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Active CO_2 Transport by the Green Alga *Chlamydomonas reinhardtii*[1]

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ABSTRACT

Mass spectrometric measurements of dissolved free $^{13}CO_2$ were used to monitor CO_2 uptake by air grown (low CO_2) cells and protoplasts from the green alga *Chlamydomonas reinhardtii*. In the presence of 50 micromolar dissolved inorganic carbon and light, protoplasts which had been washed free of external carbonic anhydrase reduced the $^{13}CO_2$ concentration in the medium to close to zero. Similar results were obtained with low CO_2 cells treated with 50 micromolar acetazolamide. Addition of carbonic anhydrase to protoplasts after the period of rapid CO_2 uptake revealed that the removal of CO_2 from the medium in the light was due to selective and active CO_2 transport rather than uptake of total dissolved inorganic carbon. In the light, low CO_2 cells and protoplasts incubated with carbonic anhydrase took up CO_2 at an apparently low rate which reflected the uptake of total dissolved inorganic carbon. No net CO_2 uptake occurred in the dark. Measurement of chlorophyll *a* fluorescence yield with low CO_2 cells and washed protoplasts showed that variable fluorescence was mainly influenced by energy quenching which was reciprocally related to photosynthetic activity with its highest value at the CO_2 compensation point. During the linear uptake of CO_2, low CO_2 cells and protoplasts incubated with carbonic anhydrase showed similar rates of net O_2 evolution (102 and 108 micromoles per milligram of chlorophyll per hour, respectively). The rate of net O_2 evolution (83 micromoles per milligram of chlorophyll per hour) with washed protoplasts was 20 to 30% lower during the period of rapid CO_2 uptake and decreased to a still lower value of 46 micromoles per milligram of chlorophyll per hour when most of the free CO_2 had been removed from the medium. The addition of carbonic anhydrase at this point resulted in more than a doubling of the rate of O_2 evolution. These results show low CO_2 cells of *Chlamydomonas* are able to transport both CO_2 and HCO_3^- but CO_2 is preferentially removed from the medium. The external carbonic anhydrase is important in the supply to the cells of free CO_2 from the dehydration of HCO_3^-.

Green algae and cyanobacteria possess a high apparent affinity for DIC[3] when grown at low DIC concentrations (low CO_2 cells: 2, 5, 9, 17), and DIC accumulation has been demonstrated for these organisms (2, 4, 9, 15). In the case of cyanobacteria, both HCO_3^- and CO_2 are substrates for active transport (2, 3, 6, 7, 14, 15) with CO_2 being selectively and preferentially used by the cells (2, 6, 16). In *Chlamydomonas*, HCO_3^- is actively transported (4, 25, 29), but CO_2 uptake has been considered to be passive (18, 20). Carbon dioxide, however, is taken up from the medium faster than HCO_3^- by *Chlamydomonas* (13, 28, 29) and several authors (13, 29) have considered the possibility of active CO_2 transport.

Studies on the DIC transport mechanism of green algae are complicated by their cellular compartmentation. Recently, it was shown that isolated chloroplasts of low CO_2 *Chlamydomonas reinhardtii* were able to accumulate DIC (19) and a model was presented where the only active DIC transport mechanism was located on the chloroplast envelope (18, 19). In that model the plasma membrane was suggested to be only a diffusion barrier for CO_2 generated by external carbonic anhydrase. In contrast, by comparison of the apparent affinities for DIC of whole cells and purified chloroplasts, Sültemeyer *et al.* (26) came to the conclusion that active transport by the chloroplast alone may not be responsible for the photosynthetic characteristics of whole cells.

Another difficulty in examining the DIC species taken up by whole cells is the presence of an external carbonic anhydrase (10) which catalyzes the rapid equilibrium between CO_2 and HCO_3^-, thus making a direct discrimination between CO_2 and HCO_3^- uptake impossible (7, 13). However, using inhibitors for external carbonic anhydrase or the cell-wall less mutant CW-15, some authors came to the conclusion that CO_2 and not HCO_3^- (13) or that both CO_2 and HCO_3^- (29) were actively transported.

Confusion about which DIC species is actively taken up from the medium may also be caused by methods which only measure total rates of transport rather than transport of CO_2 or HCO_3^- individually. Using MS, which measures free dissolved gases in liquid, several authors presented direct evi-

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[3] Abbreviations: DIC, dissolved inorganic carbon; AZA, acetazolamide; BTP, 1,3-bis-(tris[hydroxymethyl]methylamino)-propane; F_{max}, maximum fluorescence yield; k_D, dehydration rate constant of HCO_3^- to CO_2; F_V, variable fluorescence yield; $K_{½}$ (DIC), concentration of DIC required to maintain O_2 evolution at one-half its maximum rate; $K_{½}$ (CO_2), CO_2 concentration where O_2 evolution is half maximum; PFR, photon fluence rate; Q, primary electron acceptor of photosystem II; q_E, quench coefficient for nonphotochemical quenching; q_Q, quench coefficient for photochemical quenching; Rubisco, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; TAPS, tris[hydroxymethyl] methyl amino propane sulfonic acid.

dence consistent with an active CO_2 transport in cyanobacteria (2, 6, 16). We have used mass spectrometry (2, 16) for measuring CO_2 and O_2 exchange in *C. reinhardtii*. To overcome the problem of external carbonic anhydrase we used protoplasts of low CO_2 *C. reinhardtii* which were washed free of external carbonic anhydrase activity. The results presented in this paper will show that both CO_2 and HCO_3^- are actively taken up from the medium but that CO_2 is preferentially used. Further, we shall present evidence that the function of external carbonic anhydrase is to supply the cells with free CO_2.

MATERIALS AND METHODS

Growth of Algae

Chlamydomonas reinhardtii (Dangeard) strain 11/32b (obtained from the Sammlung für Algenkulturen, Göttingen, FRG) was grown in 50 mL glass tubes at 32°C in a medium described by Kreuzberg *et al.* (12), except that 50 mM TAPS was added in order to maintain pH 8.0. The cultures were either gassed with air (low CO_2 cells) or with air enriched with 5% CO_2 (high CO_2 cells). During growth, the algae were continuously illuminated by a PFR of 150 μmol photons m^{-2} s^{-1}.

Preparation of Cells and Protoplasts

For each measurement, an aliquot of algae containing about 30 μg of total Chl was harvested and washed three times by centrifugation in 1 mL of 25 mM BTP/HCl buffer (pH 8.0). This buffer contained only small amounts (15 μM) of DIC (14).

For preparations of protoplasts, an aliquot of low CO_2 grown cells (total Chl about 30 μg) was harvested, washed in 25 mM Hepes KOH (pH 7.8), and incubated in 5 mL of autolysin which was prepared according to Schlösser *et al.* (22). At room temperature protoplast formation was completed within 10 min. Isolated protoplasts were washed six times in 1 mL BTP/HCl-buffer (pH 8.0) to ensure complete removal of external carbonic anhydrase activity (Fig. 1).

Fluorometry

Fluorescence measurements were carried out with a pulse modulated Chl fluorometer (24) at the same time as O_2 and $^{13}CO_2$ exchanges were measured by MS. The pulsed measuring beam (100 kHz) and the actinic light to drive $^{13}CO_2$ uptake and O_2 evolution were about 1 and 220 μmol photons m^{-2} s^{-1}, respectively. The latter was provided by a tungsten-halogen projector lamp. The oxidation state of Q was monitored at 10 s intervals with a high intensity light flash (1600 μmol photons m^{-2} s^{-1}) for 1 s. F_{max} was obtained by pulsing dark adapted cells with one single high intensity light flash and the quenching coefficients for photochemical (q_Q) and nonphotochemical (q_E) fluorescence quenching were calculated using the formulas of Schreiber *et al.* (24).

Mass Spectrometry

The uptake of $^{13}CO_2$ (m/e = 45) and the release of O_2 (m/e = 32) by cells and protoplasts were continuously recorded using the closed system described by Miller *et al.* (16). It consists of a water-jacketed cuvette which is coupled to a magnetic sector MS (VG Gas Analysis, model MM 14-80 SC; Middlewitch, England) via an aqueous membrane inlet system.

Washed cells and protoplasts were resuspended in 6 mL BTP/HCl-Buffer (pH 8.0; final Chl content 4–6 μg mL^{-1}) and introduced into the cuvette thermostated at 30°C. The chamber was closed with a plexiglas stopper which contained a small capillary and was darkened for temperature equilibration. The suspension was continuously stirred during the measurements.

Measurements of Carbonic Anhydrase

The activity of external carbonic anhydrase was monitored by MS by following the time course of $^{13}CO_2$ formation after addition of $K_2{}^{13}CO_3$. The chamber was filled in the dark at 30°C with 6 mL of BTP/HCl buffer (pH = 8.0) containing the cell material (final Chl concentration = 4–6 μg mL^{-1}) and closed. Subsequently, 3 μL of $K_2{}^{13}CO_3$ stock solution (100 mM) were added to yield an initial concentration of 50 μM and the increase in the signal at mass 45 ($^{13}CO_2$) was followed. For a further comparison, buffer without cells was measured either in the absence or presence of bovine carbonic anhydrase (50 Wilbur-Anderson units mL^{-1}).

Chemicals

Bovine carbonic anhydrase and BTP were obtained from Sigma Chemical Co. $K_2{}^{13}CO_2$ (99 atom % ^{13}C) was purchased from MSD Isotopes, Montreal, Canada. Other chemicals were of reagent grade.

RESULTS

The possibility of measuring selective CO_2 uptake by mass spectrometry (2, 16) in low CO_2 grown *C. reinhardtii* is complicated by the presence of an external carbonic anhydrase (trace b, Fig. 1). This activity could be abolished by addition of 50 μM AZA, a potent inhibitor of carbonic anhydrase (trace e, Fig. 1) where the rate of CO_2 formation was similar to that in BTP buffer without cells (trace c, Fig. 1). Similar to AZA treated low CO_2 cells, high CO_2 grown algae and washed protoplasts from low CO_2 grown *C. reinhardtii* did not show any measurable carbonic anhydrase activity (traces d and f, Fig. 1). The initial rates of CO_2 appearance in traces c to f (Fig. 1) showed a rate constant k_D of 0.88×10^{-3} s^{-1}, which is in close agreement to values reported previously (6, 16) for the uncatalyzed dehydration of HCO_3^-.

To examine the ability for CO_2 uptake, cells and protoplasts were illuminated after being incubated in the dark for several minutes with 50 μM $K_2{}^{13}CO_3$ (0.8 μM $^{13}CO_2$ at pH 8.0). High CO_2 cells could use the inorganic carbon only to a small degree (trace d, Fig. 2), whereas low CO_2 algae were more effective (trace b, Fig. 2) indicating a higher affinity for DIC. This is supported by the $K_{1/2}$ (DIC) values which were 12 μM DIC (0.19 μM CO_2 at pH 8.0) and 332 μM DIC (5.2 μM CO_2 at pH 8.0) for low and high CO_2 cells, respectively (not shown). When 50 μM AZA was added to low CO_2 cells to inhibit the

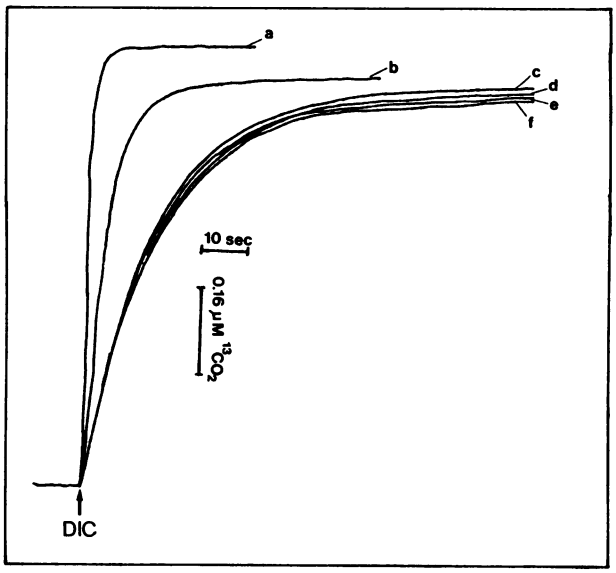

Figure 1. Time course of $^{13}CO_2$ formation in the dark after injection of 50 μM $K_2{}^{13}CO_3$ (DIC) as measured by mass spectrometry at 30°C and pH 8.0. a, BTP-buffer + carbonic anhydrase (50 Wilbur-Anderson units mL^{-1}); b, low CO_2 grown cells; c, 25 mM BTP/HCl buffer; d, high CO_2 grown cells; e, low CO_2 grown cells treated with 50 μM AZA; f, washed protoplasts from low CO_2 grown cells.

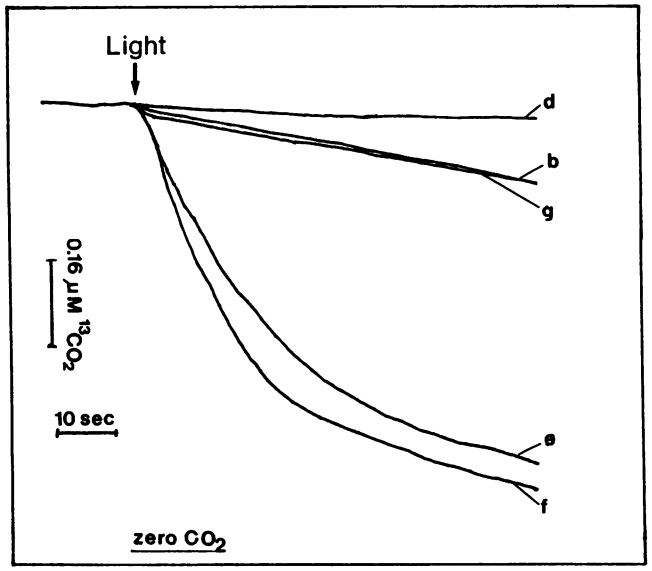

Figure 2. Measurement of $^{13}CO_2$ uptake by mass spectrometry during illumination at pH 8.0 in the presence of 50 μM $K_2{}^{13}CO_2$ (0.8 μM $^{13}CO_2$). b, Low CO_2 grown cells; d, high CO_2 grown cells; e, AZA (50 μM) treated low CO_2 grown cells; f, washed protoplasts from low CO_2 grown cells; g, washed protoplasts from low CO_2 grown cells incubated with carbonic anhydrase (50 Wilbur-Anderson units mL^{-1}).

external carbonic anhydrase (Fig. 1) a rapid decrease in the extracellular $^{13}CO_2$ concentration was observed (trace e, Fig. 2). A similar drop of the external $^{13}CO_2$ content was obtained with washed protoplasts of low CO_2 algae (trace f, Fig. 2) which did not possess external carbonic anhydrase activity (Fig. 1). However, this latter result with washed protoplasts could be changed to a pattern similar to that observed with low CO_2 cells by the addition of bovine carbonic anhydrase (50 Wilbur-Anderson units mL^{-1}; trace g, Fig. 2).

In order to investigate the nature of the rapid $^{13}CO_2$ disappearance from the external medium, further experiments were conducted with washed protoplasts from low CO_2 cells. Complications due to AZA, such as possible inhibition of intracellular carbonic anhydrase (29) could thus be avoided.

After a period of $^{13}CO_2$ uptake in the light, darkening of the protoplasts resulted in a reappearance of $^{13}CO_2$ in the medium (Fig. 3A). Estimation of the velocity by which $^{13}CO_2$ was formed after darkening (Fig. 3A) led to a k_D value similar to that for spontaneous dehydration of HCO_3^- to CO_2 (Fig. 1). Thus, the reappearance of $^{13}CO_2$ in the medium upon darkening was due mainly to reequilibration of the HCO_3^-/CO_2 system. Addition of carbonic anhydrase in the light caused a rapid increase in the $^{13}CO_2$ concentration in the external medium (Fig. 3B) indicating a disequilibrium of the HCO_3^-/CO_2 system prior to carbonic anhydrase addition similar to that observed in cyanobacteria (6, 16). Because the fixation period (1.5 min) was short, dark respiration was not likely to contribute to the reappearance of $^{13}CO_2$ and because of the kinetics in the absence and presence of carbonic anhydrase, leakage of $^{13}CO_2$ from the internal pool could at most make a minor contribution to the reappearance of $^{13}CO_2$.

Since carbonic anhydrase catalyzes a fast equilibration between HCO_3^- and CO_2, it appears from these results that at least one function of carbonic anhydrase is to ensure the availability of CO_2. In order to check this hypothesis, we compared the O_2 evolution, CO_2 uptake, and fluorescence quenching patterns of whole cells and protoplasts (Figs. 4–6).

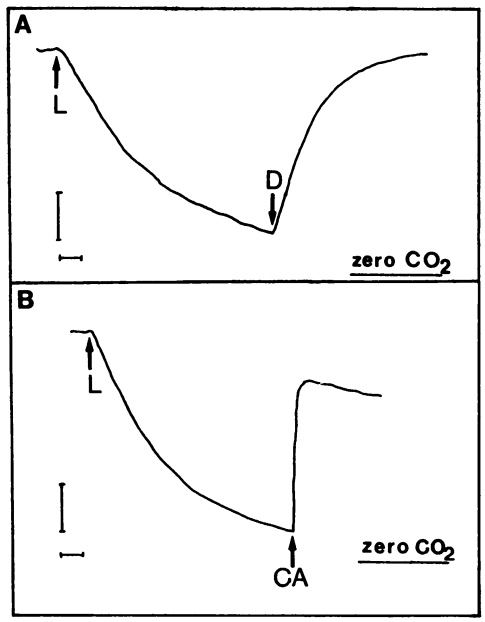

Figure 3. Time courses of $^{13}CO_2$ uptake by washed protoplasts of low CO_2 cells of *C. reinhardtii*. Protoplasts were incubated with 50 μM $K_2{}^{13}CO_3$ (0.8 μM $^{13}CO_2$) for several minutes in the dark. The transport of $^{13}CO_2$ was induced by switching the light on as indicated (L). Reequilibration was achieved by: A, dark; B, addition of carbonic anhydrase (50 Wilbur-Anderson units mL^{-1}). Each horizontal and vertical bar represents 10 s and 0.16 μM $^{13}CO_2$, respectively.

In the presence of 50 μM DIC, low CO_2 cells reached CO_2 compensation (about 50–60 nM CO_2) within 8 min following illumination (Fig. 4). This was indicated by the cessation of O_2 evolution and CO_2 uptake. Simultaneously, the nonphotochemical quenching (q_E) drastically increased from 0.2 (at maximal photosynthesis) to 0.76 (at CO_2 compensation) which is indicative of an enhanced thylakoid membrane energization at CO_2 limitation (24). The photochemical quenching (q_Q) was not affected over the same time period.

In the presence of carbonic anhydrase, rapid equilibration between HCO_3^- and CO_2 occurs, and the [CO_2] measured by MS then represents the uptake of total DIC. At a known pH (pH = 8.0), it is possible to calculate rates of DIC uptake from the decline of the CO_2 trace. DIC uptake was 93 μmol mg^{-1} Chl h^{-1} and O_2 evolution was 108 μmol mh^{-1} Chl h^{-1} in low CO_2 cells (Fig. 4). When protoplasts were incubated with carbonic anhydrase (50 Wilbur-Anderson units mL^{-1}), they exhibited similar rates (93 and 102 μmol mg^{-1} Chl h^{-1}) of DIC uptake and O_2 evolution (Fig. 5) as low CO_2 cells (Fig. 4). The fluorescence yield pattern in protoplasts with carbonic anhydrase (Fig. 5) was essentially the same as that in whole

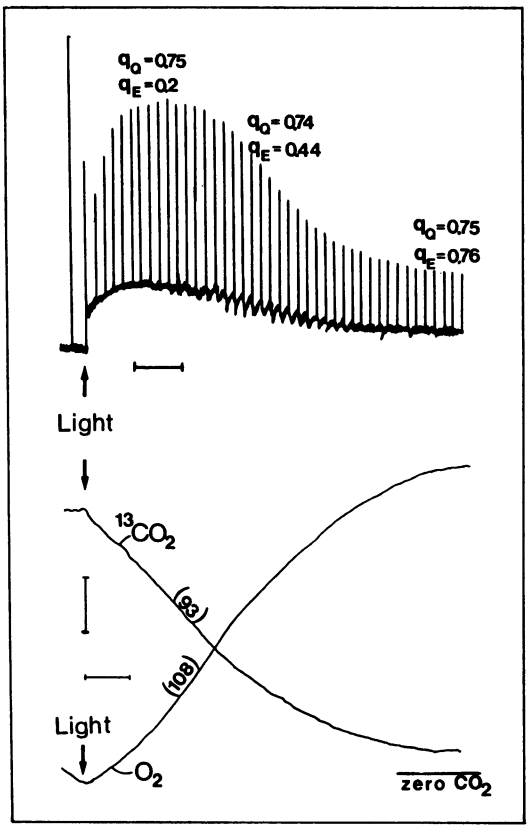

Figure 4. Changes in the fluorescence, $^{13}CO_2$ concentration and total O_2 content during illumination in low CO_2 cells of *C. reinhardtii* in the presence of 50 μM DIC. The coefficients q_Q and q_E are indicated when maximal photosynthesis, declining photosynthesis and CO_2 compensation occurred. The fluorescence yield of dark-adapted cells after the first light flash was taken as F_{max} according to Schreiber *et al.* (24). Numbers in parentheses indicate the rates of total DIC uptake and O_2 evolution expressed as μmol mg^{-1} Chl h^{-1}. Horizontal bars represent 1 min and the vertical bar indicates 0.16 μM CO_2 and 9.2 μM O_2, respectively.

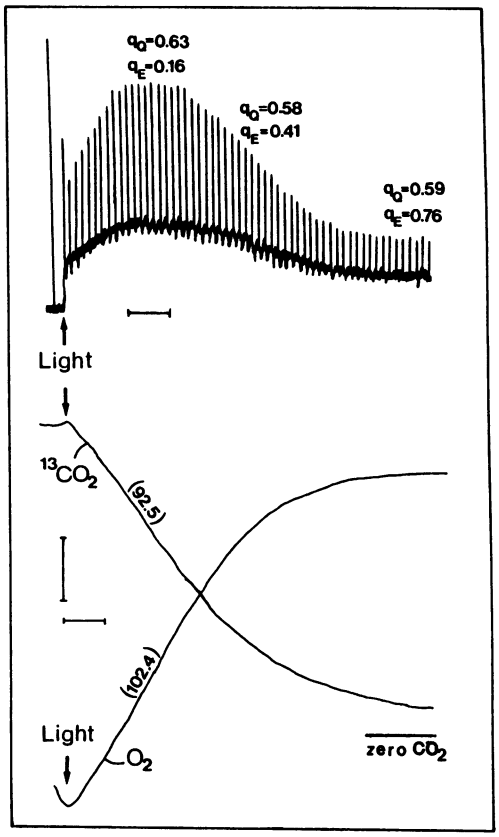

Figure 5. Changes in fluorescence, $^{13}CO_2$ concentration and total O_2 content during illumination by washed protoplasts of *C. reinhardtii* in the presence of 50 μM DIC and carbonic anhydrase (50 Wilbur-Anderson units mL^{-1}). Horizontal bars represent 1 min and the vertical bar indicates 0.16 μM CO_2 and 8 μM O_2, respectively. For further details see Figure 4.

cells (Fig. 4) showing an increase in q_E when the CO_2 compensation point was approached. Again, q_Q was little changed.

Figure 6 shows the changes of fluorescence yield and $^{13}CO_2$ and O_2 concentrations during photosynthesis in the absence and presence of carbonic anhydrase by washed protoplasts. Without carbonic anhydrase, $^{13}CO_2$ was initially taken up at a rate of about 16 μmol mg^{-1} Chl h^{-1} (since CO_2 and HCO_3^- were not in equilibrium this rate cannot be expressed in terms of DIC). Simultaneously, O_2 was evolved but at a lower rate (83 μmol mg^{-1} Chl h^{-1}) than with whole cells and carbonic anhydrase-treated protoplasts (Figs. 4 and 5). Before addition of carbonic anhydrase, the rate of O_2 production had declined to 46 μmol mg^{-1} Chl h^{-1}. Oxygen evolution increased about 2.5-fold after addition of carbonic anhydrase (Fig. 6). Carbonic anhydrase reequilibrated the CO_2/HCO_3^- system and this reequilibration indicated that about one-half of the initial [DIC] (50 μM) had been taken up and fixed by the cells.

As was observed for the CO_2 and O_2 gas exchanges, the fluorescence yield patterns also responded to the addition of carbonic anhydrase (Fig. 6). Without carbonic anhydrase, q_E was relatively high (q_E = 0.45) 2 min after onset of illumination (Fig. 6) compared to whole cells and carbonic anhydrase treated protoplasts (Figs. 4 and 5). It slowly increased during CO_2 depletion and cessation of O_2 evolution and reached 0.58

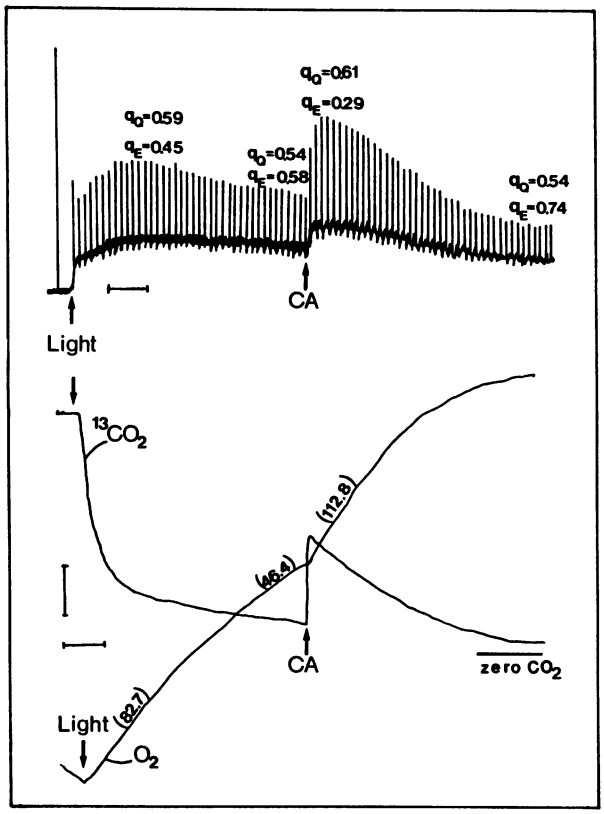

Figure 6. Changes in fluorescence, $^{13}CO_2$ concentration and total O_2 content during illumination of washed protoplasts from *C. reinhardtii*. Five and one-half min after onset of illumination carbonic anhydrase (50 Wilbur-Anderson units mL^{-1}) was added as indicated. The quenching coefficients q_Q and q_E are shown for maximal and limited photosynthesis before and after carbonic anhydrase addition. Horizontal bars represent 1 min and the vertical bar indicates 0.16 μM CO_2 and 8 μM O_2, respectively. For further details see Figure 4. The initial rate of CO_2 uptake was 16.3 μmol mg^{-1} Chl h^{-1}.

just before the addition of carbonic anhydrase. After addition of carbonic anhydrase, q_E immediately declined to 0.29 within 1 min. Thereafter, it increased to 0.74 at CO_2 compensation. The q_Q was little changed by carbonic anhydrase addition (Fig. 6). The q_E was always about 0.75 when CO_2 compensation was reached (Figs. 4–6).

DISCUSSION

The adaptation of *C. reinhardtii* to low DIC concentrations is accompanied by an increased affinity for DIC (5), which is correlated with increased levels of carbonic anhydrase (10) and an increased ability to actively accumulate DIC (4, 18, 20, 25). Most of the carbonic anhydrase activity that appears is due to increased amounts of extracellular carbonic anhydrase (10). It has been suggested that the function of the external carbonic anhydrase is to provide the cells with CO_2 (8, 18, 20, 28) but photosynthesis could be sustained when the external carbonic anhydrase was inhibited (13, 29). The question of the role of the external carbonic anhydrase is strongly associated with the question of which DIC species is taken up by the cells. Some authors (18–20, 28) propose that only CO_2 diffuses across the plasmalemma but other authors propose that both HCO_3^- and CO_2 may be actively accumulated (13, 25, 29).

Removal of the extracellular carbonic anhydrase activity is necessary in order to investigate which forms of DIC are transported into the cells (6, 7, 16). This was accomplished by two methods. In the first method the cells were treated with AZA, a potent inhibitor of carbonic anhydrase (Fig. 1, trace e). In the second method protoplasts were made from low CO_2 cells and washed extensively until no carbonic anhydrase activity could be detected (Fig. 1, trace f).

In the absence of external carbonic anhydrase, cells and protoplasts rapidly removed CO_2 from the medium with an initial rate of about 16 μmol CO_2 mg^{-1} Chl h^{-1} (Figs. 2, 3, 6). CO_2 uptake occurred faster than CO_2 could be supplied to the medium by HCO_3^- dehydration and the CO_2 concentration dropped to the level of the CO_2 compensation point (50–60 nM CO_2). That the CO_2/HCO_3^- system was removed from equilibrium was shown by the increase in mass 45 ($^{13}CO_3$) upon addition of carbonic anhydrase (Figs. 3B and 6). Maintenance of the CO_2/HCO_3^- system out of equilibrium cannot be explained by simple diffusion of CO_2 across the plasma membrane (18–20, 25, 28) but could only occur by the input of energy, presumably to a CO_2 transport system.

The specific and rapid removal of CO_2 by protoplasts cannot be attributed to passive uptake of CO_2 into a more alkaline compartment because the external pH of 8.0 was higher than the intracellular pH values (7.1–7.5) that have been reported (4, 25), and such a mechanism of initial CO_2 removal could not maintain the CO_2/HCO_3^- system out of equilibrium for any length of time. It also cannot be attributed to fixation by Rubisco because with a $K_m(CO_2)$ of 57 μM (5) the activity of this enzyme would be slight at the CO_2 concentration of 0.8 μM and of even less significance at the CO_2 compensation point of 50 to 60 nM CO_2. Moreover, high CO_2 cells without carbonic anhydrase activity (Fig. 1) could not remove CO_2 from the medium (Fig. 2) even though the $K_m(CO_2)$ of Rubisco in these cells is similar to that in low CO_2 cells and the total activity is higher (5).

The location of active CO_2 transport is not known, and results such as those presented would be obtained if a CO_2 transporter(s) was located either on the plasma membrane or the chloroplast envelope or both. The results could also be obtained through the diffusion of CO_2 to the cytoplasm, rapid conversion to HCO_3^-, and active transport of HCO_3^- at the chloroplast envelope if the rate of transport of HCO_3^- at the chloroplast envelope was sufficient to maintain the HCO_3^- concentration in the cytoplasm at a level less than 1 μM. Such a low concentration would be required to obtain a cytoplasmic CO_2 concentration that would maintain the diffusion gradient for CO_2 from the external medium where the CO_2 concentration is about 50 nM. Such a mechanism would require an extremely efficient HCO_3^- transporter in the chloroplast envelope. While we think this mechanism is unlikely, until the capability of the chloroplast for inorganic carbon transport is known, the possibility cannot be ruled out.

The possibility of active CO_2 transport by *Chlamydomonas* was proposed by Marcus *et al.* (13) and Williams and Turpin (29). The latter authors demonstrated that low CO_2 cells treated with AZA accumulated DIC up to 25 times the

external DIC concentration. In terms of CO_2 the accumulation was at least 100-fold the external CO_2 concentration assuming an internal pH of 7.5 (25), internal equilibrium between CO_2 and HCO_3^-, and an external CO_2 content of 0.8 μM. The internal DIC pool reached a maximum value within 30 s after the onset of illumination (29), which is the same as the time course of the rapid CO_2 uptake by AZA-treated cells and protoplasts (Figs. 2, 3, and 6). Thus, the observed CO_2 transport undoubtedly occurred against a concentration gradient. Since there is no reason to assume that the fast CO_2 disappearance of AZA-treated cells is different from that observed with carbonic anhydrase free protoplasts, we conclude from the present data that CO_2 transport is an active uptake mechanism coupled to metabolic energy. Preliminary studies with inhibitors suggest that it is photosynthetic electron flow rather than mitochondrial respiration which is involved in providing the energy for the CO_2 transport (DF Sültemeyer, unpublished results).

The measurement of fluorescence showed that inorganic carbon transport and fixation primarily affected nonphotochemical quenching (q_E) rather than photochemical quenching (q_Q) (Figs. 4, 5, and 6). As expected (11, 24) q_E was smaller when carbon was available for fixation and increased to a high value (0.75) when the inorganic carbon supply was exhausted (Figs. 4, 5, and 6). In the absence (Fig. 6) of carbonic anhydrase, q_E was larger (0.45) than in the presence (Fig. 5) of carbonic anhydrase (0.16) about 2 min after the start of the experiment, indicating that the DIC was not as effectively used when carbonic anhydrase was not present. The decrease in q_E to 0.29 when carbonic anhydrase was added to the protoplasts (Fig. 6) supports this view. When the [CO_2] in the medium was close to zero before the addition of carbonic anhydrase (Fig. 6), q_E only increased to 0.58 and not to 0.75, the value that was observed when all the inorganic carbon had been used (Figs. 4 and 5). This value (0.58) probably reflects not only the use of CO_2 formed from the dehydration of HCO_3^- but also the direct transport and utilization of the HCO_3^- ion. The small changes in photochemical quenching (q_Q) are puzzling. The high values of q_Q (0.54–0.75), even at the CO_2 compensation point, indicate that oxidized Q was available and that there still must exist a substantial flow of electrons from it to an oxidant. In the absence of inorganic carbon, a high rate of O_2 uptake was observed in *C. reinhardtii* (27) suggesting that, in these circumstances, oxygen may be reduced as has been proposed for higher plants (23).

In the absence of carbonic anhydrase, the rates of CO_2 uptake measured with *Chlamydomonas* (Fig. 6) were similar to those reported for cyanobacteria under similar conditions (6, 16). Unfortunately, it is not yet possible to prevent the use of HCO_3^- in *Chlamydomonas*, and the rate of O_2 evolution (83 μmol mg^{-1} Chl h^{-1}) compared to the rate of CO_2 use (16 μmol mg^{-1} Chl h^{-1}) suggests that in *Chlamydomonas* the direct uptake of the HCO_3^- ion contributed about 4 times as much carbon to photosynthesis as did the CO_2. The direct transport of HCO_3^- was also supported by the high rate of O_2 evolution (46 μmol mg^{-1} Chl h^{-1}) that was observed when the [CO_2] had been reduced to close to zero (Fig. 6, just before addition of carbonic anhydrase). Under these conditions the maximum rate of CO_2 supply from the spontaneous dehydration of HCO_3^- (calculated according to Espie *et al.* [6]) could only be responsible for about 29% of the observed rate of O_2 evolution, again suggesting that the transport of HCO_3^- ion supplied the additional carbon. Consequently, while we have directly demonstrated the active transport of CO_2 (Figs. 2, 3, and 6), our results are fully consistent with earlier publications (8, 13, 29) that suggested the active transport of both CO_2 and HCO_3^- in *Chlamydomonas* and other green algae.

In the presence of carbonic anhydrase where CO_2 and HCO_3^- are kept in equilibrium, it is impossible to determine the proportion of photosynthesis sustained by CO_2 transport and that sustained by HCO_3^- transport. In the absence of carbonic anhydrase, when the CO_2 concentration was less than 0.1 μM (Fig. 6, just before the addition of carbonic anhydrase), the maximum rate of CO_2 supply by spontaneous dehydration of HCO_3^- (78 nmol mL^{-1} h^{-1}) could only support 29% of the observed rate of photosynthesis (273 nmol mL^{-1} h^{-1}). In these circumstances, HCO_3^- transport was presumably supplying the remaining carbon for photosynthesis. As has been discussed previously (6), the proportion of CO_2 or HCO_3^- that will be used by the cells for photosynthesis will not be constant but will depend upon the availability of each species. The availability of each species will, in turn, depend upon the DIC concentration, the pH, and the presence or absence of carbonic anhydrase. The proportion of CO_2 or HCO_3^- used will also depend upon whether or not there is a separate transporter for each species and the affinity and maximum capacity of the transporters.

Although Palmqvist *et al.* (21) used a method for measuring DIC uptake that depends upon external carbonic anhydrase maintaining the CO_2/HCO_3^- equilibrium, they and other authors (1, 18, 20, 25, 28, 29) have questioned the essentiality of the external carbonic anhydrase to photosynthesis of low CO_2 grown *Chlamydomonas* and other green algae. Our results show that, indeed, the external carbonic anhydrase was not essential for a measurable rate of photosynthesis (Fig. 6, just before the addition of carbonic anhydrase) and, of course, it would not be required at all in the special case where, because of low cell density or low photosynthetic rates (29), the rate of CO_2 removal from the medium was equal to or less than the rate of spontaneous dehydration of HCO_3^- to CO_2. But, in our experimental conditions, when carbonic anhydrase was added, the rate of photosynthesis was more than doubled (Fig. 6, just after addition of carbonic anhydrase) and the rate of photosynthesis at the beginning of the experiments was 25% higher when carbonic anhydrase was present (Figs. 4 and 5) than when carbonic anhydrase was absent (Fig. 6). These results, then, show that while the external carbonic anhydrase is not essential for photosynthesis of low CO_2 grown *Chlamydomonas*, it is essential for the maintenance of maximum rates of photosynthesis under low DIC conditions by ensuring that both the CO_2 transporter and the HCO_3^- transporter are operating at their maximum capacity under the existing conditions. In the absence of external carbonic anhydrase, the CO_2 transporter could be limited by a lack of substrate so that, as Findenegg (8) stated, the external carbonic anhydrase is "an important factor in the regulation of the use of HCO_3^- and CO_2 for photosynthesis."

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LITERATURE CITED

1. **Aizawa K, Miyachi S** (1984) Carbonic anhydrase located on the cell surface increased the affinity for inorganic carbon in photosynthesis of *Dunaliella tertiolecta.* FEBS Lett **173:** 41–44
2. **Badger MR, Andrews TJ** (1982) Photosynthesis and inorganic carbon usage by the marine cyanobacterium *Synechococcus* sp. Plant Physiol **70:** 517–523
3. **Badger MR, Bassett M, Cairns HN** (1985) A model for HCO_3^- accumulation and photosynthesis in the cyanobacterium *Synechococcus* sp. Plant Physiol **77:** 465–471
4. **Badger MR, Kaplan A, Berry JA** (1980) Internal inorganic carbon pool of *Chlamydomonas reinhardtii*: evidence for a carbon dioxide-concentrating mechanism. Plant Physiol **66:** 407–413
5. **Berry J, Boynton J, Kaplan A, Badger M** (1976) Growth and photosynthesis of *Chlamydomonas reinhardtii* as a function of CO_2 concentration. Carnegie Inst Wash Year Book **75:** 423–432
6. **Espie GS, Miller AG, Birch DG, Canvin DT** (1988) Simultaneous transport of CO_2 and HCO_3^- by the cyanobacterium *Synechococcus* UTEX 625. Plant Physiol **87:** 551–554
7. **Espie GS, Owttrim GW, Colman B** (1984) A comparative study of inorganic carbon transport in photosynthetic cells. *In* C Sybesma, ed, Advances in Photosynthesis Research, Vol III. Martinus Nijhoff, Dr. W. Junk, The Netherlands, pp 681–684
8. **Findenegg GR** (1976) Correlations between accessibility of carbonic anhydrase for external substrate and regulation of photosynthetic use of CO_2 and HCO_3^- by *Scenedesmus obliquus.* Z Pflanzenphysiol **79:** 428–437
9. **Kaplan A, Badger MR, Berry JA** (1980) Photosynthesis and inorganic carbon pool in the blue green alga *Anabaena variabilis*: response to external CO_2 concentration. Planta **149:** 219–226
10. **Kimpel DL, Togasaki RK, Miyachi S** (1983) Carbonic anhydrase in *Chlamydomonas reinhardtii* I. Localization. Plant Cell Physiol **24:** 255–259
11. **Krause GH, Vernotte C, Briantais J-M** (1982) Photoinduced quenching of chlorophyll fluorescence in intact chloroplasts and algae: resolution into two components. Biochim Biophys Acta **679:** 116–124
12. **Kreuzberg K, Klöck G, Grobheiser D** (1987) Subcellular distribution of pyruvate-degrading enzymes in *Chlamydomonas reinhardtii* studied by an improved protoplast fractionating procedure. Physiol Plant **69:** 481–488
13. **Marcus Y, Volokita M, Kaplan A** (1984) The location of the transporting system for inorganic carbon and the nature of the form translocated in *Chlamydomonas reinhardtii.* J Exp Bot **35:** 1136–1144
14. **Miller AG, Canvin DT** (1985) Distinction between HCO_3^- and CO_2-dependent photosynthesis in the cyanobacterium *Synechococcus leopoliensis* based on the selective response of HCO_3^- transport to Na^+. FEBS Lett **187:** 29–32
15. **Miller AG, Colman B** (1980) Active transport and accumulation of bicarbonate by a unicellular cyanobacterium. J. Bacteriol **1143:** 1253–1259
16. **Miller AG, Espie GS, Canvin DT** (1988) Active transport of CO_2 by the cyanobacterium *Synechococcus* UTEX 625. Plant Physiol **86:** 677–683
17. **Miller AG, Turpin DH, Canvin DT** (1984) Growth and photosynthesis of the cyanobacterium *Synechococcus leopoliensis* in HCO_3^- limited chemostats. Plant Physiol **75:** 1064–1070
18. **Moroney JV, Husic HD, Tolbert NE** (1985) Effect of carbonic anhydrase inhibitors on inorganic carbon accumulation by *Chlamydomonas reinhardtii.* Plant Physiol **79:** 177–183
19. **Moroney JV, Kitayama M, Togasaki RK, Tolbert NE** (1987) Evidence for inorganic carbon transport by intact chloroplasts of *Chlamydomonas reinhardtii.* Plant Physiol **83:** 460–463
20. **Moroney JV, Tolbert NE** (1985) Inorganic carbon uptake by *Chlamydomonas reinhardtii.* Plant Physiol **77:** 253–258
21. **Palmqvist K, Sjöberg S, Samuelsson G** (1988) Induction of inorganic carbon accumulation in the unicellular green algae *Scenedesmus obliquus* and *Chlamydomonas reinhardtii.* Plant Physiol **87:** 437–442
22. **Schlösser UG, Sachs H, Robinson DG** (1976) Isolation of protoplasts by means of a species specific autolysin in *Chlamydomonas reinhardtii.* Protoplasma **88:** 51–64
23. **Schreiber U, Bilger W** (1985) Rapid assessment of stress effects on plant leaves by chlorophyll fluorescence measurements. Presented at NATO Advanced Research Workshop, Sesimbra, Portugal (Paper supplied by H. Walz, D-8521 Effeltrich, FRG)
24. **Schreiber U, Schliwa U, Bilger W** (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth Res **10:** 51–62
25. **Spalding MH, Portis AR** (1985) A model of carbon assimilation in *Chlamydomonas reinhardtii.* Planta **169:** 308–320
26. **Sültemeyer DF, Klöck G, Kreuzberg K, Fock HP** (1988) Photosynthesis and apparent affinity for dissolved inorganic carbon by cells and chloroplasts of *Chlamydomonas reinhardtii* grown at high and low CO_2 concentrations. Planta **176:** 256–260
27. **Sültemeyer DF, Klug K, Fock HP** (1987) Effect of dissolved inorganic carbon on oxygen evolution and uptake by *Chlamydomonas reinhardtii* suspension adapted to ambient and CO_2-enriched air. Photosynth Res **12:** 25–33
28. **Tsuzuki M** (1983) Mode of HCO_3^--utilization by the cells of *Chlamydomonas reinhardtii* grown under ordinary air. Z Planzenphysiol **110:** 29–37
29. **Williams TG, Turpin DH** (1987) The role of external carbonic anhydrase in inorganic carbon acquisition by *Chlamydomonas reinhardtii* at alkaline pH. Plant Physiol **83:** 92–96