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The Role of External Carbonic Anhydrase in Inorganic Carbon Acquisition by *Chlamydomonas reinhardii* at Alkaline pH[1]

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ABSTRACT

The role of external carbonic anhydrase in inorganic carbon acquisition and photosynthesis by *Chlamydomonas reinhardii* at alkaline pH (8.0) was studied. Acetazolamide (50 micromolar) completely inhibited external carbonic anhydrase (CA) activity as determined from isotopic disequilibrium experiments. Under these conditions, photosynthetic rates at low dissolved inorganic carbon (DIC) were far greater than could be maintained by CO_2 supplied from the spontaneous dehydration of HCO_3^- thereby showing that *C. reinhardii* has the ability to utilize exogenous HCO_3^-. Acetazolamide increased the concentration of DIC required to half-saturate photosynthesis from 38 to 80 micromolar, while it did not affect the maximum photosynthetic rate. External CA activity was also removed from the cell-wall-less mutant (CW-15) by washing. This had no effect on the photosynthetic kinetics of the algae while the addition of acetazolamide to washed cells (CW-15) increased the $K_{1/2}^{DIC}$ from 38 to 80 micromolar. Acetazolamide also caused a buildup of the inorganic carbon pool upon $NaHCO_3$ addition, indicating that this compound partially inhibited internal CA activity. The effects of acetazolamide on the photosynthetic kinetics of *C. reinhardii* are likely due to the inhibition of internal rather than a consequence of the inhibition of external CA. Further analysis of the isotopic disequilibrium experiments at saturating concentration of DIC provided evidence consistent with active CO_2 transport by *C. reinhardii*. The observation that *C. reinhardii* has the ability to take up both CO_2 and bicarbonate throws into question the role of external CA in the accumulation of DIC in this alga.

Chlamydomonas reinhardii, like many algae, changes its photosynthetic affinity for DIC[2] in response to the DIC levels on which it was grown (2, 6, 12, 14). Cells grown under air concentrations of CO_2 (0.03%) exhibit high affinity photosynthetic kinetics (low values of $K_{1/2}^{DIC}$) while cells grown on 3 to 5% CO_2 exhibit low affinity photosynthetic kinetics (high values of $K_{1/2}^{DIC}$) (6, 12, 14). Recent work with cyanobacteria has shown that this adaptation occurs in response to the total [DIC] rather than the level of CO_2 (18).

In algae that produce external CA, decreases in $K_{1/2}^{DIC}$ are correlated with increases in the activity of CA (1, 4, 12). The induction of CA in *C. reinhardii* has been well documented (5, 7, 8, 15, 27), yet there is no consensus as to its role in the supply of carbon for photosynthesis (23). Findenegg (12) suggested that decreases in $K_{1/2}^{DIC}$ in response to low DIC by *Scenedesmus*, were facilitated by increases in external CA. Further studies on *C. reinhardii* suggested the only species of carbon that can cross the plasmamembrane is CO_2 (1, 16, 21, 22, 29). If so, periplasmic CA could then facilitate resupply of CO_2 from HCO_3^- at pH values where bicarbonate is the dominant species. These results are not unequivocal and as Imamura *et al.* (13) have suggested, the evidence does not preclude the occurrence of either CA facilitated CO_2 transport or bicarbonate transport. If HCO_3^- can be taken up in support of photosynthesis (26) then the function of external CA is called into question.

Internal CA appears to facilitate the CO_2 supply to Rubisco from the internal HCO_3^- pool (24, 25). Spalding *et al.* (24, 25) showed that a mutant of *C. reinhardii* lacking internal CA exhibited high rates of photorespiration and accumulated large concentrations of intracellular inorganic carbon. Their conclusion was that an internal CA was required to facilitate CO_2 supply to Rubisco from actively transported HCO_3^-. Proponents of the theory that CO_2 is the only species of carbon to cross the plasmalemma suggest that active transport of bicarbonate occurs at the chloroplast envelope and that the internal CA is located within the chloroplast (21, 22).

In this report we provide evidence that *C. reinhardii* has the ability to take up either CO_2 or bicarbonate and that the magnitude of each flux is dependent upon the experimental conditions. Our results suggest that both CO_2 and HCO_3^- accumulation are active and occur at the plasmalemma. We also provide evidence that external CA is not necessary for efficient photosynthesis at alkaline pH.

MATERIALS AND METHODS

Algal Cultures. *Chlamydomonas reinhardii* (127 wt+) and a cell-wall-less mutant (CW-15) were kindly donated by Dr. J. R. Coleman, Botany Department, University of Toronto. Cultures were grown axenically in carbon-limited chemostat vessels (25°C) as previously described (20). A modification of Surzycki's medium (28) was employed. Modifications included the use of 11.6 mM K_2HPO_4 as the phosphate source while iron was supplied from Allen's (3) stock solution with a 3-fold increase in the citrate concentration. Inorganic carbon was supplied in the inflow medium as 10 mM $NaHCO_3$. Cultures were maintained at pH 8.0± 0.05 by a pH-stat (Cole Palmer 5652-00) with a titrant of 0.5 N HCl. Inorganic carbon in the chemostats was measured as previously described (20). All experiments were carried out on DIC limited cells exhibiting a steady state growth rate of 0.8 d^{-1}.

Carbonic Anhydrase Activity. Culture aliquots were removed aseptically from the chemostat vessel and resuspended in 20 mM ice cold phosphate buffer. The CA activity was measured electrochemically by determining the time taken for the pH to drop from 8.0 to 7.0 upon addition of 1 ml of ice cold CO_2 saturated distilled H_2O. One unit of activity was defined as $(t_o/t - 1) \cdot mg^{-1}$

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[2] Abbreviations: DIC, dissolved inorganic carbon; AZA, acetazolamide; CA, carbonic anhydrase; $K_{1/2}^{DIC}$, concentration of DIC required to maintain half maximal photosynthetic rate; P_{max}, maximum photosynthetic rate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

Chl where t_o was the time taken for the control and t was the time taken with cells.

Distinction between internal and external CA activity in wild type cells was made by observing the increase in CA activity in lysed cells (French pressure press 14,000 p.s.i.) over that of whole cells. Because of the problems associated with distinguishing small increases in the CA activity in the lysed cell preparation over the already high activity of the whole cells, the cell-wall-less mutant was also used. All measurable external CA activity in whole cells of the mutant could be removed by washing 4 ml of cells two times in 50 ml of phosphate buffer. Increases in CA activity upon lysing the washed cells were then readily detectable (Chl = 20 $\mu g \cdot ml^{-1}$).

Photosynthetic Kinetics. DIC dependent O_2 evolution was measured in a Clark type O_2 electrode (Hansatech CBD-1). Cells were withdrawn from culture vessels in a sterile syringe and resuspended in low [DIC] 20 mM Na^+ Hepes/HCl (pH 8.0) buffer before being placed in the electrode chamber and allowed to reach compensation point. Photosynthetic kinetics were calculated as previously described (19). The CA inhibitor, AZA, was dissolved in 95% ethanol and used at a concentration of 50 μM. Controls showed that there was no effect of ethanol on photosynthesis. As washing of the CW-15 mutant removed all exogenous CA activity, the photosynthetic kinetics of the unwashed mutant were measured following the addition of sufficient low [DIC] buffer concentrate (300 mM) to bring the buffer concentrations to 20 mM. Bovine CA was obtained from Sigma and was added at 2.0 μg/ml. Maximal rates of CO_2 supply from spontaneous dehydration of HCO_3^- to CO_2 were calculated from the equations of Miller and Colman (19).

Inorganic Carbon Accumulation and Isotopic Disequilibrium. Inorganic carbon accumulation was determined using the silicone fluid centrifugation technique as described elsewhere (5, 9, 14). Terminating solution (100 μl of 2 N NaOH in 20% methanol) was placed in the bottom of a 400 μl microfuge tube and layered with 100 μl of silicone fluid (Ar20:Ar200 = 13.2:5, Wacker Chemical Co; FDR). A 100 μl aliquot of a cell suspension (20 μg Chl ml^{-1}), which had been allowed to reach compensation point in an O_2 electrode, was layered over this mixture and covered with 100 μl of degassed mineral oil. Each tube was allowed to incubate in the light for 2 min before the assay was carried out. Assays were initiated by pulsing the algal layer with 10 μl of 10 mM $NaHCO_3$ (4 $\mu Ci \cdot \mu mol^{-1}$) which resulted in a concentration of 0.91 mM. The sample was stirred throughout the incubation period with the tip of a Hamilton syringe.

Isotopic disequilibrium experiments were performed at pH 7.5 following the technique of Espie and Colman (10). Steady state photosynthesis was allowed to be reached after the addition of 5.5 μl of 250 mM HCO_3^- (final concentration = 550 μM) to a 2.5 ml algal suspension contained in an O_2 electrode chamber. Isotopic disequilibrium was initiated by the addition of 4.15 nmol $^{14}CO_2$ (56 $\mu Ci \cdot \mu mol^{-1}$). At pH 7.5 this represents a 5% increase in aqueous [CO_2]. Theoretical time-courses of ^{14}C assimilation were calculated using the equations of Espie and Colman (10).

RESULTS

CA Activity. The activity of external CA varied as a function of steady state [DIC] under which the cultures were grown. CA activity was high in cells grown at low DIC (0.8 d^{-1}) (250 units) and low in cells grown at saturating levels of DIC (25 units). CA activity of lysed wild type cells was greater than that of intact cells indicating the presence of internal CA activity (data not shown). Further support for this observation was provided by the use of the cell-wall-less mutant CW-15. Unwashed cells (4 μg Chl $\cdot ml^{-1}$) exhibited activity of 412 units. Washing these cells completely removed external CA activity (<1 unit). Subsequent lysis of washed cells resulted in the detection of 7.5 units of CA activity.

Removal of External CA Activity. Treatment with 50 μM AZA completely eliminated the external CA activity as detected by the electrochemical method (data not shown). As this method has a low sensitivity (30 $ng \cdot ml^{-1}$ bovine CA) it was possible that trace levels of external CA activity still remained. This was discounted by observing the time course of ^{14}C fixation following the addition of a trace amount of $^{14}CO_2$ (Fig. 1A) to wild type cells photosynthesizing in the presence of 500 μM DIC. In the presence of 50 μM AZA the time-course of ^{14}C assimilation following $^{14}CO_2$ addition was nonlinear and quantitatively followed the time-course predicted for the uncatalyzed rate of CO_2-HCO_3^- interconversion (10). In the absence of AZA, incorporation of ^{14}C was substantially reduced indicating that catalytic enhancement of the CO_2-HCO_3^- interconversion decreased the time required to attain isotopic equilibrium (10, 11). Isotopic disequilibrium experiments also showed that washing alone effectively removed external CA activity from the CW-15 mutant (Fig. 1B).

Kinetics of Photosynthesis. Cells grown at pH 8.0 exhibited values of $K_{1/2}^{DIC}$ and P_{max} of 38 μM and 119 $\mu mol\ O_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$. AZA (50 μM) increased the $K_{1/2}^{DIC}$ from 38 μM to 80 μM but

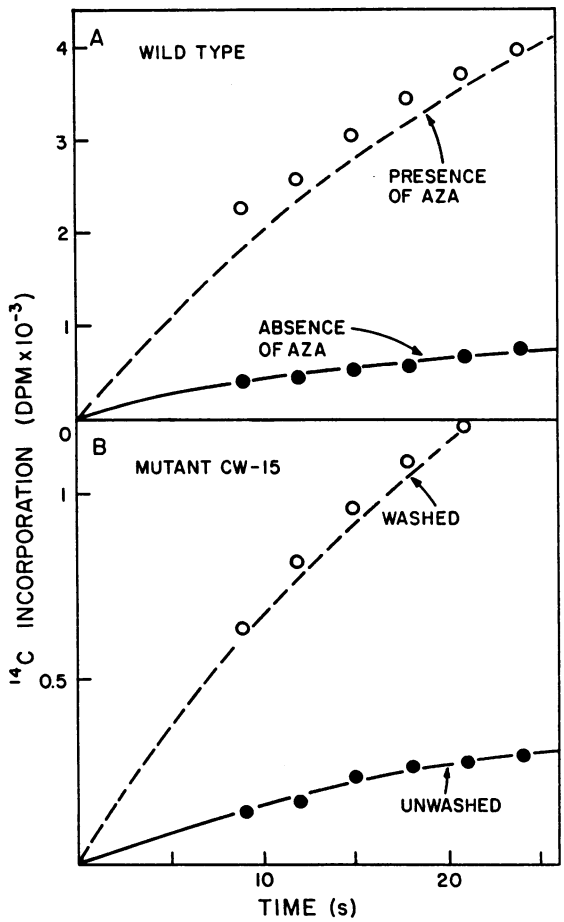

FIG. 1. Comparison between the theoretical time-course of ^{14}C fixation during isotopic disequilibrium with the observed patterns in the absence (●) and presence (○) of 50 μM AZA, for wild type (A) and for the unwashed (●) and washed (○) CW-15 mutant (B). Isotopic disequilibrium was initiated by addition of 0.0041 μM $^{14}CO_2$ to cells photosynthesizing at a steady rate (119 $\mu mol\ O_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$) in the presence of 500 μM DIC. The theoretical pattern of ^{14}C incorporation assuming CO_2 use alone and the total absence of CA activity was calculated using the method of Espie and Colman (10) and is represented by (- - -).

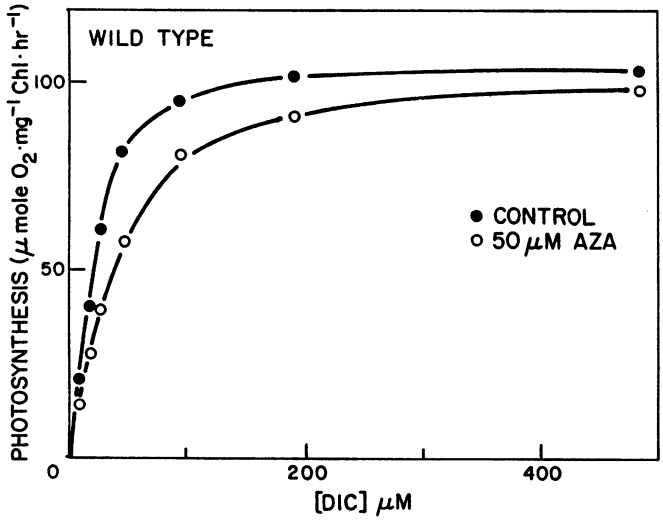

FIG. 2. The effect of 50 μM AZA on the photosynthetic kinetics of *C. reinhardii*. The value of P_{max} was 107 μmol $O_2 \cdot mg^{-1}$ Chl·h^{-1} and was unaffected by AZA. $K_{1/2}^{DIC}$ increased from 38 to 80 μM following AZA treatment.

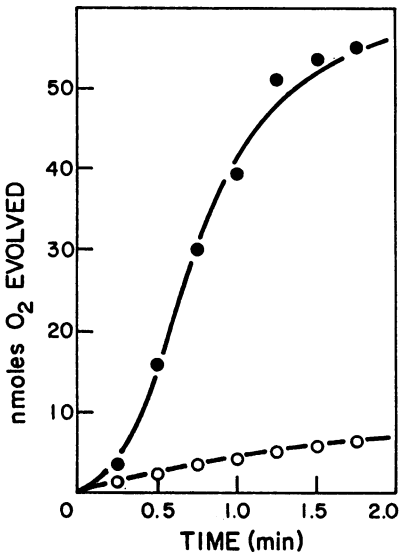

FIG. 3. A comparison of the observed rate of O_2 evolution in the presence of 100 μM AZA with the theoretical rate of O_2 evolution that could be maintained by spontaneous dehydration of HCO_3^- to CO_2. O_2 evolution (●) was initiated by pulsing a suspension of cells (pH = 8.0, Chl = 35 μg·ml^{-1}) with 50 μM $NaHCO_3$ at time zero. Theoretical O_2 evolution (○) was derived from the equations of Miller and Colman (19).

had no effect on P_{max} (Fig. 2). At low levels of DIC (50 μM) and in the absence of external CA activity (AZA inhibited) the rate of photosynthetic O_2 evolution by wild type cells was over 10 times the rate of spontaneous dehydration of HCO_3^- to CO_2 (Fig. 3).

The cell-wall-less strain (CW-15) exhibited values of $K_{1/2}^{DIC}$ and P_{max} of 38 μM and 21 μmol $O_2 \cdot mg^{-1}$ Chl·h^{-1}, respectively. Washing the cell-wall-less mutants free of external CA activity or the subsequent addition of 2 μg bovine CA·ml^{-1}, had no effect on the kinetics of photosynthesis (Fig. 4). The addition of 50 μM AZA to washed CW-15 caused an increase in $K_{1/2}^{DIC}$ from 38 to 80 μM (Fig. 4).

The Effects of AZA on Carbon Uptake and Accumulation. Pulsing with $NaHCO_3$ resulted in a rapid increase in the internal DIC pool until a constant level was reached after about 20 s. The

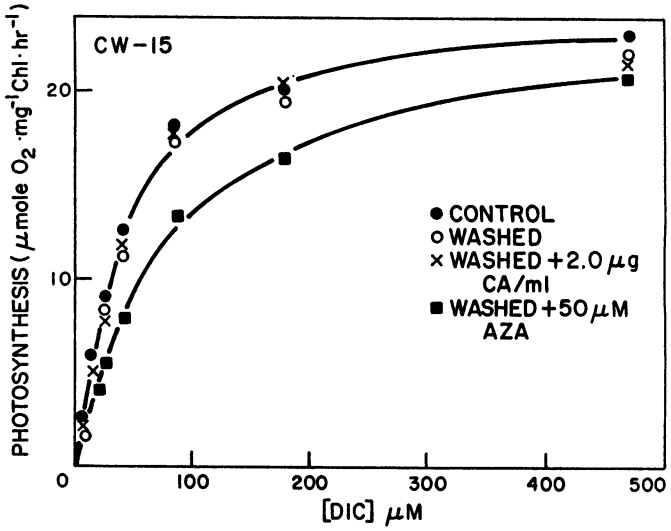

FIG. 4. The effects of removal of external CA activity on the photosynthetic kinetics of the CW-15 cell-wall-less mutant of *C. reinhardii*. The value of P_{max} was 21 μmol $O_2 \cdot mg^{-1}$ Chl·h^{-1} and was unaffected by subsequent treatments. Control cells (●) exhibited a $K_{1/2}^{DIC}$ of 38 μM. Removal of external CA by washing (○) and readdition of 2.0 μg/ml bovine CA (×) had no effect on the photosynthetic kinetics. Addition of 50 μM AZA (■) to washed cells increased the $K_{1/2}^{DIC}$ from 38 to 80 μM.

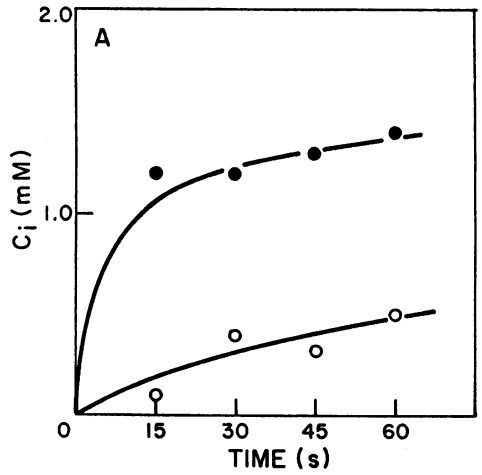

FIG. 5. The effect of AZA on inorganic carbon accumulation in wild type cells of *C. reinhardii* at pH 8.0. The internal pool of DIC over time upon pulsing with 0.91 mM DIC (see "Materials and Methods" for details) in the presence (●) and absence (○) of 50 μM AZA.

presence of AZA resulted in an increase in the internal DIC pool relative to the control (Fig. 5).

DISCUSSION

In *Chlamydomonas reinhardii* adaptation to low levels of DIC is manifested by both a decrease in $K_{1/2}^{DIC}$ and in induction of periplasmic CA (8, 30). Our results confirm the existence of this phenomenon for chemostat grown cells as well as the presence of internal CA activity. It has been assumed that since the induction of external CA coincides with an increase in affinity for DIC, CA must play a role in the supply of inorganic carbon to these organisms (12). If external CA facilitates carbon uptake, its exact function must hinge upon the species of carbon traversing the plasmamembrane. There is evidence consistent with the theory that CO_2 is the only species of carbon to cross the membrane of *C. reinhardii* (17, 21, 22, 29). Much of this evi-

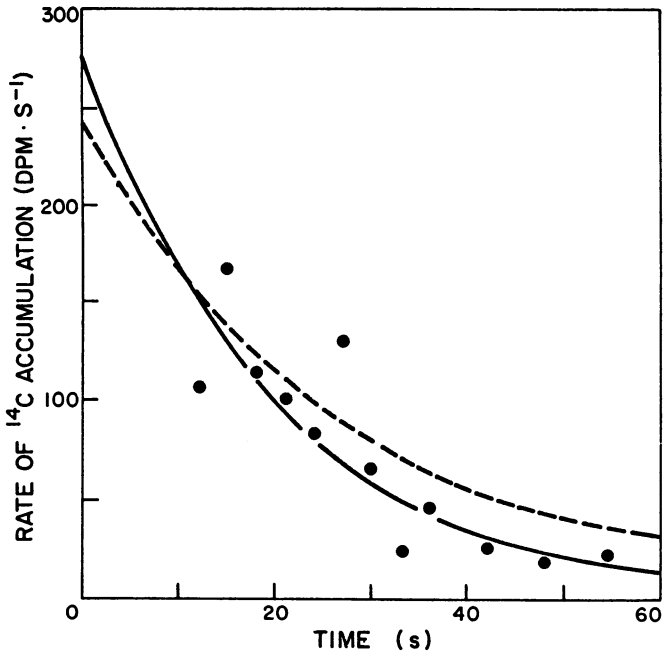

FIG. 6. Comparison between the theoretical time course of rates of ^{14}C accumulation and observed rates during isotopic disequilibrium. Theoretical rates, assuming CO_2 was in instantaneous equilibrium across the plasmalemma, were calculated using the method of Espie and Colman (9, 10) and are represented by (- - -). Observed rates (●) were taken as the slope between two adjacent points in Figure 1A and the curve fitted to these data ($Y = 279.5e^{-0.0516t}$, $r = 0.888$; ——) was produced using a curve fit program (Curve Fitter for ADALAB).

dence, however, does not preclude the uptake of HCO_3^- in addition to CO_2. In fact, previous workers have concluded that *C. reinhardii* can utilize both CO_2 and HCO_3^- (16) and recent models have assumed that both CO_2 and HCO_3^- can cross the plasmalemma (26).

The assessment of which species of DIC can potentially cross the plasmalemma is made much simpler if exogenous CA is inhibited by AZA. Under such conditions, photosynthesis at pH 8.0 and at low levels of DIC (50 μM), proceeded at a far greater rate than could be supported by spontaneous dehydration of HCO_3^- to CO_2 (Fig. 3). Therefore, a carbon source other than CO_2, presumably bicarbonate, must have been transported into the cells in support of photosynthesis. Moroney *et al.* (22) have reported similar results yet they assumed that AZA treatment did not fully inhibit external CA activity and the residual activity still effectively catalyzed the dehydration of HCO_3^-. We evaluated this possibility by determining the time-course of ^{14}C incorporation during isotopic disequilibrium, and comparing it to that predicted for the uncatalyzed reaction (Fig. 1A). These results showed that in the presence of AZA, disequilibrium between CO_2 and HCO_3^- was maintained for a period of time similar to that calculated for the spontaneous hydration of CO_2 to HCO_3^- (Fig. 1B). This demonstrates that AZA completely eliminated external CA activity. Consequently, photosynthetic rates in excess of the uncatalyzed rate of CO_2 supply from HCO_3^- must be a result of carbon supply from HCO_3^-.

Insight into the role of CO_2 in support of photosynthesis may be obtained by further analysis of the isotopic disequilibrium results reported in Figure 1A. The rates of ^{14}C fixation between each sampling time over the duration of the time-course were calculated (Fig. 6). A theoretical prediction of this disequilibrium data can be obtained using the models of Espie and Colman (9, 10) assuming that CO_2 rapidly equilibrates across the membrane. This model provides a reasonable fit to the observed data. Given that $^{14}CO_2$ was added to a steady state photosynthesizing culture (pH 8.0, 500 μM DIC) and that exogenous CA was completely inhibited, extrapolation of a least squares fit of the data back to time zero (the point at which all ^{14}C was in the form of CO_2) allows us to predict the contribution of CO_2 uptake to total DIC acquisition at saturating levels of DIC. The intercept was determined to be 280 dpm·s^{-1} corresponding to a photosynthetic rate of 141 μmol O_2·mg^{-1} Chl·h^{-1}. This was similar to the simultaneous rate of O_2 evolution measured in the electrode chamber (119 μmol O_2·mg^{-1} Chl·h^{-1}). This evidence suggests that at saturating levels of DIC (500 μM) CO_2 alone is capable of supplying the carbon requirements of photosynthesis in *C. reinhardii* at pH 8.0. Accordingly, this analysis suggests a rapid equilibration of CO_2 occurred across the plasmamembrane. Given that the internal CO_2 pool is large (5) rapid equilibrium of CO_2 between the cells and the medium would take place against a considerable concentration gradient. This implies that active CO_2 transport may be involved.

We have demonstrated that in the absence of external CA activity CO_2 is sufficient to meet the requirements of photosynthesis at saturating carbon, yet bicarbonate acquisition is required at high cell densities and limiting levels of DIC. The observation that either species of carbon may be transported in support of photosynthesis throws into question the role of external CA in carbon acquisition by *C. reinhardii*. Although both HCO_3^- and CO_2 can be utilized in the absence of CA activity, it is conceivable that active CA would facilitate the preferred use of one form over the other. For example it may be energetically favorable to take up CO_2 as it is electroneutral. If this were true, one would expect to see a change in the kinetics of photosynthesis with respect to DIC following inhibition of external CA activity. At pH 8.0 AZA caused an increase in $K_{1/2}^{DIC}$ from 38 to 80 μM (Fig. 2). Similar results have been interpreted by others as evidence that CA is required for resupply of CO_2 from HCO_3^- (21, 22).

Another approach to this problem is to remove all exogenous CA activity from the cell-wall-less mutant by washing (confirmed by isotopic disequilibrium, Fig. 1B). Under these conditions the absence of CA activity had no effect on the kinetics of photosynthesis (Fig. 4), nor did the readdition of 2.0 μg·ml^{-1} bovine CA. The subsequent addition of 50 μM AZA, however, caused an increase in $K_{1/2}^{DIC}$ from 38 μM to 80 μM (Fig. 4). We must therefore conclude that AZA affects photosynthesis in some manner other than the inhibition of external CA. One possibility is that trace amounts of AZA are entering the cell and inhibiting intracellular CA activity. In the presence of AZA internal DIC pools increased above those of controls (Fig. 5). Thus, it may be that AZA was entering the cells and at least partially inhibiting internal CA. A buildup in the internal DIC pool was then required in order to maintain comparable rates of CO_2 supply from HCO_3^- (21, 22, 24, 25). These results suggest that the effect of AZA on photosynthetic kinetics was mediated through inhibition of internal CA and not due to any effects on the activity of the external enzyme. Consequently, it is reasonable to assume the external CA activity is not an absolute requirement for efficient DIC utilization at alkaline pH by *C. reinhardii* in liquid culture.

In summary, we have provided evidence suggesting that DIC limited cells of *C. reinhardii* at saturating levels of DIC can meet all the carbon demands of photosynthesis by active CO_2 accumulation. When extracellular CO_2 is limiting these cells have the capability to utilize HCO_3^-. The complete removal of external CA activity by washing the cell-wall-less mutant does not affect the kinetics of photosynthesis. The addition of AZA increases the value of $K_{1/2}^{DIC}$ in both CW-15 and wild type cells but these effects are attributable to AZA inhibition of internal CA. We are therefore unable to show a relationship between the occurrence of exogenous CA and the kinetics of photosynthesis in *C. reinhardii*.

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