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Role of Photosynthetic Reactions in the Activity of Carbonic Anhydrase in *Synechococcus* sp. (UTEX 2380) in the Light[1]

INHIBITOR STUDIES USING THE ^{18}O-EXCHANGE IN $^{13}C/^{18}O$-LABELED BICARBONATE

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ABSTRACT

The role of the photosystems in the exchange of ^{18}O between species of inorganic carbon and water was studied in suspensions of the cyanobacterium *Synechococcus* sp. (UTEX 2380) using membrane-inlet mass spectrometry. This ^{18}O exchange is caused by the hydration-dehydration cycle of CO_2 and is catalyzed by carbonic anhydrase. We observed the complex ^{18}O exchange kinetics including dark-light-dark transients in suspensions of whole cells and found these to be identical to the ^{18}O exchange kinetics of physiologically fully active spheroplast preparations. There was no enhancement effect of inorganic nitrogen on inorganic carbon accumulation. Membrane preparations exhibited no uptake of inorganic carbon and very little carbonic anhydrase activity, although these membranes were photosynthetically fully competent. DCMU, the inhibitor of photosystem II, eliminated almost entirely the ^{18}O exchange activity of whole cells in the light. But this effect of DCMU could be reversed by addition of the electron donor couple 3,6-diaminodurene/ascorbate, suggesting the involvement of photosystem I in the events leading to ^{18}O exchange. Iodoacetamide, an inhibitor of CO_2 fixation, enhanced the ^{18}O exchange in whole cell suspensions and inhibited neither the uptake of inorganic carbon nor the dehydration of bicarbonate in the light. The proton carrier carbonylcyanide *m*-chlorophenylhydrazone and the inhibitors diethylstilbestrol and *N,N'*-dicyclohexyl carbodiimide affecting the membrane potential, totally abolished ^{18}O exchange in the light. From ^{18}O-labeled inorganic carbon experiments we conclude that one of the roles of photosystem I is to provide the active uptake of inorganic carbon into the cells, where carbonic anhydrase catalyzes the interconversion between CO_2 and HCO_3^- resulting in the ^{18}O exchange from inorganic carbon to water.

CA[2] catalyzes the reaction between CO_2 and HCO_3^- at high rates in plant and animal systems (11, 16, 17). Although this enzyme was detected and partially characterized in a variety of lower and higher plants (3, 6, 8), its function in photosynthetic organisms is still unresolved, partly because the enzyme found in cyanobacteria and many other plant systems exhibits lower overall activity as compared to enzymes from animal systems (2). The possible association of CA with Rubisco in CO_2 fixation (6) raises still another question, since the pH environment of Rubisco is even higher than pH 8, an environment, where most of the inorganic carbon exists as HCO_3^-. One might conclude that under these slightly alkaline conditions the main reaction for the generation of CO_2 would be the dehydration of HCO_3^-, provided the bulk of inorganic carbon is present as bicarbonate. If this assumption is correct, the question arises of how the photosynthetic cell manages to continuously produce an excess of CO_2 in an alkaline environment. Although the alkalization of the medium during photosynthesis was previously reported for another cyanobacterium *Coccochloris* (13), the participation of the photosystems in this reaction needs to be explored.

We have used the ^{18}O-exchange of labeled bicarbonate ($H^{13}C^{18}O_3^-$) to follow the activity of carbonic anhydrase in light-dark transients of *Synechococcus*. We have further used photosynthetically active spheroplast preparations of *Synechococcus* and membrane preparations directly derived from these spheroplasts to clarify the role of the pool of inorganic carbon in the complex mass-spectra obtained with whole cell preparations. We demonstrate that carbonic anhydrase activity is observed when inorganic carbon is accumulated in *Synechococcus* in a process supported by PSI. This CA activity can be detected even in whole cells under conditions when CO_2 fixation is blocked and electron transport is uncoupled by methylamine. The role of rapid pH changes taking place in dark-light-dark transients and their effects on the pools of inorganic carbon as discussed. Inhibitors of energy transduction blocked both the uptake of inorganic carbon and the observable ^{18}O exchange catalyzed by the *Synechococcus* cells.

MATERIALS AND METHODS

Organism and Growth Conditions. *Synechococcus* sp. (UTEX 2380) is a marine, unicellular, nonnitrogen fixing cyanobacterium that was obtained from the University of Texas Culture Collection. Cells were grown as described previously (19), except that tricine buffer (from Sigma) was substituted for bicine at pH 8.2.

Preparation of Spheroplasts and Assay for Photosynthetic Oxygen. To obtain fragile spheroplasts of *Synechococcus*, the original method developed for *Anabaena* (18) was modified to increase sorbitol to 0.65 M. The incubation medium for lysozyme digestion of the cells contained tricine 10 mM, phosphate 5 mM, $MgCl_2$ 5 mM, and lysozyme 2 mg/ml, and the pH was 7.8. Cells were incubated at a cell concentration of 70 μl pcv per ml, at 30°C. The progress of lysozyme digestion was followed over time by periodically taking aliquots of 1 ml, packing the cells by centrifugation at low speed, and resuspending the packed cells immediately in reaction medium. The latter consisted of 10 mM tricine at pH 7.8, including 0.05% BSA, 5 mM phosphate. Mg^{2+}

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[2] Abbreviations: CA, carbonic anhydrase; C_i, inorganic carbon (CO_2 + HCO_3^- + CO_3^{2-}); ClCCP, carbonylcyanide *m*-chlorophenylhydrazone; DAD, 3,6-diaminodurene; DES, diethylstilbestrol; DCCD, *N,N'*-dicyclohexyl carbodiimide, DMBQ, 2,6-dimethyl-benzoquinone; EZ, ethoxzolamide; Fecy, ferricyanide; IA, iodoacetamide; MA, methylamine; pcv, packed cell volume; Rubisco, ribulose 1,5-biphosphate carboxylase; W-A, Wilbur-Anderson units.

ion was added at 10 mM before initiating photosynthetic O_2 evolution. O_2 and pH measurements were carried out simultaneously using an O_2 and pH electrode fitted in a transparent tube holding 10 ml of cell suspension and kept in a temperature-controlled water bath. O_2 evolution was followed in the absence and in the presence of ferricyanide (3 mM). The extent of cell lysis after incubation with lysozyme was followed as the release of phycocyanin, monitored as the absorbance increase at 620 nm in the supernatant after osmotic shock (10), and by monitoring O_2 evolution of lysozyme-treated cells with Fecy as electron acceptor. Cell lysis was complete when both phycocyanin release and O_2 evolution reached a maximum, indicating full access of ferricyanide to membranes. Prolonged incubation beyond the maximum for O_2 evolution did not increase phycocyanine release further, but rather resulted in lower O_2 evolution rates due to further cell and membrane lysis. Incubation of whole cells or spheroplasts in isotonic assay medium in the electrode chamber usually required the inclusion of DMBQ to attain maximum activity of O_2 evolution. DMBQ serves as an electron carrier shuttle, capable of crossing the plasmalemma. For oxygen depletion experiments and for photosynthetic reactions a 1000 W slide projector was used, whose light was passed through a red filter of 2 mm thickness, with a cutoff (89% T) at 610 nm. Incident light intensity on the reaction vessel was 500 to 600 $\mu E \times m^{-2} \times s^{-1}$. The packed cell volume of the assay suspension varied from 4 to 12 μl pcv/ml depending on the type of experiment.

^{18}O-Exchange Method. This method was extensively described in a previous communication (19), and uses a mass spectrometer with a gas-permeable membrane inlet to measure the ^{18}O content of CO_2 in solution as well as in a suspension of cells. The measured variable is the ^{18}O content of CO_2, in our case $^{13}CO_2$. We used ^{13}C-containing CO_2 so as not to be susceptible to errors arising from the CO_2 generated in the cells by photorespiration which would contain the naturally predominant ^{12}C (natural abundance 98.9%).

$$^{18}O \text{ atom fraction in } {}^{13}CO_2 = \frac{(^{13}C^{16}O^{18}O) + 2(^{13}C^{18}O^{18}O)}{2[(^{13}C^{16}O^{16}O) + (^{13}C^{16}O^{18}O) + (^{13}C^{18}O^{18}O)]} = \frac{(47) + 2(49)}{2(45 + 47 + 49)}$$

where 45, 47, 49 are the heights of the corresponding mass peaks. The ^{18}O content of CO_2 and HCO_3^- in solution decreases with time because of the hydration-dehydration cycle:

$$^{13}C^{18}O^{18}O + H_2{}^{16}O \rightleftharpoons H^{13}C^{18}O^{18}O^{16}O^- + H^+ \rightleftharpoons {}^{13}C^{18}O^{16}O + H_2{}^{18}O$$

The ^{18}O appearing in water is very greatly diluted by $H_2{}^{16}O$ (natural abundance of ^{16}O is 99.76%). The rate of the ^{18}O exchange between CO_2 and water is enhanced by carbonic anhydrase which catalyzes the reaction. The decrease in ^{18}O atom fraction of $^{13}CO_2$ is a first-order kinetic process with a rate constant Θ_{13} in a homogeneous solution (16, 17).

When we applied this method using whole cells, values of Θ_{13} in cell suspensions during the three major phases of the light period were compared to Θ_{13} of the uncatalyzed reaction. The ratio of the catalyzed to the uncatalyzed rate gives an estimate of the ^{18}O exchange activity, and hence the carbonic anhydrase activity, under the conditions used.

Carbonic Anhydrase Assay, CO_2 Hydration. The activity of CA was measured by the Wilbur-Anderson electrometric method (20). To the reaction mixture of 7.5 ml veronal buffer 15 mM (pH 8.3), 0.1 to 0.2 ml spheroplast suspension of *Synechococcus* was added in a closed reaction vessel that was kept at 2°C in a thermo-controlled bath. The reaction was started by injecting 1.5 ml of CO_2-saturated water kept also at 2°C. The activity of CA was determined measuring the time required for a pH change from 8.3 to 7.0 with heated and nonheated spheroplast material. Enzyme activity was calculated in W-A units:

$$\text{Activity} = \frac{T_{Co} - T_S}{T_S} \times 10$$

where T_{Co} represents the time recorded for the uncatalyzed pH change, T_S the time taken for the fresh sample reaction to effect the same pH change.

RESULTS

^{18}O-Exchange Activity in Whole Cells in Light, Effect of Inorganic Nitrogen. In seawater at pH 8.3, the amount of free CO_2 is estimated at about 0.5% of the total carbon present (1). In order to maintain the observed high rates of CO_2 fixation (*e.g.* 200 μmol × mg Chl^{-1} × h^{-1}) marine cyanobacteria such as *Synechococcus* need to use bicarbonate as the carbon source for CO_2 fixation as previously reported for *Coccochloris* (13). Our previous results using ^{18}O-labeled $H^{13}CO_3^-$ suggested some uptake of inorganic carbon (19). Under natural growth conditions, however, either ammonia or nitrate would be present. Since cyanobacteria store excess organic nitrogen in phycobiliproteins, the question was investigated whether the addition of ammonia or nitrate enhanced the accumulation of external inorganic carbon inside the cell also in *Synechococcus* (Figs. 1 and 2). All samples were preilluminated to minimize the dilution of ^{13}C label by endogenous C_i.

Measurements were made using suspensions containing ammonium ions (Fig. 1) or nitrate ions (data not shown). Under the conditions employed (a photosynthetic rate of 200 μmol O_2 evolved per ml pcv per h in the steady state of photosynthesis and an initial photosynthetic uptake of C_i near 800 μmol per ml

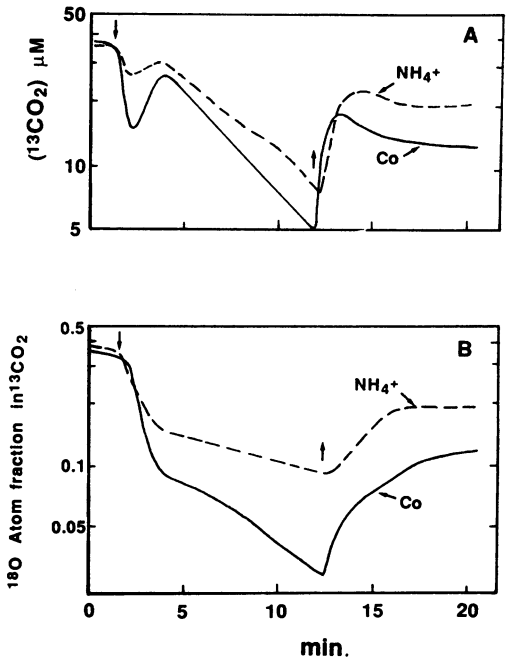

FIG. 1. Transient kinetics of inorganic carbon uptake and ^{18}O exchange kinetics of labeled CO_2 in whole cell suspensions of *Synechococcus*. Midlog phase, air-grown cells were washed and resuspended in ASN III medium, buffered with tricine 5 mM, phosphate 5 mM (pH 8), and after preillumination, transferred to the reaction vessel; pcv was 6 μl/ml. HCO_3^- added was 1 mM. A, $^{13}CO_2$ uptake and efflux; B, ^{18}O atom fraction in $^{13}CO_2$; (——) Co, control, no inorganic N addition; (- - -), ammonia 3 mM added; (↓), light on; (↑), light off.

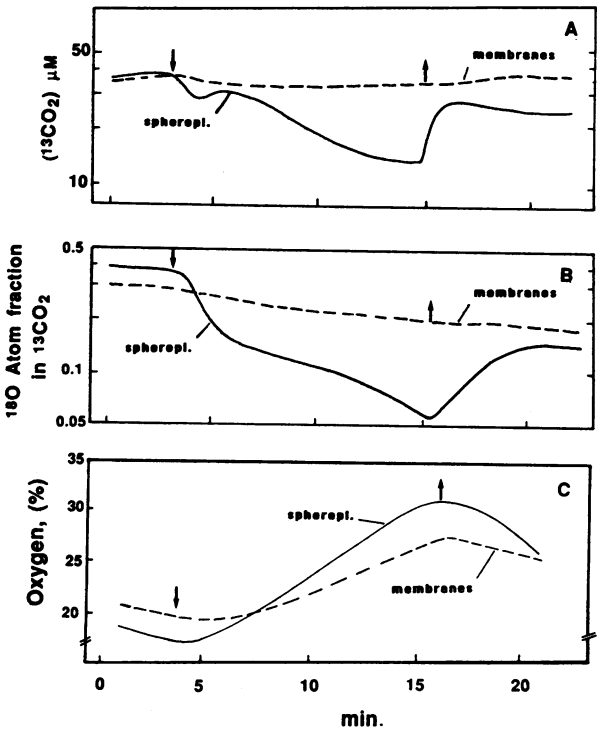

FIG. 2. Transient kinetics of inorganic carbon uptake and ^{18}O exchange of spheroplast preparations and membranes derived from spheroplasts of *Synechococcus*. Spheroplasts and membranes were prepared as described in "Materials and Methods." HCO_3^- 0.5 mM added, pcv at 6.8 μl/ml; the reaction medium was either isotonic for intact spheroplasts (0.65 M sorbitol), or hypotonic to produce osmotically shocked spheroplasts in the reaction medium, containing 10 mM tricine buffer (pH 7.8), phosphate 5 mM, and $MgCl_2$ 10 mM. A, Total $^{13}CO_2$; B, ^{18}O atom fraction in $^{13}C^{18}O_2$; C, oxygen evolution with Fecy at 3 mM as electron acceptor. (——), Spheroplasts; (– – –), membranes; (↓), light on; (↑), light off.

pcv per h was measured), an estimated 0.36 μmol/ml inorganic carbon or 36% of the C_i present was taken up and/or fixed during the experimental time period. These data were independently confirmed using ^{14}C-bicarbonate (20).

In contrast to samples containing inorganic nitrogen, the control, which was devoid of inorganic nitrogen, exhibited a rather distinct peak of CO_2 efflux right after the CO_2 uptake phase (Fig. 1A). In the nitrate case kinetic patterns of ^{18}O exchange almost identical to Figure 1 were observed. Also, the suspensions containing ammonia and nitrate showed almost identical patterns of oxygen evolution. In these experiments we observed that the magnitude of changes in $^{13}CO_2$ concentration and in ^{18}O atom fraction in $^{13}CO_2$ upon turning the light on and off were greater for the control than for the ammonium containing suspensions. These conclusions were confirmed quantitatively by comparing the rate constants Θ_{13} for each of these regions. The Θ_{13} values of the linear part of the steady state light period appeared to be clearly greater in the control sample (Fig. 1B). In the dark, for both NH_4^+-containing sample and for the control, the ^{18}CO exchange rate was very close to the uncatalyzed rate.

Upon turning the light off there was a rapid increase of both $^{13}CO_2$ concentration and ^{18}O atom fraction measured in the suspending solution (Fig. 1). This kinetic phase has been shown to be dominated by the uncatalyzed return to equilibrium outside the cells with $HC^{18}O_3^-$ being converted to $C^{18}O_2$ (19). Overall the presence of inorganic N did not enhance the accumulation of external C_i inside the cells.

Uptake of C_i by Spheroplasts and by Membranes Derived from Spheroplasts of *Synechococcus*. The interpretation of mass-spectra of species of CO_2 in whole cell suspensions is complicated by the presence of an ever changing internal pool of C_i, and the rapid changes of pH on each transition from dark to light and back to dark. Also when considering possible locations for the pool of C_i, both the cytosol and the intrathylakoid space are candidates for the movement and deposition of inorganic carbon. In order to evaluate these possibilities, known methods of spheroplast preparation were modified to prepare physiologically active spheroplasts and intact photosynthetic membranes (15, 18). The use of spheroplasts in place of whole cells has the advantage that in an isotonic or slightly hypertonic medium, material with partially digested cell walls still contains intact membranes with functional ion uptake mechanisms. On the sudden dilution in a hypotonic medium, however, these spheroplasts rupture, possibly also losing the pools of inorganic carbon. The thylakoids of osmotically shocked spheroplasts of cyanobacteria, however, are photosynthetically fully active (15, 19).

The progress of the lysozyme digestion of *Synechococcus* cells was followed by measuring O_2 evolution with Fecy as electron acceptor, and by monitoring phycocyanine release into the supernatant after osmotic shock. Intact cells require Fecy and the further addition of the lipid-soluble electron carrier DMBQ to attain a maximum rate of O_2 evolution. Broken cells of *Synechococcus* release soluble phycocyanine similar to *Anabaena* (10). After 70 min lysozyme incubation more than 90% of the cells had been converted to spheroplasts, characterized by high oxygen evolution rates after osmotic shock in the presence of Fecy. This material was used to determine the uptake of inorganic carbon and the O_2 evolution properties of spheroplasts and of photosynthetic membranes derived from spheroplasts (Fig. 2, A–C).

The mass-spectrum kinetics of dark/light/dark transients of spheroplasts (Fig. 2, A and B) are very similar to the ones obtained with whole cell preparations (Fig. 1). Inorganic ^{13}C uptake, $^{13}CO_2$ production in the light, and the return to chemical equilibrium upon transition to the dark was nearly the same. Fecy addition did not alter the kinetic patterns of the mass spectra of spheroplasts in hypertonic medium, indicating that the partial digestion of the exterior cell wall had hardly impaired the CO_2-exchange properties of the cells.

Shocked spheroplasts preparations, containing mainly photosynthetic and respiratory membranes, exhibited only a negligible change of the $^{13}CO_2$ concentration upon turning the light on or off (Fig. 2A). An identical membrane preparation tested in the same manner but lacking Fecy showed no oxygen evolution. This result suggests that CO_2 was not reduced due to the dilution of the intracellular components of the CO_2 fixation system by osmotic shock. The ^{18}O exchange by membranes, although slightly greater than the uncatalyzed rate, also did not show major differences between light and dark at the pH and under the conditions employed (Fig. 2B). The substitution of the natural electron acceptor CO_2 by ferricyanide however, resulted in a considerable rate of O_2 evolution by membranes, amounting to ~60% of the rate obtained with intact spheroplasts, indicating that the thylakoids were photosynthetically fully active (Fig. 2C).

These kinetics suggest that little if any C_i was taken up by membranes of *Synechococcus*. Furthermore no efflux of CO_2 from membranes was observed, when the light-dark controlled pH of the cytosol was replaced with a buffered medium lacking pools of inorganic carbon.

Effect of the Inhibitor of Photosystem II, DCMU, on the ^{18}O-Exchange Kinetics. Since the ^{18}O exchange between species of CO_2 and water in suspensions of cells and spheroplasts is enhanced by light, it is relevant to determine which of the two photosystems is involved. The presence of DCMU, an inhibitor of PSII, not only blocked the uptake of inorganic carbon, but

also inhibited nearly all of the catalyzed ^{18}O exchange (data not shown), leaving an ^{18}O exchange rate similar to the uncatalyzed rate. Energization of membranes and electron flow from PSII seemed to be a prerequisite for enhanced ^{18}O exchange, resulting from either (a) the active uptake of C_i into the cells or (b) the activation by light of the activity of CA in the cells. This is clearly evident from the steady state phase of the light period, which displayed a constant rate for Θ_{13} before the light was turned off (Fig. 1B).

To investigate further, we added to whole cell suspensions the artificial electron carrier DAD, which is capable of traversing biological membranes, and ascorbate to keep DAD reduced. In such an experiment, inorganic carbon seemed to be taken up initially in the light (Fig. 3A), but then was released back to the medium resulting in much less net uptake of C_i than in the control. On the other hand, the ^{18}O exchange pattern for the PSII-inhibited and the control sample were nearly identical (Fig. 3B). The implication of this observation is that the uptake of C_i to the site of intracellular CA can be driven by PSI alone. In fact, the data suggest that PSI can do this as efficiently as both photosystems, provided a suitable electron donor can feed electrons into PSI.

It seems therefore that the ^{18}O exchange, characteristic of C_i uptake and CA activity, can operate independent of the electron flow originating from the inner thylakoid region, since DCMU is known to completely block electron flow from PSII.

Effect of IA on C_i Utilization in Dark-Light Transients of CO_2 in *Synechococcus*. Iodoacetamide is known to block pathways to CO_2 fixation (14). In a previous publication (19) we have reported that IA, by blocking CO_2 fixation, causes an intracellular accumulation of CO_2 which passes out of the *Synechococcus* cells causing an increase of the CO_2 concentration in the external medium. In the following experiments we set different goals for the effects of IA, namely to obtain a complete dark-light-dark cycle (Fig. 4) in order to simulate a closed C_i system without the complex reactions of CO_2 assimilation. We further monitored the evolution of O_2 simultaneously with pH changes to see whether the dehydration of bicarbonate proceeded without O_2 generation (Fig. 4). MA was also used in order to induce the uncoupling of photosynthetic electron transport and to study the effect of a reduced pH change in the light on the C_i equilibrium. The cells were preilluminated before the experiment to deplete the pools of internal C_i.

^{13}C-carbon was taken up by the cells in the presence of IA in the light (Fig. 4b), however, labeled CO_2 diffused out of the cells into the surrounding medium reaching a concentration level much higher than the equilibrium concentration of CO_2 at start of the light period. When the light was turned off, the level of free CO_2 gradually returned to its equilibrium concentration. The effect of CO_2 production and loss to the medium was even more increased, almost doubled, by including MA at 8 mM, but the final equilibrium level of CO_2 at the end of the second dark period was the same as without amine uncoupler.

The kinetics of the ^{18}O exchange exhibited three major phases in all samples: phase 1, the uptake of C_i; phase 2, the steady state light period; phase 3, the reequilibration in the dark (Fig. 4, a, b, and c). In the steady state light period only the samples with inhibitors showed a steady loss of ^{18}O, with the MA sample exhibiting the greatest loss of ^{18}O label, much larger than the ^{18}O exchange in the dark, which was very close to the uncatalyzed rate.

Since *Synechococcus* is known to undergo distinct pH changes during dark-light shifts similar to pH changes in *Plectonema* (12), it was of interest to check oxygen evolution and pH changes in the absence and in the presence of the inhibitors IA and MA (Fig. 5). The samples were again preilluminated and 0.5 mM bicarbonate was added in the dark after the addition of inhibitor. Net O_2 was evolved in significant amounts only in the control sample, following the initial extrusion of protons after 30 s. Surprisingly, the sample with IA also exhibited a pronounced pattern

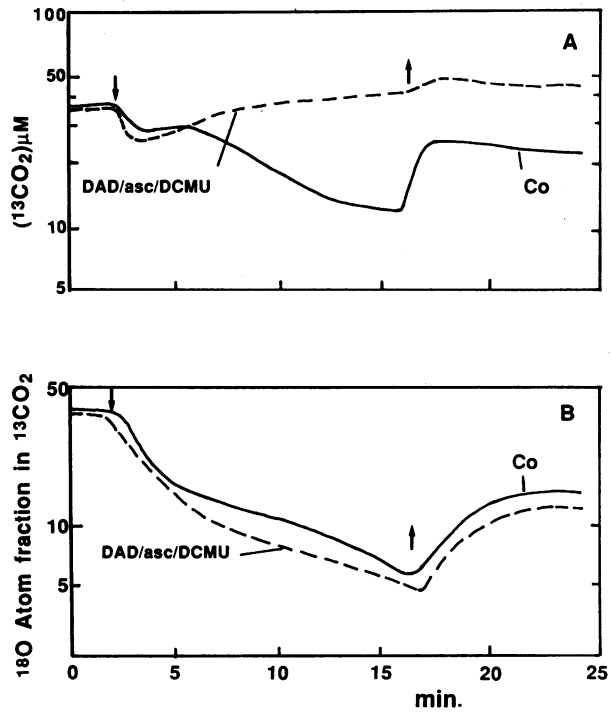

FIG. 3. Uptake and ^{18}O exchange kinetics of $H^{13}C^{18}O_3^-$ in the presence of DCMU in whole cells of *Synechococcus*. ASN III medium, buffered with tricine 10 mM, phosphate 4 mM (pH 7.8); pcv at 8 μl/ml. Labeled bicarbonate, 1 mM; DCMU, 10 μM; DAD, 40 μM; Na^+ ascorbate 10 mM. A, Uptake and efflux of $^{13}CO_2$; B, ^{18}O exchange of $H^{13}C^{18}O_3^-$; (——) Co, control, no addition; (– – –), plus DCMU, DAD/ascorbate; (↓), light on; (↑), light off.

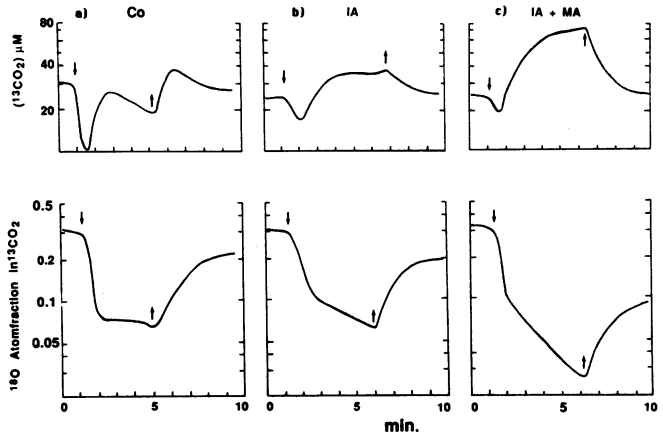

FIG. 4. Effect of IA and MA on inorganic carbon uptake and ^{18}O exchange kinetics in dark-light transients of whole cells of *Synechococcus*. An air-grown culture of pcv 18 μl/ml and medium pH of 8.25 was washed and resuspended in ASN III medium, buffered with tricine 12 mM, phosphate 12 mM at pH 7.75. To this assay medium, cells were added to 25 μl/ml assay, and HCO_3^- 1 mM. Samples were preilluminated for IC depletion for 25 min. a, Co, Control, no further addition; b, addition of IA; 2.5 mM; c, addition of 2.5 mM IA and 8 mM MA; (↓), light on; (↑), light off.

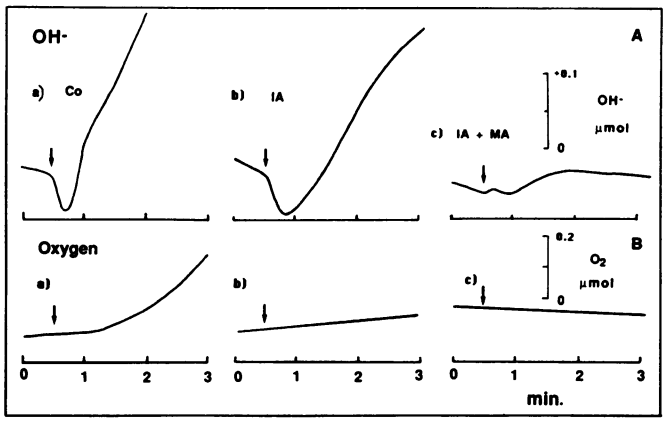

FIG. 5. Effect of IA and MA on bicarbonate-dependent pH change and O_2 evolution, monitored simultaneously, in whole cell suspensions of *Synechococcus*. Midlog phase cells of pcv 2 were washed twice and resuspended in seawater in the absence of buffer, supplemented with 0.5 mM phosphate (pH 7.8) and KCl 20 mM. Cell concentration pcv 8 μl/ml. A, OH^- generation; B, O_2 evolution. Samples: a, Co, control, no addition; b, addition of 5 mM IA; c, addition of 5 mM IA; and 8 mM MA; (↓), light on.

of proton translocation, followed by a high rate of steady alkalization of the medium for 3 to 5 min, with only a minimal rate of O_2 evolved that tapered off after about 3 min. The third sample, containing MA and IA, experienced a severe reduction of both phases of the pH change, with no net OH^- production apparent after 2 min. Neither was any net O_2 generated. This latter sample also showed a slight rise in pH after turning the light off, indicating that the internal pH of the cells during illumination was actually higher than that of the external medium. It seems that the dehydration of HCO_3^-, monitored by its product OH^-, can operate without the concomitant evolution of oxygen, even in whole cells. The internal pH of the sample containing IA was determined to be 8.4 ± 0.1 in the light using a method described previously (12), which is close to the internal pH of the cells without inhibitor in the light (pH 8.6).

Activity of CA in Cell Lysates and in Whole Cells of *Synechococcus* using the CO_2 Hydration Assay and the ^{18}O Exchange. The overall low activity of CA in *Anabaena* prompted other investigators to question the function of CA *in vivo* in some cyanobacteria (7). We studied this aspect by measuring the hydration of CO_2 (20), using fragile spheroplast preparations of *Synechococcus*. Our standard conditions employed 3 to 6 μg Chl equivalent of spheroplast suspension per 7.5 ml veronal buffer (12 mM) of pH 8.3. The addition of 1.5 ml CO_2-saturated water resulted in a pH change of >0.5 units over a 5 min time period. The inclusion of 5 μg Chl/ml of spheroplasts cut the reaction time in half, yielding 10 W-A units (applying the formula in "Materials and Methods"). Following calibration with 0.01 N HCl, 1 μmol H^+ was released in this process. Since for each mole of CO_2 hydrated 1 mol H^+ is generated and taking into account the amount of Chl used (50 μg Chl), the catalyzed rate of CO_2 hydration amounted to 480 μmol mg^{-1} Chl h^{-1}. The same material exhibited a photosynthetic rate of 150 μmol O_2 evolved mg^{-1} Chl h^{-1}. Therefore under appropriate conditions of C_i accumulation, the rate of CO_2 generation is not limiting. Badger *et al.* (2) reported CA activity of *Synechococcus*, using presumably homogenate fractions and obtaining CA activity of 43 and 52 W-A units per mg Chl. Using spheroplasts of *Synechococcus* and checking CA activity as a function of Chl concentration, we obtained a maximum specific activity for CA of 170 W-A units per mg Chl, which is close to values reported for *Anabaena* M3 (21).

The ^{18}O exchange with water of labeled bicarbonate monitors the complete hydration-dehydration cycle of CO_2 and HCO_3^- under equilibrium conditions. This method was applied to both cell lysates and whole cell suspensions. Using cell lysate equivalent to 100 μl pcv per assay, the ^{18}O exchange reached a maximum with 10 mM bicarbonate at pH 7.69, with $\theta_{13} = 3 \times 10^{-3} \times s^{-1}$. The ratio of the catalyzed to uncatalyzed rate was 7.2 under these conditions, resulting in enhancement of the CO_2/HCO_3^- interconversion by a factor of 72/ml pcv. The ^{18}O exchange of cell lysates was not enhanced by illumination of the lysate.

The rate of ^{18}O loss from ^{18}O-labeled bicarbonate was experimentally obtained from the steady state of the photosynthetic light period, using whole cells. θ_{13} approached the value of $2 \times 10^{-3} \times s^{-1}$ under CO_2 fixing conditions and using a cell density of 5 μl pcv/ml. This value represents a factor of 4.8 for the speeding up of the uncatalyzed rate at the pH given. Since this activity, in the absence of external CA, has to be placed into the internal cell volume, and further taking into account the amount of cells or Chl used, the interconversion between CO_2 and HCO_3^- was increased by a factor of 960 over the uncatalyzed rate in the cells. These data are consistent with the higher values (factor 1000) for CA of a previous model for *Synechococcus* (2).

Effect of Mediators and Inhibitors of Electron Transport and Phosphorylation on the Exchange of ^{18}O in Whole Cells of *Synechococcus*. The intracellular action of CA can be further characterized by the effect of mediators and inhibitors of photosynthetic reactions on the light-dependent ^{18}O exchange (Table I). Rates of the ^{18}O-exchange were taken from the late steady state in the light, when the dehydration of bicarbonate resulted in a constant rate of O_2 evolution concomitant with OH^- excretion into the medium (phase 2 of the light period). It should also be noted that the uncatalyzed rate, measured as the exchange of ^{18}O, increases with lower pH. IA addition in all cases resulted in a considerable increase in ^{18}O exchange rate. MA, at the concentration used, acted as an uncoupler of electron transport and further enhanced the rate. Ethoxzolamide effectively decreased the activity of carbonic anhydrase, but only to a level of just over 50% of the control. DCMU at low concentrations completely eliminated all activity, indicating that the uptake of C_i and subsequent catalyzed ^{18}O exchange was dependent on linear photosynthetic electron transport. When PSII was blocked, the electron donor couple DAD-ascorbate reversed the inhibition of the ^{18}O exchange, indicating that the uptake of C_i and ^{18}O exchange activity can be driven by PSI alone. The ^{18}O-exchange activity is inhibited by ClCCP, since photosynthesis is mediated by proton gradients across membranes and ClCCP acts as an artificial proton carrier, short-circuiting existing proton gradients. Both DES and DCCD are known to decrease or eliminate the membrane potential of biological membranes (4), and they effectively also inhibited the ^{18}O exchange at low concentrations of inhibitor by more than 90%.

DISCUSSION

An extensive literature deals with the accumulation of C_i in plant systems (3, 8–10). Whereas the existence of large pools of C_i in plant cells is undisputed, the nature of these pools and their mobilization is almost unknown. Furthermore, even the role of the key enzyme in C_i conversion, CA, has been widely underestimated since comparably low levels of activity were detected (7). While the activity of CA in the dark is confined to the reversible hydration of CO_2, the association of CA with photosynthetic processes has remained more or less unexplored. In a marine, highly saline environment of high pH, the bulk of C_i exists as HCO_3^-, and it is conceivable that conversion mecha-

Table I. *Effect of Mediators and Inhibitors of Electron Transport and Phosphorylation on ^{18}O Exchange in Whole Cells of Synechococcus*

Conditions were as described in "Materials and Methods," pH and rate readings were taken from values obtained in the late steady state phase in the light. IA, 3 mM; ClCCP, 20 μM; DCMU, 10 μM; DAD, 50 μM, sodium ascorbate, 10 mM; DCCD, 30 μM; DES, 20 μM; ethoxzolamide, 40 μM; Fecy, 3 mM; DMBQ, 10 μM; MA, 8 mM; cat, catalyzed; uncat, uncatalyzed.

Addition	pH	$(\Theta_{13})_{cat} \times 10^3\ s^{-1}$	Activity cat/uncat	Inhibition
			Ratio	*%*
None	7.56	1.66	3.18	0
IA	7.97	1.69	6.76	0
IA, methylamine	7.57	2.84	5.45	0
Fecy, DMBQ	7.81	1.04	3.01	5
Ethoxzolamide	7.58	1.08	2.06	51
DCMU	7.80	0.24	0.77	100
DCMU, DAD/asc.	7.80	1.01	2.93	0
DES	7.94	0.23	0.75	100
DCCD	7.85	0.46	1.47	90
ClCCP	8.02	0.27	1.12	97

nisms involving energized membranes developed to rapidly convert the bulk of HCO_3^- into CO_2, the only form usable for Rubisco.

In plant systems which include cyanobacteria inorganic nitrogen is often a limiting growth nutrient and amino nitrogen is usually stored in the form of phycobiliproteins. In *Synechococcus* an enhancement by ammonia of the accumulation of external C_i into the cells was not observed, different from processes in red cells (11) and the ^{18}O-exchange in the light was even smaller in the samples with inorganic nitrogen. In a tightly coupled photosynthetic system, fixed CO_2 might be assimilated as soon as it was generated from HCO_3^-, resulting in low rates of ^{18}O loss.

The pool size of inorganic carbon was estimated from the efflux pattern of CO_2 after a light-to-dark shift using mass spectrometry by Badger *et al.* (2). Ogawa *et al.* (14) also monitored small CO_2 changes associated with dark/light transients in *Anacystis* using an infrared gas analyzer to assess the effects of photosynthesis on the CO_2 concentrating mechanism. It is difficult though to make an estimate of the pool size of internal C_i based solely on the magnitude of these changes in either account, since distinct internal pH changes have been reported to occur in dark/light transients in higher plant chloroplasts and in *Anacystis* (5) and these pH changes would certainly influence the amount of CO_2 either diffusing out into the medium or being converted into HCO_3^- inside the cells, depending on the direction of the pH change.

Masamoto and Nishimura (12) using the H^+-carrying uncoupler ClCCP to monitor pH transients between cells and medium, verified cell pH data on *Anacystis* reported by others (5) and applied this method to determine the cytosol pH in the marine cyanobacterium *Plectonema*. Using the latter method, we found the intracellular pH of *Synechococcus* in the light to be 8.6 and in the dark 7.45. It is reasonable to assume that each pH shift inside the cell might have an effect on the CO_2/HCO_3^- equilibrium in the cytosol pool of C_i. It is worth noting that in ruptured spheroplasts in the absence of the outer cell membrane and with a finitely diluted pool of C_i both the uptake of CO_2, indicative of transport, and the efflux of CO_2 are nearly eliminated (Fig. 3). The transient change in CO_2 concentration observed at the onset of the light period in whole cells of *Synechococcus* can be regarded as a result of the C_i taken up, as well as of the amount of CO_2 converted inside the cell to HCO_3^- following a pH shift upwards. Upon return to dark conditions after a light period two processes are superimposed, (a) a return to equilibrium outside the cells controlled by uncatalyzed processes, resulting in transient CO_2 release from bicarbonate as reported earlier (19), (b) upon return to dark conditions the cell metabolism switches to dark respiration, and excess protons are exported to maintain a stable pH of 7.45 inside the cell. The pH transient changes in *Synechococcus* are similar to observations made by Masamoto and Nishimura (12) using *Plectonema*: the acidification of the medium started when the light was turned off, but the kinetics of the pH transition from light to dark was slow and it took about 2 min for the rate of pH decrease to stabilize. The CO_2 efflux occurred within the same time frame (Figs. 1, 3, and 4). Process 1 can be accelerated considerably by adding CA externally, resulting in an apparent efflux of CO_2 as shown earlier (2), even though the predominant inorganic carbon species at the pH employed is bicarbonate. However, since a pH shift downwards by, *e.g.* 1.2 units would release only a fraction of the C_i present in the cytosol, the pool size of C_i can hardly be determined by CO_2 efflux without using a calibration method.

The question arose whether the intrathylakoid space was a possible site for the location of C_i. However, the acidification of this site by proton accumulation in the light would convert most of the HCO_3^- to CO_2 in a rather short time period. Although CO_2 probably diffused also into the intrathylakoid space of the membrane preparations we used, neither a light-mediated transport of inorganic carbon nor a major change in the concentration of CO_2 was observed in the early light phase. Since an accumulation of C_i in thylakoids is highly unlikely, the pool of C_i in *Synechococcus* is probably predominantly located in the cytosol.

The effect of DCMU on the uptake and ^{18}O-exchange kinetics of *Synechococcus* suggests that the uptake of C_i and possibly the subsequent conversion of HCO_3^- to CO_2 requires photosynthetic energy. Without DCMU the uptake and accumulation of C_i is driven by linear electron transport from water and by ATP generated through cyclic electron flow as shown by Ogawa *et al.* (14). The same authors showed that the postillumination burst of CO_2 was severely inhibited by DCMU in *Anacystis*. They suggested that cyclic electron transport mediated by PSI was essential for the transport of C_i. Our data using mass spectrometric kinetics support their view, and suggest that upon introducing another electron source feeding into PSI, DAD/ascorbate, both the C_i uptake activity and the ^{18}O-exchange activity can be completely restored. It also seems reasonable to relate

the postillumination burst reported by these authors to the post-light-phase rise in CO_2 observed in mass spectra of *Synechococcus*. However, we consider this phenomenon in the absence of CA added externally to be due mainly to uncatalyzed processes outside the cells.

Iodoacetamide blocks the fixation of CO_2, at possibly more than one step of the CO_2 assimilation pathway. When CO_2 fixation was blocked, the initial uptake of C_i was hardly affected, yet a fraction of this C_i was converted to CO_2 and diffused back into the medium (Fig. 4). The question then arises, whether the inhibitor effected a change in pH and this in turn caused a chemical conversion of HCO_3^- to CO_2. We found the internal pH in the presence of IA to be 8.4 ± 0.1 (*cf.* 12). Therefore the observed increase in CO_2 concentration in the presence of IA in the light was most likely not the result of an internal pH change (Fig. 5b).

In the absence of CO_2 fixation an increase in the transport of inorganic carbon into the cells is not expected. Yet the concentration of CO_2 in the medium in the light reached levels of 150% of the starting level in the dark. The intracellular concentration of CO_2 was estimated at 2.5 times the starting level. One explanation for this effect is that the accumulation of C_i at the intracellular site of CA would lead to increased production of CO_2 and its increased efflux out of the cells. The higher C_i level inside the cell then would increase the CA activity and also OH^- production by dehydration as observed (Fig. 5b).

The inclusion of the uncoupler methylamine, however, eliminated both the proton extrusion step in the light and the alkalization of the medium. This is consistent with other reports that MA or ammonium ion at medium concentrations of >5 mM equalizes the ΔpH across photosynthetic membranes (15, 18). This effect explains the increase of the CO_2 concentration to 65 μM by the action of (IA + MA), since the internal pH of these cells would be close to the assay pH of 7.7 (Fig. 5c).

In our ^{18}O exchange experiments using *Synechococcus* cells in the dark, we have not observed any detectable ^{18}O exchange rate above the uncatalyzed rate. In the light, however, high rates of ^{18}O exchange were observed. These effects bring us to the critical conclusion of our study. Have we observed the PSI-dependent transport of C_i as reported by Ogawa *et al.* (14) or some other effect? The low ^{18}O exchange activity in the dark can be due to the very low uptake of inorganic carbon by cells in the dark, a rate so slow that the uncatalyzed dehydration outside the cell predominates over CO_2 taken up by the cells, and neither the concentration of CO_2 nor its ^{18}O content was detectably perturbed in the dark. One role of PSI then is not to influence CA activity directly, but provide the ATP for the active transport of C_i into the cells. The ATP levels then would be reduced by the action of the uncouplers DCCD and ClCCP as shown. In this context we have confirmed the uptake of inorganic carbon driven by PSI by a new method, which extends the interpretation of Ogawa *et al.* (14) as it relates to the role of carbonic anhydrase.

There is yet a second hypothesis which is consistent with and not excluded by our data, the light-activation of the CA activity in the cells. When we look at the kinetics of inorganic carbon assimilation in *Synechococcus*, we notice three steps. During the first minute of light the concentration of CO_2 is lowered dramatically by the uptake and conversion of CO_2 into bicarbonate. The observed uptake of bicarbonate is not balanced by OH^- export, but rather proton extrusion is observed (Fig. 5). In step 2, O_2 evolution is coincidental with the build up of CO_2 from the dehydration of bicarbonate simultaneously with OH^- excretion. In step 3, the uptake of HCO_3^- is balanced by the fixation of CO_2 into cell material. Thus, the dehydration of bicarbonate occurs after its accumulation in the internal pool of C_i and both OH^- and O_2 production can be blocked by the CA inhibitor ethoxzolamide (reported elsewhere). Light activation of purified CA is very unlikely based on what we know of the mechanism of animal and plant CA. However, the catalysis of the hydration-dehydration reaction of CO_2 by CA is known to be highly pH-dependent in the region of pH 6 to 8 and very responsive to the concentration of buffer species (17). The high pH environment during the dehydration process of bicarbonate in the light observed in *Synechococcus* would indeed create special conditions for catalysis of CA activity in the cytosol, so as to export the excess OH^- ions generated into the medium. One support for this suggestion is that we observed no ^{18}O exchange above the uncatalyzed rate in the dark, as mentioned above. In contrast, in the light and in the presence of iodoacetamide there was rapid ^{18}O exchange and CO_2 readily passed any barriers between intracellular CA and the external solution.

Thus, the data presented are consistent with both the exchange of ^{18}O enhanced by light-mediated uptake of inorganic carbon and with the light-dependent interconversion of inorganic carbon species during the bicarbonate dehydration reaction, but we cannot at present distinguish which of the two processes predominates. It would be useful to isolate the carbonic anhydrase enzyme to measure its catalytic activity under conditions that simulate the intracellular environment in the light and dark.

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