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Ethoxyzolamide Differentially Inhibits CO_2 Uptake and Na^+-Independent and Na^+-Dependent HCO_3^- Uptake in the Cyanobacterium *Synechococcus* sp. UTEX 625[1]

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The effects of ethoxyzolamide (EZ), a carbonic anhydrase inhibitor, on the active CO_2 and Na^+-independent and Na^+-dependent HCO_3^- transport systems of the unicellular cyanobacterium *Synechococcus* sp. UTEX 625 were examined. Measurements of transport and accumulation using radiochemical, fluorometric, and mass spectrometric assays indicated that active CO_2 transport and active Na^+-independent HCO_3^- transport were inhibited by EZ. However, Na^+-independent HCO_3^- transport was about 1 order of magnitude more sensitive to EZ inhibition than was CO_2 transport (50% inhibition = 12 μM versus 80 μM). The data suggest that both the active CO_2 (G.D. Price, M.R. Badger [1989] Plant Physiol 89: 37–43) and the Na^+-independent HCO_3^- transport systems possessed carbonic anhydrase-like activity as part of their mechanism of action. In contrast, Na^+-dependent HCO_3^- transport was only partially (50% inhibition = 230 μM) and noncompetitively inhibited by EZ. The collective evidence suggested that EZ inhibition of Na^+-dependent HCO_3^- transport was an indirect consequence of the action of EZ on the CO_2 transport system, rather than a direct effect on HCO_3^- transport. A model is presented in which the core of the inorganic carbon translocating system is formed by Na^+-dependent HCO_3^- transport and the CO_2 transport system. It is argued that the Na^+-independent HCO_3^- utilizing system was not directly involved in translocation, but converted HCO_3^- to CO_2 for use in CO_2 transport.

For use in photosynthesis, cyanobacteria concentrate C_i internally through the action of light-dependent transport systems that utilize CO_2 and/or HCO_3^-. In *Synechococcus* sp. UTEX 625 (PCC 6301) and *Synechococcus* sp. PCC 7942, three physiologically distinct modes of C_i transport have been identified that contribute differently to the formation of the intracellular C_i pool, depending on the level of external C_i present during growth (Kaplan et al., 1990; Miller et al., 1990; Badger and Price, 1992). For cells grown on high levels of C_i, active CO_2 transport is the major means of C_i acquisition, whereas under growth-limiting C_i conditions, CO_2 transport, along with Na^+-dependent and Na^+-independent HCO_3^- transport, contribute variably to the supply of intracellular C_i (Kaplan et al., 1990; Miller et al., 1990; Badger and Price, 1992; Espie and Kandasamy, 1992, 1994).

The C_i transport modes are distinguished from one another based on their substrate (CO_2/HCO_3^-) specificity, their requirement for Na^+ for C_i transport, their transport kinetics, and their response to various types of chemical inhibitors. Active CO_2 uptake by *Synechococcus* sp. UTEX 625 involves a saturable transport system with a $K_{0.5}(CO_2)$ of 0.4 μM and a maximum transport rate that ranges from 400 to 700 μmol C mg^{-1} Chl h^{-1} (Espie et al., 1991; Salon et al., 1996). Transport of $^{12}CO_2$ is inhibited by $^{13}CO_2$, but not by $H^{13}CO_3^-$, indicating a high specificity for CO_2 as the substrate (Espie et al., 1991). CA inhibitors such as EZ, H_2S, and COS (also a CO_2 analog) also inhibit active CO_2 transport, suggesting that the CO_2 transport system has CA-like properties (Volokita et al., 1984; Espie et al., 1989; Miller et al., 1989; Price and Badger, 1989a). This suggestion is supported further by the observation that although CO_2 is the substrate for transport, it is HCO_3^- that is delivered to the cytoplasm (Volokita et al., 1984; Marcus et al., 1986; Price and Badger, 1989b).

Active HCO_3^- transport by cyanobacteria also involves a saturable system with a $K_{0.5}(HCO_3^-)$ of about 10 to 40 μM and a maximum transport rate near 210 μmol C mg^{-1} Chl h^{-1} at pH 8.0 (Kaplan et al., 1990; Salon et al., 1996). Na^+-dependent HCO_3^- transport is distinguished from Na^+-independent HCO_3^- transport and CO_2 transport by its requirement for up to 25 mM extracellular Na^+ for maximum activity (Kaplan et al., 1990; Miller et al., 1990). Li^+, which competitively inhibits Na^+-dependent HCO_3^- transport, has little effect on the other modes of C_i uptake (Espie et al., 1988; Espie and Kandasamy, 1994). Na^+-dependent HCO_3^- transport appears to involve a Na^+/HCO_3^- symport that is energized by the Na^+ electrochemical potential, as evidenced by a strong inhibition of transport by monensin. In contrast, monensin has only a

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Abbreviations: BTP, 1,3-bis-(tris [hydroxymethyl] methylamino)-propane; CA, carbonic anhydrase; Chl, chlorophyll; C_i, dissolved inorganic carbon (CO_2 + HCO_3^- + CO_3^{2-}); EZ, ethoxyzolamide; F_M*, near-maximum fluorescence yield; F_O, chlorophyll fluorescence arising from dark-adapted cells illuminated with a pulse-modulated light beam; F_V*, variable fluorescence; $K_{0.5}$, substrate concentration required for one-half the maximun rate.

slight effect on Na^+-independent HCO_3^- transport and CO_2 transport (Espie and Kandasamy, 1994).

Several models describing the relationship among the various C_i transport modes have been proposed. Miller et al. (1989) and Espie et al. (1989) proposed that CO_2 and Na^+-dependent HCO_3^- transport occur through separate transport processes, based on the distinguishing characteristics outlined above. Strong evidence also has been presented for a single, common transport complex, in which a central CO_2 pump operates in conjunction with an inducible, HCO_3^--utilizing accessory component that supplies CO_2 to the central pump (Price and Badger, 1989a). Alternatively, the central pump may accept both CO_2 and HCO_3^- in the active site with variable substrate affinities (Volokita et al., 1984; Badger and Price, 1992). However, the most compelling evidence for a single transport complex is that EZ and COS directly inhibit both CO_2 and HCO_3^- transport (Ogawa and Togasaki 1988; Price and Badger, 1989a; Badger and Price, 1990, 1992). There are, however, conflicting results about the effects of these inhibitors on HCO_3^- transport. Miller et al. (1989) and Espie et al. (1989) found that neither COS nor H_2S greatly inhibited Na^+-dependent HCO_3^- transport in *Synechococcus* sp. UTEX 625 at a concentration that substantially inhibited CO_2 uptake. In contrast, Ogawa and Togasaki (1988), Price and Badger (1989a), and Badger and Price (1990) reported that COS and EZ substantially reduced HCO_3^- uptake, with EZ causing an equal inhibition of CO_2 and HCO_3^- uptake in *Synechococcus* sp. PCC 7942 (Yu et al., 1994b). Unfortunately, these studies did not determine whether COS and EZ inhibited Na^+-dependent HCO_3^- transport and/or Na^+-independent HCO_3^- transport.

In our previous work (Espie et al., 1989; Miller et al., 1989), we deliberately grew *Synechococcus* sp. UTEX 625 under conditions that excluded Na^+-independent HCO_3^- transport to avoid this additional complication. It is possible, therefore, that the effects of H_2S, COS, and EZ on total HCO_3^- transport may vary considerably, depending on the proportion of the two HCO_3^- utilizing systems present and their relative sensitivities to the inhibitors. This idea is supported by the finding that H_2S reduces Na^+-independent HCO_3^- transport in *Synechococcus* sp. UTEX 625 (Espie and Kandasamy, 1992) more effectively than it reduces Na^+-dependent HCO_3^- transport (Espie et al., 1989).

To clarify the effects of EZ on C_i transport, we examined the effects of this inhibitor on active CO_2 transport, Na^+-dependent HCO_3^- transport, and Na^+-independent HCO_3^- transport in the cyanobacterium *Synechococcus* sp. UTEX 625. Our results confirm the inhibitory effect of EZ on CO_2 transport previously reported by Price and Badger (1989a). Our results also show that HCO_3^- uptake by *Synechococcus* sp. UTEX 625 was affected differently by EZ, depending on the conditions for growth, the presence or absence of Na^+ during assays, and the mode of HCO_3^- utilization employed by the cells. Na^+-independent HCO_3^- transport is even more sensitive to EZ inhibition than is CO_2 transport, whereas Na^+-dependent HCO_3^- transport is much less affected.

MATERIALS AND METHODS

Organism and Growth

The unicellular cyanobacterium *Synechococcus* sp. UTEX 625 (University of Texas Culture Collection, Austin, TX), also known as *Synechococcus* sp. PCC 6301, was grown in unbuffered Allen's medium at 30°C as described previously (Espie and Canvin, 1987; Espie and Kandasamy, 1992). Cells grown by bubbling cultures with air (70 mL min^{-1}) containing 0.035% (v/v) CO_2 are referred to as air-grown cells and were capable of active CO_2 and Na^+-dependent HCO_3^- transport. Cells grown in standing culture, in which diffusion of atmospheric CO_2 into the culture was the sole means of CO_2 delivery, were capable of active CO_2 and Na^+-independent HCO_3^- transport (McKay et al., 1993). Cells were harvested at a [Chl] of 4 to 6 $\mu g\ mL^{-1}$ with the culture pH between 9.0 and 10.5.

Experimental Conditions

Cells were washed three times by centrifugation (1 min at 12,000*g*; Microfuge E, Beckman) and resuspended in 25 mM BTP/23.5 mM HCl buffer, pH 8.0, relatively free of C_i (15 μM) and Na^+ (5 μM). All experiments were conducted at pH 8.0 and 30°C. The [Chl] of cell suspensions was determined spectrophotometrically at 665 nm after extraction in methanol (MacKinney, 1941) and ranged from 7.5 to 9.5 $\mu g\ mL^{-1}$.

Aqueous stock solutions of CO_2 were prepared by continuously bubbling ice-cold, acidified (2 mM HCl), distilled H_2O with 5% (v/v) CO_2 in N_2. The concentration of CO_2 was measured by MS and found to be 3.6 to 3.9 mM (Espie et al., 1991). Stock solutions of C_i (HCO_3^-) were made from $KHCO_3$.

Fluorometry and Photosynthesis

Indirect measurements of CO_2 and HCO_3^- transport and intracellular C_i accumulation were made by monitoring the rate and extent of Chl *a* fluorescence quenching following the addition of CO_2 or HCO_3^- to an illuminated cell suspension at the CO_2 compensation point (Espie et al., 1991; Miller et al., 1991; McKay et al., 1993; Crotty et al., 1994). The Chl *a* fluorescence yield was measured with a pulse amplitude modulation fluorometer (PAM 101, H. Walz, Effeltrich, Germany) (Schreiber et al., 1986) as described previously (Crotty et al., 1994). Changes in fluorescence yield are expressed as a percentage of F_V^*, where $F_V^* = F_M^* - F_O$. F_M^* and F_O were determined as in McKay et al. (1993). Alternatively, the rates and the extents of fluorescence quenching were expressed as a percentage of the control. The measurements of fluorescence yield and photosynthetic O_2 evolution were made simultaneously (Crotty et al., 1994) with washed cells (1.5 mL) suspended in the reaction chamber of a Clark-type O_2 electrode (Hansatech, Norfolk, UK). Experiments were initiated by the addition of 10 μM CO_2 or various concentrations of HCO_3^- to illuminated cells. At the CO_2 compensation point, the fluorescence yield of cell suspensions was near F_M^* (Miller et al., 1991).

Inorganic Carbon Uptake and Fixation

Direct measurements of $^{14}CO_2$ and $H^{14}CO_3^-$ transport, C_i accumulation, and fixation were performed using the silicone fluid filtering centrifugation technique (Espie et al., 1988; Miller et al., 1988). Reactions were initiated by the addition of either $^{14}CO_2$ or $H^{14}CO_3^-$. These species were prepared by the addition of $NaH^{14}CO_3$ to either 10 mM phthalic acid (pH 4.0) or C_i-free 25 mM BTP buffer (pH 8.0), respectively. Intracellular [C_i] was calculated using an internal volume of 48.5 or 62 μL mg^{-1} Chl for air-grown and standing culture cells, respectively (Espie and Kandasamy, 1992).

MS and CO_2 Transport

CO_2 uptake by cell suspensions was measured using a magnetic sector mass spectrometer (MM 14–80 SC, VG Gas Analysis, Middlewich, UK) connected to a membrane inlet system as described by Espie et al. (1988). Transport was initiated by providing illuminated cells with a pulse of pure CO_2.

Chemicals

BTP, EZ, and the sodium salt of monensin were obtained from Sigma. Monensin was dissolved in ethanol, and stock solutions of EZ were prepared in DMSO just prior to experiments. The [DMSO] in cell suspensions did not exceed 0.2% (v/v) and had no effect on photosynthesis, C_i transport, or Chl *a* fluorescence.

RESULTS

The Effect of EZ on CO_2 Transport

Increasing concentrations of EZ progressively inhibited the ability of *Synechococcus* sp. UTEX 625 air-grown cells to transport CO_2, as indicated by a progressive decrease in the initial rate of Chl *a* fluorescence quenching following the addition of CO_2 (Fig. 1A). The inhibitory effect of EZ on CO_2 transport was confirmed by both silicone fluid centrifugation assays and by MS (Fig. 1, B and C). All assays in the absence of 25 mM Na^+ indicated that CO_2 transport was reduced 90 to 100% by 400 μM EZ. In control experiments (without EZ), CO_2-induced Chl *a* fluorescence quenching paralleled in time the initial rate of C_i accumulation mediated by the CO_2 transport system (Fig. 1, A and B). The maximum extent of fluorescence quenching also closely corresponded in time with the maximum level of C_i accumulation. The relationships between CO_2 transport and fluorescence quenching persisted in the presence of EZ (Fig. 1, A and B), indicating that fluorescence measurements provided a good means for monitoring CO_2 transport and C_i accumulation (Espie et al., 1991; Miller et al., 1991; Crotty et al., 1994). Previous work by Price and Badger (1989a) using MS showed that EZ also inhibits CO_2 transport in *Synechococcus* sp. PCC 7942. Our results with *Synechococcus* sp. UTEX 625, which were obtained under conditions in which HCO_3^- transport was inhibited by the lack of Na^+, are in complete agreement with theirs.

Effect of EZ on C_i Transport in Air-Grown Cells

In air-grown cells in which Na^+-dependent HCO_3^- uptake had been prevented by removing extracellular Na^+, CO_2 transport was progressively inhibited by increasing [EZ] as monitored by the fluorescence quenching assay (Figs. 1A and 2, A and B). Approximately 50% of CO_2 transport activity was inhibited at 75 μM EZ and 90% was inhibited at 150 μM (Fig. 2A). In the presence of 25 mM Na^+, a very similar pattern of inhibition of CO_2 transport by EZ was observed. Only about 10% of the residual activity remained at and beyond 200 μM EZ, indicating that Na^+ did not relieve EZ inhibition of CO_2 transport. The 10 μM CO_2 provided to the cells in these experiments was 25 times greater than the $K_{0.5}(CO_2)$ of the CO_2 transport system (0.4 μM; Espie et al., 1991) and, therefore, increasing

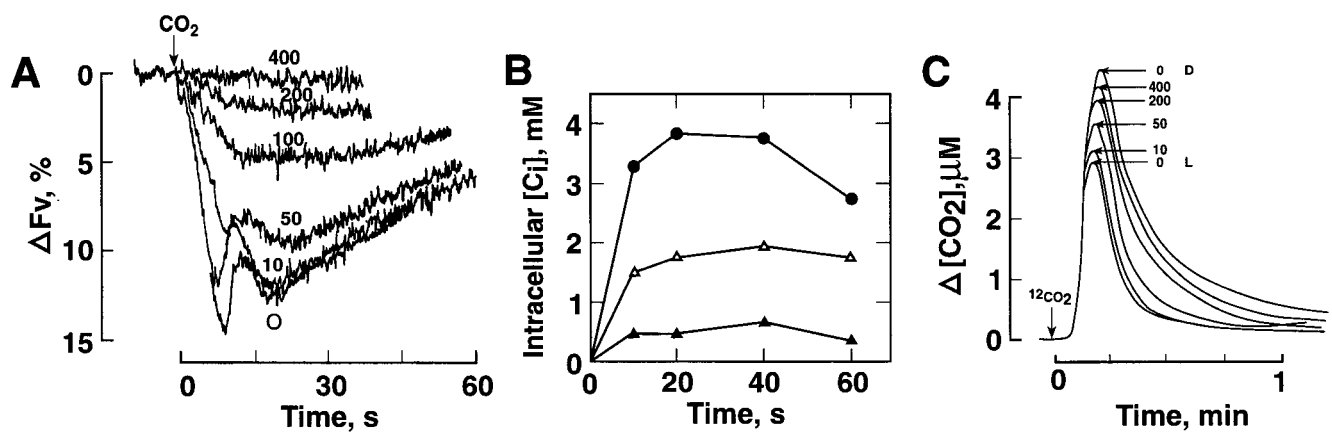

Figure 1. The effect of EZ on CO_2 transport in air-grown cells of *Synechococcus* sp. UTEX 625. CO_2 transport was initiated by the addition of 10 μM CO_2 and was measured indirectly using the fluorescence quenching method (A) or directly using the silicone fluid filtering centrifugation technique (B) or MS (C). In C, the difference in peak heights between dark (D) and light (L) controls (0 EZ) was taken as a measure of CO_2 transport. Experiments were conducted in 25 mM BTP buffer, pH 8.0, containing 100 μM NaCl to preclude Na^+-dependent HCO_3^- transport. EZ was varied between 0 and 400 μM. In B, ●, control; △, 75 μM EZ; ▲, 400 μM EZ.

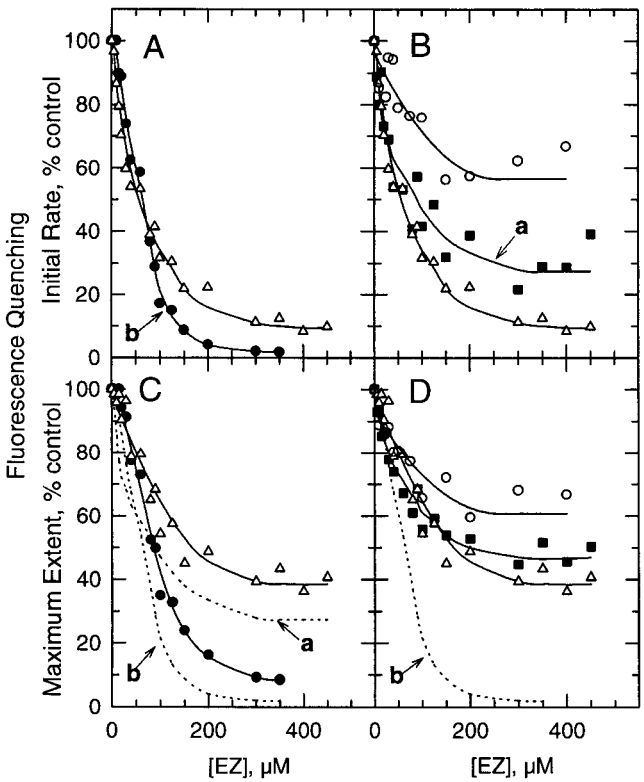

Figure 2. The effect of [EZ] on CO_2 and Na^+-dependent HCO_3^- transport and accumulation in air-grown cells of *Synechococcus* sp. UTEX 625. Transport and accumulation were measured indirectly using the initial rate of Chl *a* fluorescence quenching (A and B) to estimate transport and using the extent of Chl *a* fluorescence quenching (C and D) to estimate intracellular C_i accumulation. The initial rate of fluorescence quenching was measured during the first 10 s of the experiment after providing the cells with 10 μM CO_2, or 10 or 20 μM HCO_3^- in the presence of Na^+ (as NaCl). The maximum extent of fluorescence quenching occurred 0.5 to 1 min after the addition of CO_2 or HCO_3^-. For comparison, the regression lines denoted a and b from panels A and B are also shown in C and D. The data are the average of five separate experiments. In the absence of Na^+, HCO_3^- additions caused little or no quenching, and the data are not shown. A and C (10 μM CO_2), △, 25 mM Na^+; ●, 100 μM Na^+. B and D (25 mM Na^+), ■, 10 μM HCO_3^-; △, 10 μM CO_2; ○, 20 μM HCO_3^-.

the CO_2 concentration further would likely have had little effect on the pattern or magnitude of the EZ inhibition of CO_2 transport.

In air-grown cells, little HCO_3^- transport occurred in the absence of extracellular Na^+ (e.g. Kaplan et al., 1990; Miller et al., 1990). In the presence of 25 mM Na^+, Na^+-dependent HCO_3^- uptake also was apparently inhibited by [EZ] (Fig. 2B). But at 10 or 20 μM HCO_3^-, approximately 30 to 60% of the initial rate of Na^+-dependent HCO_3^- uptake was found to be insensitive to 400 μM EZ (Fig. 2B), a concentration that almost completely inhibited CO_2 transport (Fig. 2, A and B). When 10 μM HCO_3^- was provided to the cells, this concentration would support no more than 50% of the maximum rate of HCO_3^- transport (Fig. 3A). Thus, the effects of EZ on CO_2 and Na^+-dependent HCO_3^- transport were quantitatively and qualitatively different.

The extent of fluorescence quenching provides a reliable indicator of the size of the internal C_i pool achieved during steady-state photosynthesis (Espie et al., 1991; Miller et al., 1991; Crotty et al., 1994). The extent of fluorescence quenching following the addition of CO_2 or HCO_3^- was therefore determined as a function of [EZ] (Fig. 2, C and D). This measurement was made after equilibration between CO_2 and HCO_3^- had occurred (approximately 1 min after C_i addition). Consequently, contributions from both CO_2 and HCO_3^- transport to the internal C_i pool were expected, regardless of the C_i species initially added. In air-grown cells, in which only CO_2 transport was allowed (without Na^+), the ability of cells to accumulate an internal C_i pool when CO_2 was provided was markedly reduced by increasing [EZ] as measured by the radiochemical or fluorescence quenching assay (Figs. 1B and 2C). The EZ-induced decline in C_i accumulation closely paralleled the decline in the initial rate of CO_2 transport (Fig. 2C, curve b). However, in the presence of 25 mM Na^+, the effect of EZ on C_i accumulation was substantially reduced (Fig. 2C). Although C_i accumulation declined in the presence of EZ when CO_2 was provided to the cells, the decline paralleled the decrease in the initial rate of Na^+-dependent HCO_3^- transport (Fig. 2C, curve a), rather than CO_2 transport (Fig. 2C, curve b). C_i accumulation to about 40% of the control level occurred at a time (1–2 min after CO_2 addition) when very little CO_2 remained in the solution and under conditions (400 μM EZ) in which CO_2 transport was virtually inactive (Fig. 2C).

When 10 or 20 μM HCO_3^- was provided to the cells in the presence of 25 mM Na^+, the EZ-induced decline in C_i accumulation (Fig. 2D) paralleled the decline in the initial rate of HCO_3^- transport (Fig. 2B). As was the case with Na^+-dependent HCO_3^- transport (Fig. 2B), 45 to 65% of C_i accumulation (Fig. 2D) was also insensitive to 400 μM EZ. In the presence of EZ and 25 mM Na^+, the addition of both 10 μM CO_2 and 10 μM HCO_3^- resulted, after equilibration of the C_i species, in equivalent levels of C_i accumulation (Fig. 2D). However, CO_2 transport was more sensitive to EZ inhibition than was HCO_3^- transport (Fig. 2, A and B). Therefore, the EZ-insensitive component of C_i accumulation must be a consequence of the Na^+-dependent HCO_3^- transport system activity.

Kinetic Characterization of EZ Inhibition of Na^+-Dependent HCO_3^- Transport

Figure 3A shows the effect of increasing HCO_3^- concentration on the initial rate of Na^+-dependent HCO_3^- transport at four EZ concentrations, as determined by the Chl *a* fluorescence quenching assay. Double-reciprocal plots of rate versus substrate concentration (Fig. 3B) indicated a $K_{0.5}(HCO_3^-)$ of about 10 μM, and that EZ acted as a noncompetitive inhibitor with a K_i of 230 μM.

Figure 3C shows the effect of EZ on the level of C_i accumulation as a function of HCO_3^- concentration. In this experiment, C_i accumulation was mediated primarily by the Na^+-dependent HCO_3^- transport system. Intracellular accumulation was measured indirectly using the Chl *a* fluorescence assay, and it is expressed as a percentage of

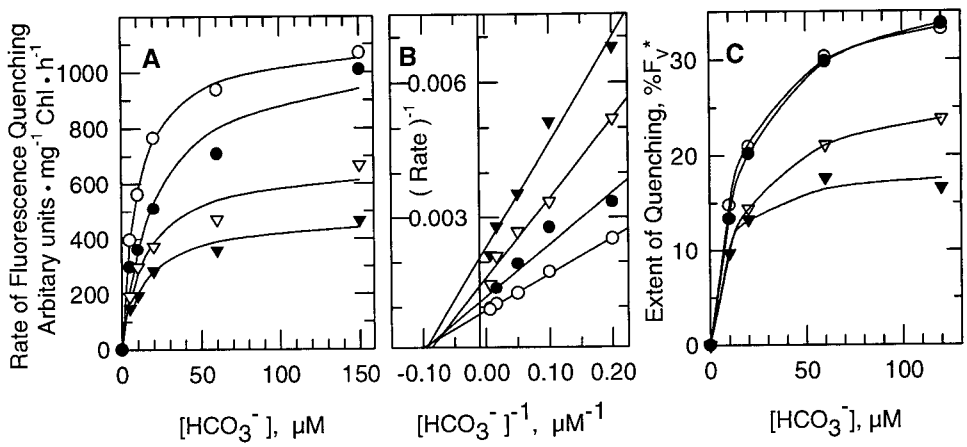

Figure 3. The effect of EZ on the kinetics of Na^+-dependent HCO_3^- transport in air-grown cells of *Synechococcus* sp. UTEX 625. Transport and accumulation were measured indirectly using the initial rate of Chl *a* fluorescence quenching (A and B) to estimate transport and the extent of Chl *a* fluorescence quenching (C) to estimate intracellular C_i accumulation. The initial rate of fluorescence quenching was measured in arbitrary units during the first 10 s of the experiment after providing cells with HCO_3^-. The regression lines in A were fitted to the data using the Michaelis-Menten equation. Double-reciprocal plots of the data in A are shown in B. Cells (7.5–9.0 μg Chl mL^{-1}) were suspended in 25 mM BTP/HCl buffer, pH 8.0, containing 25 mM NaCl and EZ as indicated. ○, ●, ▽, and ▼ are 0, 75, 200, and 400 μM EZ, respectively.

the variable fluorescence quenched. In the absence of EZ, intracellular C_i accumulation increased with increasing HCO_3^-, saturating after about 100 μM external HCO_3^-. In contrast to C_i accumulation mediated by CO_2 transport (Fig. 1B), EZ at 75 μM only slightly reduced the steady-state level of C_i accumulation mediated by the Na^+-dependent HCO_3^- transport system. At 200 and 400 μM, the effect of EZ was more evident and reduced C_i accumulation to about 30 and 50% of the control level, respectively, regardless of the HCO_3^- concentration.

EZ Inhibition of Na^+-Independent HCO_3^- Transport

Synechococcus sp. UTEX 625 cells grown in standing culture differ from air-grown cells in that they are capable of HCO_3^- transport that results in substantial HCO_3^--induced fluorescence quenching in the absence of millimolar levels of Na^+ (Espie and Kandasamy, 1992, 1994; Crotty et al., 1994). Consequently, the effect of EZ on CO_2 and Na^+-independent HCO_3^- transport was studied in these cells in the presence of 100 μM Na^+ or 25 mM Na^+. EZ inhibited CO_2 transport (Fig. 4, A and B) and C_i accumulation (Fig. 4C) in standing culture cells in a manner similar to that observed for air-grown cells. The Na^+-independent HCO_3^- transport system, however, was found to be much more sensitive to EZ inhibition than the CO_2 transport system when assayed at a 10 μM substrate concentration and 100 μM NaCl (Fig. 4, A and C). The initial rate of Na^+-independent HCO_3^- transport and steady-state C_i accumulation were reduced to 5 to 10% of the control at 75 μM EZ (Fig. 4, A and C), with 50% inhibition at about 12 μM (Fig. 4A). The inhibitory effect of EZ on both CO_2 and Na^+-independent HCO_3^- transport was mirrored in the steady-state level of C_i accumulation (Fig. 4, A and C).

In the presence of 25 mM Na^+, some recovery in the cell's ability to transport HCO_3^- and to accumulate C_i was observed (Fig. 4, B and D). The initial rate of HCO_3^--induced fluorescence quenching was more sensitive to EZ inhibition in the absence of Na^+ than in the presence of 25 mM NaCl (Fig. 4, A and B). This suggests that standing culture cells possess some Na^+-dependent HCO_3^- transport activity. When the effect of EZ on HCO_3^- transport and C_i accumulation was investigated at 20 μM HCO_3^- and 100 μM Na^+, the pattern of inhibition was similar to that found at 10 μM HCO_3^- (Fig. 4, A and C). However, relief of EZ inhibition by Na^+ in standing culture cells was much more pronounced at the higher HCO_3^- concentrations (Fig. 4, B and D). This effect may have been due to an increase in the cell's ability to accumulate C_i via the Na^+-dependent HCO_3^- transport system ($K_{0.5}[HCO_3^-]$ = 10 μM; Fig. 3) with increasing external $[HCO_3^-]$. When external C_i concentrations reach saturating levels for Na^+-dependent HCO_3^- transport, maximum relief from a given [EZ] was attained, as previously observed in air-grown cells (Fig. 3, A and C).

$H^{14}CO_3^-$ Transport Studies

Direct measurements of intracellular $[C_i]$ and carbon fixation were made using the silicone fluid centrifugation technique. With standing culture cells, 75 μM EZ reduced the initial rate of Na^+-independent HCO_3^- transport by 70% and C_i accumulation by 50%, whereas 400 μM EZ inhibited transport by 90% (Fig. 5A), in agreement with the fluorescence quenching assays (Fig. 4). As expected, carbon fixation also declined in parallel with the decline in the intracellular C_i concentration (Fig. 5B).

Air-grown cells were assayed for HCO_3^- transport in the presence of 25 mM NaCl to ensure a fully active Na^+-dependent HCO_3^- transport system. Seventy-five micromolar EZ had a small effect on HCO_3^- transport and accumulation, whereas 400 μM EZ resulted in a 45 to 55% inhibition of intracellular C_i accumulation (Fig. 5C) and carbon fixation (Fig. 5D); these results also agree with the

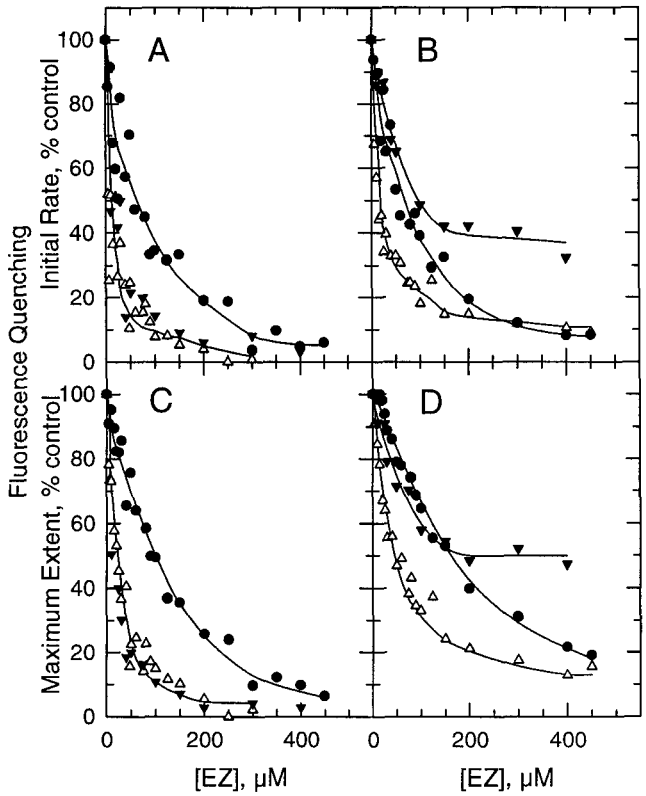

Figure 4. The effect of [EZ] on CO_2 and Na^+-independent HCO_3^- transport and accumulation in *Synechococcus* sp. UTEX 625 grown in standing culture. After providing the cells with 10 μM CO_2, or 10 or 20 μM HCO_3^-, transport and accumulation were measured indirectly, in the presence of the indicated amount of Na^+, using the initial rate of Chl *a* fluorescence quenching (A and B) to estimate transport and using the extent of Chl *a* fluorescence quenching (C and D) to estimate intracellular C_i accumulation as in Figure 2. A and C (100 μM Na^+), △, 10 μM HCO_3^-; ▼, 20 μM HCO_3^-; ●, 10 μM CO_2. B and D (25 mM Na^+), △, 10 μM HCO_3^-; ▼, 20 μM HCO_3^-; ●, 10 μM CO_2.

results from fluorescence quenching assays (Fig. 2). In general, when assayed at the same substrate concentrations, EZ differentially inhibited the C_i transport systems, with the Na^+-independent HCO_3^- transport being the most sensitive (Fig. 5A), followed by CO_2 transport (Figs. 1B and 2, A and B) and Na^+-dependent HCO_3^- transport (Fig. 5C).

Effect of EZ, Na^+, and Monensin on HCO_3^- Transport by Standing Culture Cells

To determine if standing culture cells possessed Na^+-dependent HCO_3^- transport capability in addition to Na^+-independent HCO_3^- transport (Espie and Kandasamy, 1992), experiments were conducted in the absence and presence of monensin, an inhibitor of Na^+-dependent HCO_3^- transport (Espie and Kandasamy, 1994). Advantage was taken of the fact that EZ preferentially inhibits Na^+-independent HCO_3^- transport, so Na^+-dependent activity can be observed. In the absence of Na^+, 40 μM EZ inhibited HCO_3^--induced fluorescence quenching and photosynthesis by about 64% compared with the control addition of 20 μM HCO_3^- (Fig. 6, A, B, A′, and B′). The initial rate of fluorescence quenching was usually reduced by about 25% (Fig. 6B) to 40% (not shown). In the presence of 25 mM Na^+, however, both fluorescence quenching and photosynthesis recovered to near-control levels (Fig. 6, C and C′). The recovery of these activities could not be brought about by 25 mM KCl, indicating that this effect was specific to Na^+ alone (data not shown). Monensin was a much more effective inhibitor of HCO_3^--induced fluorescence quenching and photosynthesis in the presence of EZ and Na^+ (Fig. 6, D and D′) than in the presence of Na^+ alone (see Espie and Kandasamy, 1994). This result indicates that standing culture cells have a capacity for monensin-sensitive, Na^+-dependent HCO_3^- transport, but that this capacity is normally masked by Na^+-independent HCO_3^- transport.

In other experiments (not shown), DMSO, the solvent in which EZ was dissolved, was used to see if it could sensitize standing culture cells to monensin inhibition either in the absence or presence of 25 mM NaCl. No effect of the solvent was observed, indicating that EZ caused the inhibition of Na^+-independent HCO_3^- uptake. This result also indicated that monensin did not require the presence of DMSO to facilitate its inhibitory activity. Consequently, the lack of a monensin effect on standing culture cells reported in other experiments (Espie and Kandasamy, 1994) cannot simply be attributed to an inability of monensin to gain access to the site of action. Rather, the results shown in Figure 6 suggested that monensin was able to penetrate to its site of action, but had little effect on Na^+-independent HCO_3^- transport in standing culture cells.

After 15 min of exposure to monensin, recovery of fluorescence quenching and photosynthetic activity to a level similar to that found in the presence of EZ alone was observed (Fig. 6, B, B′, E, and E′). However, full recovery of activity was not achieved even after 60 min of exposure (not shown). The addition of 1 mM C_i did result in substantial quenching of fluorescence and a high rate of photosynthesis (Fig. 6, E and E′). This was an indication that the cell's capacity for photosynthesis was relatively unimpaired under these conditions.

Previous work has shown that monensin, in the absence of Na^+, causes a partial but transient reduction in Na^+-independent HCO_3^- transport and photosynthesis (Espie and Kandasamy, 1994). Full activity is recovered within 15 min of exposure to monensin (Espie and Kandasamy, 1994), and under these conditions the pattern of HCO_3^--induced fluorescence quenching in the presence of 40 μM EZ was similar (not shown) to that shown in Figure 6B.

DISCUSSION

Radiochemical, fluorometric, and MS assays (Fig. 1) indicated that active CO_2 transport in *Synechococcus* sp. UTEX 625 was inhibited by EZ, a known inhibitor of CA. The initial rate of CO_2 transport was reduced to 5 to 10% of the control rate by 400 μM EZ, and this effect was not reversed by 25 mM Na^+. These results confirm and extend a previous study by Price and Badger (1989a), who found that

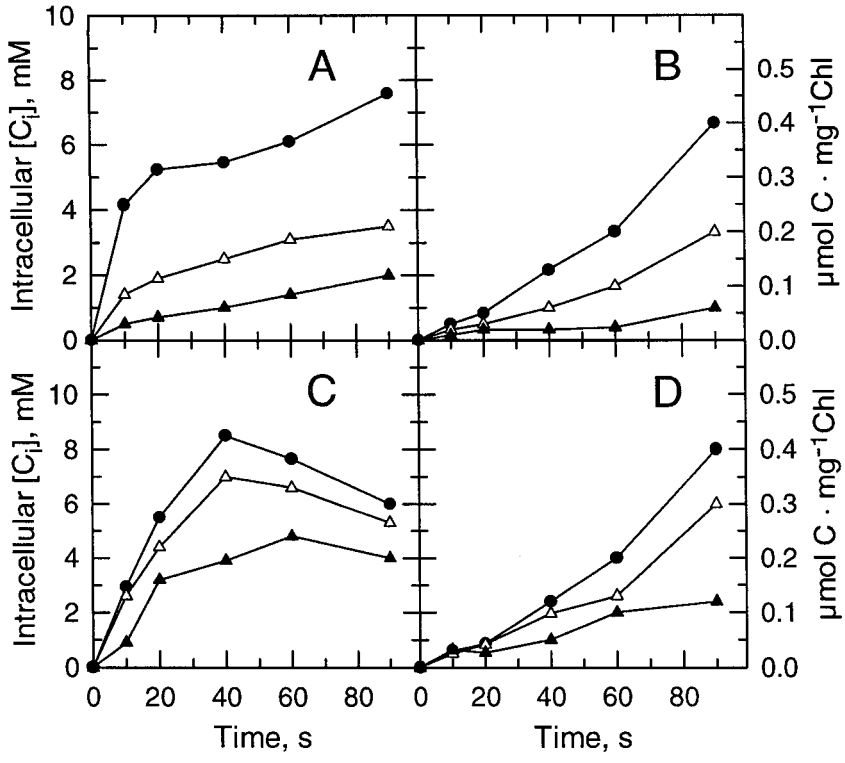

Figure 5. The effect of EZ on intracellular C_i accumulation (A and C) and photosynthetic carbon fixation (B and D) as measured by the silicone fluid centrifugation technique. The assays were initiated in the presence of EZ (as indicated) by adding 10 μM $H^{14}CO_3^-$ to either standing culture cells (100 μM NaCl) capable of Na^+-independent HCO_3^- transport (A and B) or to air-grown cells (25 mM NaCl) capable of Na^+-dependent HCO_3^- transport (C and D). The data are the average of four replicates from a representative experiment. ●, △, and ▲ are 0, 75, and 400 μM EZ, respectively.

high concentrations of EZ inhibited active CO_2 transport in *Synechococcus* sp. PCC 7942 without an apparent inhibition of PSII or intracellular CA. In the latter case, it seems that EZ does not enter the cells in sufficient quantity to cause inhibition of intracellular functions (Price and Badger, 1989a). Based on the observation that EZ inhibited CO_2 transport in *Synechococcus* sp. PCC 7942 and that HCO_3^- arrives on the inner side of the membrane (Volokita et al., 1984; Price and Badger, 1989a, 1989b), Price and Badger (1989a) concluded that CO_2 transport involves a CA-like component that catalyzes the conversion of CO_2 to HCO_3^- during the transport process. A similar conclusion also appears to be warranted for *Synechococcus* sp. UTEX 625 based on the observations that EZ (Fig. 1) and other CA inhibitors such as COS (Miller et al., 1989) and H_2S (Espie et al., 1989) inhibit CO_2 transport in this organism.

Na^+-Dependent HCO_3^- Transport

The effects of EZ on HCO_3^- transport were complex and were different for cells grown with air bubbling or in standing culture (Figs. 2 and 4). These differences reflect the different modes of HCO_3^- transport utilized by cells grown under different levels of C_i (Espie and Kandasamy 1992, 1994; McKay et al., 1993).

Although Na^+-dependent HCO_3^- transport and accumulation was reduced by EZ, a large component (40–60%) of transport was also insensitive to EZ inhibition at a concentration that nearly abolished CO_2 transport. These observations indicate that the concurrent operation of the CO_2 transport system is not a prerequisite for the Na^+-dependent, HCO_3^--transport-mediated C_i accumulation and that, therefore, the two transport systems are not mechanistically linked through a common carrier.

Kinetic analysis indicated that EZ is a noncompetitive inhibitor of Na^+-dependent HCO_3^- transport (Fig. 3), suggesting that it acts at a site other than the HCO_3^- binding site of the carrier. This result was unexpected, since we had assumed that, if effective, EZ would act to prevent HCO_3^- binding and its subsequent conversion to CO_2 (Coleman, 1975; Maren and Sanyal, 1983; Price and Badger, 1989a). The inconsistency of the kinetic data with the known mechanism of EZ action makes it seem unlikely that EZ directly inhibited Na^+-dependent HCO_3^- transport. An alternative, unifying explanation of the data is that EZ, rather than directly inhibiting Na^+-dependent HCO_3^- transport, reduced the ability of the cells to retain recently transported C_i through its direct action on the CO_2 transport system. In this scenario, some of the intracellular HCO_3^- delivered to the cytosol via Na^+-dependent transport converts to CO_2 and then leaks into the periplasm, causing a net reduction in HCO_3^- accumulation. Normally, the high-affinity CO_2 transport system would efficiently transport the leaked CO_2 back into the cell, thereby maintaining the C_i pool at a level higher than possible with Na^+-dependent HCO_3^- transport alone. However, in the presence of EZ the CO_2 leaked to the periplasm would not be efficiently transported back into the cell, but would be lost to the medium. Consequently, the loss of recently transported C_i from the internal pool would lead to the appearance that EZ had inhibited Na^+-dependent HCO_3^- transport. This suggestion is supported by previous work that showed that when CO_2 transport is inhibited by COS or H_2S under conditions in which Na^+-dependent HCO_3^- transport is the major

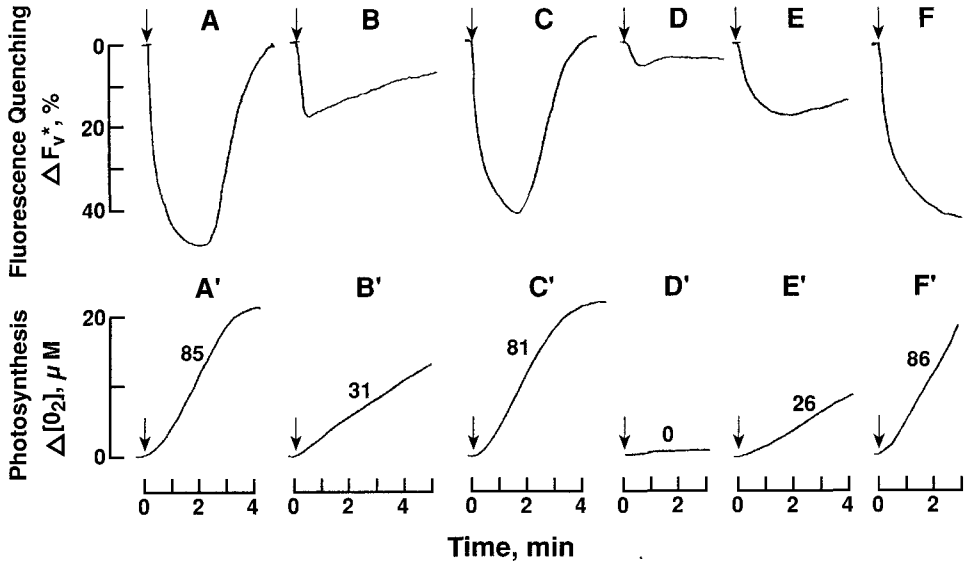

Figure 6. The effect of EZ, Na^+, and monensin on Chl *a* fluorescence yield (top, A–F) and photosynthesis (bottom, A'–F') in *Synechococcus* sp. UTEX cells grown in standing culture. The experiments were started by the addition (↓) of 20 μM $KHCO_3$ to cells at the CO_2 compensation point and near F_M*. Simultaneous measurements were made sequentially with cells suspended in buffer without Na^+ (A and A'); containing 40 μM EZ (B and B'); 40 μM EZ + 25 mM NaCl (C and C'); 40 μM EZ + 25 mM NaCl + 50 μM monensin (D and D'); as in D and D' 15 min later (E and E'); as in D and D', but 1 mM $KHCO_3$ was added to start the reaction (F and F'). The rates of photosynthesis in μmol O_2 mg^{-1} Chl h^{-1} are indicated beside each trace in the bottom.

route for C_i accumulation, large amounts of recently transported HCO_3^- reappear in the medium (as CO_2) (Espie et al., 1989; Miller et al., 1989; Badger and Price, 1990). That CO_2 leaks from the cells in the light was also demonstrated by inhibiting $^{12}CO_2$ transport with $^{13}CO_2$ (Espie et al., 1991). In addition, recent transport studies by Salon et al. (1995) using *Synechococcus* sp. UTEX 625 have shown that the concurrent operation of both the CO_2 and Na^+-dependent HCO_3^- transport systems is necessary to achieve maximum C_i accumulation. Thus, the evidence suggests that EZ reduces net Na^+-dependent HCO_3^- transport solely through its action on CO_2 transport.

Na^+-Independent HCO_3^- Transport

The effect of EZ on Na^+-independent HCO_3^- transport differed markedly from its effect on Na^+-dependent HCO_3^- transport (Figs. 2, 4, and 5). Na^+-independent HCO_3^- transport was more than 1 order of magnitude more sensitive to EZ than Na^+-dependent HCO_3^- transport and lacked the EZ-insensitive component. In addition, doubling the HCO_3^- concentration did not partially relieve EZ inhibition as was found for Na^+-dependent HCO_3^- transport. Na^+-independent HCO_3^- transport also was more sensitive to EZ inhibition (50% inhibition = 12 μM) than CO_2 transport. Consequently, the effect of EZ on Na^+-independent HCO_3^- transport cannot be ascribed to the effect of EZ on CO_2 transport, which was the case for Na^+-dependent HCO_3^- transport, suggesting that EZ directly inhibited Na^+-independent HCO_3^- transport. Thus, there appears to be a second site of EZ inhibition within the C_i transport system that is distinct from the CO_2 transport system.

CA catalyzes the reversible interconversion between HCO_3^- and CO_2 (e.g. Silverman, 1991). EZ, a well-characterized inhibitor of CA, interacts at the active site and prevents substrate binding (Coleman, 1975; Maren and Sanyal, 1983). Our discovery that EZ directly inhibited Na^+-independent HCO_3^- transport suggests that a CA-like conversion of HCO_3^- to CO_2 may be part of the transport mechanism. The conversion of HCO_3^- to CO_2 during transport across the membrane seems unlikely, however, given the evidence indicating that HCO_3^- is the C_i species that arrives in the cytosol following transport (Volokita et al., 1984; Marcus et al., 1986; Price and Badger, 1989b). Alternatively, Na^+-independent transport may convert HCO_3^- to CO_2 for its coupled and immediate utilization by the CO_2 transport system (Fig. 7). This proposal is similar in concept to the "front-end" mechanism for HCO_3^- transport suggested by Price and Badger (1989a). However, a CA-like component, rather than a region of localized acidity, may be responsible for the conversion of HCO_3^- to CO_2.

A Model for C_i Transport

The scheme (Fig. 7) depicting the relationship between the CO_2 and HCO_3^- transport systems of *Synechococcus* sp. UTEX 625 encompasses the major elements of a model previously proposed by Espie et al. (1989) and Miller et al. (1989). Two C_i-translocating elements are shown: a constitutive CO_2 transport system and an inducible Na^+/HCO_3^-

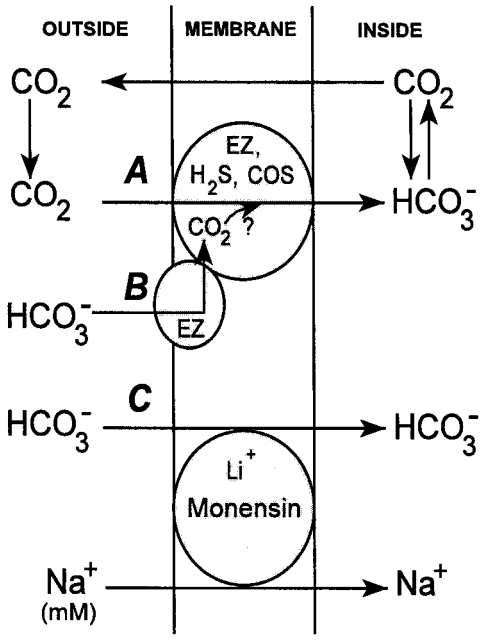

Figure 7. Schematic model depicting the relationship between CO_2 and HCO_3^- transport in low-C_i-grown cells of *Synechococcus* sp. UTEX 625. A, CO_2 transport system; B, Na^+-independent HCO_3^- utilizing system; C, Na^+-dependent HCO_3^- transport system. Inhibitors of the various transport processes are indicated.

symport system (Espie and Kandasamy, 1994). Based on the physiological evidence discussed here and elsewhere, these transport systems are considered to be substrate-specific, separate, and mechanistically independent. Additional support for this view is found in the SC mutant of *Synechocystis* sp. PCC 6803, which is impaired in CO_2 but not HCO_3^- transport (Ogawa, 1993), and in the Tm17 mutant of *Synechococcus* sp. PCC 7942, which is able to transport CO_2 but not HCO_3^- (Yu et al., 1994a). The CO_2 transport system of *Synechococcus* sp. UTEX 625 is inhibited by H_2S (Espie et al., 1989), COS (Miller et al., 1989), and, in agreement with the work of Price and Badger (1989a), EZ. Ultimately, the system delivers HCO_3^- to the cytoplasm. Distinct from CO_2 transport, the Na^+-dependent HCO_3^- transport system is inhibited by Li^+, monensin, and the lack of Na^+ (Espie et al., 1989). EZ, COS, and H_2S do not inhibit Na^+-dependent HCO_3^- transport directly, but cause an apparent, partial inhibition through their action on CO_2 transport.

The new component to our model is the Na^+-independent HCO_3^- utilizing system. This is distinguished from Na^+-dependent HCO_3^- transport by its lack of sensitivity to both monensin and Li^+ inhibition (Espie and Kandasamy, 1994). It is distinguished from the CO_2 transport system by its use of HCO_3^- and its higher sensitivity to EZ (Fig. 4). As proposed above, the Na^+-independent HCO_3^- utilizing system does not directly transport HCO_3^-, but converts it to CO_2 for the coupled and immediate use of the CO_2 transport system; this is analogous to the front-end mechanism proposed by Price and Badger (1989a). Cells grown on high C_i (McKay et al., 1993) or with air bubbling (Espie and Canvin, 1987; Espie and Kandasamy, 1992) lack high-affinity, Na^+-independent HCO_3^- uptake.

We have, therefore, assumed that this ability in standing culture cells is repressed under high-C_i growth conditions and induced in response to growth under low-C_i conditions. However, it is also possible that the kinetic properties of the Na^+-independent HCO_3^- utilizing system may change from a low-affinity state to a high-affinity state in response to a lowering of the growth C_i level and, thus, be a constitutive component of the C_i transport mechanism (Yu et al., 1994b). Standing culture cells grown on low C_i retain CO_2 transport (Price and Badger, 1989a; McKay et al., 1993) and have some capacity for Na^+-dependent HCO_3^- transport (Fig. 6). The mixture between the two modes of HCO_3^- utilization also varies with the growth C_i level. Cells grown with air bubbling lack high-affinity Na^+-independent HCO_3^- uptake (Fig. 7B) but retain CO_2 and Na^+-dependent HCO_3^- transport (Fig. 7, A and C). High-C_i-grown cells possess CO_2 transport (Fig. 7A) but lack both high-affinity Na^+-independent and Na^+-dependent HCO_3^- uptake.

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