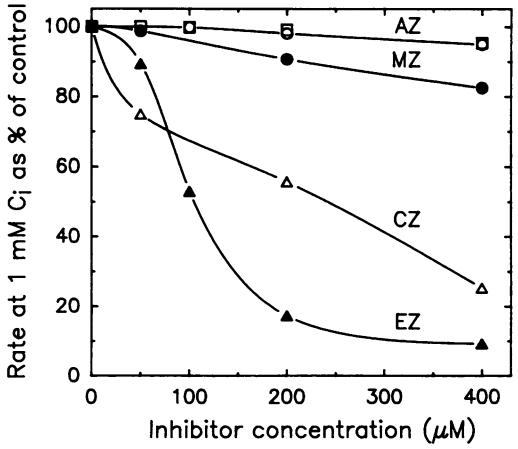

Figure 6. Concentration dependence of four CA inhibitors on photosynthetic rate in high C_i cells of *Synechococcus* PCC7942. Data represented as percentage of control rate at 1 mM C_i (pH 8.0). Control (□) rate was 345.7 ± 10.3 (SD; n = 5) μmol·mg $Chl^{-1}\cdot h^{-1}$. EZ (▲), chlorazolamide (CZ; △), methazolamide (MZ; ●) and acetazolamide (AZ; ○) were the four CA inhibitors used.

36,580), were evaluated for their ability to inhibit the photosynthetic response of high C_i cells (Fig. 6). Of the four inhibitors, EZ was the most effective, followed by chlorazolamide (Fig. 6). Methazolamide had a weak inhibitory effect, whereas acetazolamide had no apparent effect. The effectiveness of these inhibitors was roughly proportional to their lipid solubility (14). This is a further indication that the site(s) of inhibition may be within the plasma membrane or within the cytoplasmic compartment.

Effect of EZ on PSII Electron Transport

Although most CA inhibitors are quite specific, it has been shown in chloroplasts that some CA inhibitors, in addition to inhibiting CA activity, can cause a small decrease in the rate of PSII electron transport (22). Both PSI and PSII appear to be required for C_i uptake in cyanobacteria (11, 18). The possibility that EZ inhibits PSII activity was checked by measuring PSII activity in the presence of 1 mM DMQ (18). DMQ accepts electrons from plastiquinone allowing PSII activity to be measured as O_2 evolution. The addition of 200 μM EZ decreased PSII activity by 12.4%, and even at 600 μM EZ, PSII activity was reduced by only 18.2% (Table II). These levels of inhibition of PSII activity by EZ are relatively low and certainly do not explain the dramatic inhibition of photosynthesis seen in high C_i cells with 200 μM EZ, especially since much of this effect can be alleviated by the use of very high C_i levels (Fig. 2). EZ inhibition of PSII electron transport may, however, affect the maximum photosynthetic rate attained at 100 mM C_i (Fig. 2).

Reversibility of EZ Inhibition

Inhibition of photosynthesis by EZ in high C_i cells was readily reversed by either dilution or washing (Table III). Cells were preincubated with 400 μM EZ, an EZ concentration which typically inhibited photosynthesis by greater than 85%

Table II. *Effect of EZ on PSII Activity*

Table shows PSII activity in high C_i grown cells of *Synechococcus* PCC7942 as O_2 evolution in the presence of 1 mM DMQ. Data presented as a percentage decrease against the control rate of photosynthesis (absence of DMSO and EZ). Control rate was 470.1 ± 4.9 μmol O_2·mg Chl^{-1}·h^{-1} (SD; n = 7). CO_2-saturated photosynthesis (no DMQ) was 343.7 ± 8.0 μmol O_2·mg Chl^{-1}·h^{-1} (SD; n = 7). "EZ only" data (column 4) calculated as "EZ + DMSO" minus "DMSO only."

Treatment	Percentage Decrease against Control rate		
	DMSO only	EZ + DMSO	EZ only
100 μM EZ (+0.1% DMSO)	0.5	7.0	6.5
200 μM EZ (+0.2% DMSO)	1.9	14.3	12.5
300 μM EZ (+0.3% DMSO)	2.3	19.1	16.8
400 μM EZ (+0.4% DMSO)	3.3	21.4	18.1
600 μM EZ (+0.6% DMSO)	4.3	22.5	18.2

Table III. *Reversibility of EZ Inhibition*

O_2 evolution in high C_i cells of *Synechococcus* PCC7942 in the presence of 1 mM C_i (pH 8.0). Diluted cells and washed cells were resuspended to a Chl concentration of 2 μg/ml (±50 μM EZ), being initially 16 μg/ml (±400 μM EZ). Data in parentheses represented as percentage of control rate.

Treatment	Photosynthetic Rate	
	Control	Cells preincubated with 400 μM EZ
	μmol O_2·mg chl^{-1}·h^{-1}	
Cells diluted 8-fold	303.6 (100)	223.5 (73.6)
+50 μM EZ	227.2 (74.8)	
+200 μM EZ	48.2 (15.9)	46.0 (15.2)
Cells centrifuged and resuspended	301.6 (100)	304.1 (101)
+200 μM EZ	46.8 (15.5)	57.9 (19.2)

(Fig. 4B). When the cells were diluted 8-fold, the level of inhibition was equivalent to that seen in control cells with 50 μM EZ present (Table III). Also, cells preincubated with 400 μM EZ, then centrifuged and resuspended in fresh medium, showed no inhibition (Table III). These cells were inhibited normally when 200 μM EZ was added. Such results show that, in *Synechococcus,* EZ is a reversible inhibitor of possible CA-like function(s) in the cell.

Effect of EZ in Other Cyanobacterial Strains

Figure 7 depicts the photosynthetic response to C_i in high C_i grown cells of *Anabaena variabilis* M3 in the presence and absence of 200 μM EZ. The effect of EZ and its concentration dependence (Fig. 7, insert) was similar to that seen in high C_i cells of *Synechococcus* PCC7942 (Fig. 2) with inhibition being maximal around 1 mM C_i and relieved by high C_i (Fig. 7). EZ also inhibited high C_i cells of *Synechocystis* PCC6803 (Fig. 8), although the level of inhibition at limiting C_i and the concentration dependence was less pronounced than in *Synechococcus* or *Anabaena.* EZ inhibition of high C_i cells may be a common feature in many strains of cyanobacteria and may indicate that CO_2-dependent photosynthesis in cyanobacteria requires the operation of similar CA-like components.

DISCUSSION

There is now evidence that cyanobacteria possess a constitutive CO_2 transport capacity in addition to the better characterized, high affinity and inducible HCO_3^- transport/utilization system (1, 5–7, 24). Recent evidence has revealed that high C_i grown cells of *Synechococcus* PCC6301 retain the ability to transport and accumulate CO_2 but lose the ability for HCO_3^- uptake, whereas low C_i cells transport both CO_2 and HCO_3^-, although CO_2 is still transported with higher efficiency (7), It now seems likely that a C_i pump that uses CO_2 as substrate forms the key component of the CCM and that HCO_3^- usage requires front-end conversion of HCO_3^- to CO_2 (5, 7).

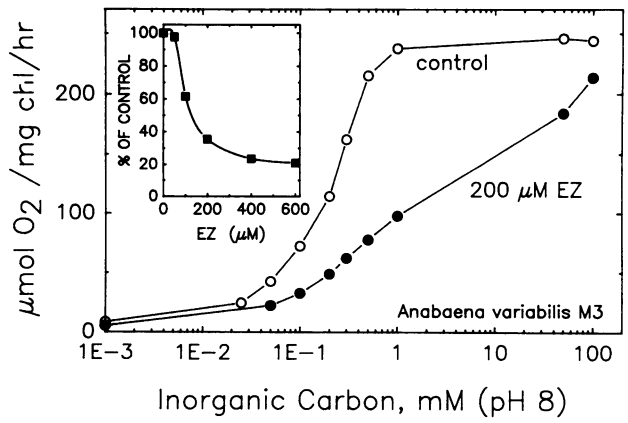

Figure 7. Effect of 200 μM EZ on the photosynthetic response of high C_i grown cells of *Anabaena variabilis* M3 to exogenous C_i (pH 8.0). Inset depicts the concentration dependence for EZ inhibition of the 1 mM photosynthetic rate as a percentage of the control rate. Control rate (inset) was 336.7 ± 1.5 (SD; n = 3) μmol·mg Chl^{-1}·h^{-1}.

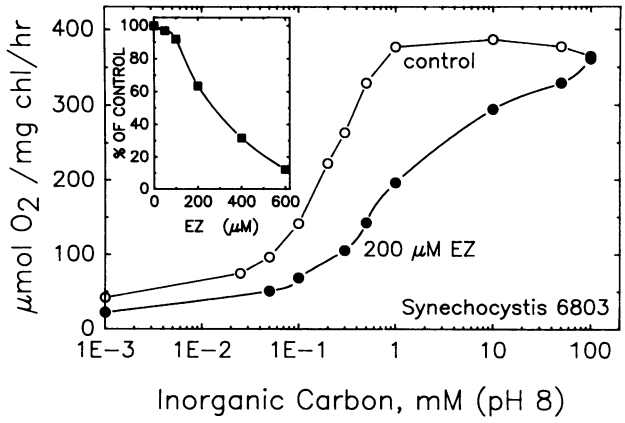

Figure 8. Effect of 200 μM EZ on the photosynthetic response of high C_i grown cells of *Synechococcus* PCC6803 to exogenous C_i (pH 8.0). Inset depicts the concentration dependence for EZ inhibition of the 1 mM photosynthetic rate as a percentage of the control rate. Control rate (inset) was 378.8 ± 2.7 (SD; n = 3) μmol·mg Chl^{-1}·h^{-1}.

This paper presents further evidence that high C_i cells utilize only CO_2 species. High C_i cells show the same photosynthetic affinity for CO_2 (*i.e.* $K_{0.5}$ (CO_2) of 3.4–5.3 μM) over the pH range 7 to 9 (Table I). This result indicates that these cells utilize CO_2 only. Miller and Canvin (16) have recently reported similar data for *Synechococcus* UTEX625 in that over the pH range 6 to 9 $K_{0.5}$ (CO_2) was similar, ranging from 7 to 20 μM. Low C_i cells of *Synechococcus* PCC7942 had the same $K_{0.5}$ (C_i) of 10 to 11 μM over the pH range 7 to 9 (Table I), indicating the ability for CO_2 and HCO_3^- utilization. Finally, in dense cell suspensions of high C_i cells the photosynthetic affinity for CO_2 was low, due to the CO_2 supply rate lagging behind demand, but the normal high affinity could be restored by the addition of exogenous CA (Fig. 1). Such an effect is consistent with high C_i cells being highly dependent on an external CO_2 supply for photosynthesis.

The addition of the CA inhibitor, EZ, to high C_i grown cells of *Synechococcus* PCC7942 had the effect of decreasing photosynthetic affinity for CO_2. This produced a result that might be expected were a CO_2 accumulating system largely inoperative, *i.e.* $K_{0.5}$ (CO_2) increased toward the K_m (CO_2) of Rubisco (Figs. 2 and 4). The addition of 200 μM EZ to high C_i cells increased $K_{0.5}$ (CO_2) from around 5 μM to greater than 155 μM (Fig. 2). P_{max} was largely restored by the addition of very high C_i levels, presumably in response to the diffusion of free CO_2 into the cells (Figs. 2 and 4). Although EZ caused a small decrease in PSII activity (Table II) the drop was too small to explain the strong inhibition of CO_2 usage caused by EZ. EZ inhibition was found to be reversible by wash or dilution (Table III). Lipophilic CA inhibitors such as EZ and chlorazolamide were found to most effectively inhibit CO_2 usage (Fig. 6). Furthermore, the pH dependence of EZ inhibition suggests that the uncharged form of EZ was most inhibitory (Fig. 4). The results above indicate that a CA-like step may be involved in photosynthetic utilization of CO_2 and that CA inhibitors may need to gain access to the cytoplasmic compartment or the interior of the cytoplasmic membrane. This is the first report that CO_2 utilization in *Synechococcus* PCC7942 can be inhibited by EZ. *Synechococcus* spp. had previously been thought to be unaffected by EZ, but earlier attempts had employed lower concentrations (10–100 μM) of the inhibitor (9). The effect of EZ on high C_i cells may be a common feature of cyanobacterial photosynthesis since, in addition to *Synechococcus* PCC7942, high C_i cells of *Anabaena variabilis* M3 and *Synechocystis* PCC6803 (Figs. 7 and 8) were sensitive to EZ.

In low C_i grown cells of *Synechococcus,* 200 μM EZ had a smaller inhibitory effect than on high C_i cells (*cf.* Figs. 2 and 3; Fig. 5) with $K_{0.5}$ (C_i) increased from 10 to 50 μM, although 400 μM EZ increased $K_{0.5}$ (C_i) to around 10 mM (Fig. 3). High concentrations of EZ were required to substantially inhibit low C_i cells (Fig. 5). It is not clear why low C_i cells should be less sensitive to EZ, but reduced access of EZ to the cell interior due to cell coat modification is a possibility. Alternatively, a $HCO_3^- \rightarrow CO_2$ front-end mechanism in low C_i cells may alter the effect of EZ on the CO_2 uptake mechanism.

The inhibitory effect of EZ on the photosynthetic affinity of high C_i cells might occur at one or both of two key CA-involving steps: *i.e.* inhibition of the CO_2 utilizing C_i pump on the plasma membrane or inhibition of internal CO_2/HCO_3^- equilibria such that the rate of supply of CO_2 to Rubisco is impeded. From evidence presented in this paper, and from other current evidence, it seems reasonable to conclude that high C_i grown *Synechococcus* spp. (6, 7, 16) and *Anabaena* spp. (1) possess a C_i pump that uses CO_2 as substrate. Current evidence, however, suggests that transported C_i arrives inside the cell as HCO_3^- (2, 24). This raises the possibility that the C_i pump has a CA-like activity ($CO_2 + OH^- \rightarrow HCO_3^-$) associated with it, allowing exogenous CO_2 to be accumulated as HCO_3^-. A demonstrated effect of the CA inhibitor, EZ, on the pump would be consistent with this view, especially since the sulfonamide group of a CA inhibitor appears to bind at the HCO_3^- site of the carbonic anhydrase enzyme (14).

The assertion that HCO_3^- is the species delivered inside the cell is quite critical to the proposal that the C_i pump has CA-like activity. It also has bearing on the prospect that EZ might, as an alternative, inhibit cytoplasmic CA activity resulting in a drop in CO_2 supply to Rubisco. Volokita *et al.* (24) have shown through active species experiments, in *Anabaena,* that when either CO_2 or HCO_3^- are presented in disequilibrium a constant linear relationship exists between internal C_i pool size and photosynthetic rate. This is interpreted to mean that a single species of C_i is delivered to the cell interior whether CO_2 or HCO_3^- is supplied externally. A more compelling argument suggesting that HCO_3^- arrives internally, comes from the analysis of mutants of *Synechococcus* that exhibit normal C_i uptake and accumulate high levels of C_i but are unable to photosynthesize normally unless ambient CO_2 is raised to the 1.0% or 5.0% level (12, 19) (GD Price, MR Badger, unpublished data). Such mutants would appear to lack internal CA activity and their inability to utilize the internal C_i pool suggests that HCO_3^- rather than CO_2 enters the cell. An essential role for internal CA in cyanobacterial photosynthesis has been identified and modeled, although the amounts required may be quite low (9). In a companion study (8) we report that low levels of CA activity can be detected in cells of *Synechococcus* PCC7942 using an ^{18}O exchange method. Internal CA activity has been detected in several other cyanobacteria (9, 23, 26).

Thus schemes whereby EZ either inhibits a CO_2 utilizing C_i pump wtih CA-like activity or inhibits an internal CA can be envisaged. An effect on either process would lead to inhibition of CO_2-dependent photosynthesis in high C_i cells of *Synechococcus* PCC7942 (or *Anabaena variabilis* M3 and *Synechocystis* PCC6803). The situation in low C_i cells may be more complicated than in high C_i cells. For instance, EZ might inhibit a front-end mechanism for converting HCO_3^- to CO_2 (5, 7) for the C_i pumps or it could inhibit a separate HCO_3^- pump. The most straightforward explanation, however, would be that EZ inhibits either the CO_2 utilizing C_i pump or an internal CA activity. In a companion study (20), however, we show that EZ does inhibit the C_i pump in high C_i cells (and low C_i cells) of *Synechococcus* PCC7942, and that EZ does not appear to inhibit internal CA in intact cells.

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