The Role of the C_4 Pathway in Carbon Accumulation and Fixation in a Marine Diatom[1]

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The role of a C_4 pathway in photosynthetic carbon fixation by marine diatoms is presently debated. Previous labeling studies have shown the transfer of photosynthetically fixed carbon through a C_4 pathway and recent genomic data provide evidence for the existence of key enzymes involved in C_4 metabolism. Nonetheless, the importance of the C_4 pathway in photosynthesis has been questioned and this pathway is seen as redundant to the known CO_2 concentrating mechanism of diatoms. Here we show that the inhibition of phosphoenolpyruvate carboxylase (PEPCase) by 3,3-dichloro-2-dihydroxyphosphinoylmethyl-2-propenoate resulted in a more than 90% decrease in whole cell photosynthesis in *Thalassiosira weissflogii* cells acclimated to low CO_2 (10 μM), but had little effect on photosynthesis in the C_3 marine Chlorophyte, Chlamydomonas sp. In 3,3-dichloro-2-dihydroxyphosphinoylmethyl-2-propenoate-treated *T. weissflogii* cells, elevated CO_2 (150 μM) or low O_2 (80–180 μM) restored photosynthesis to the control rate linking PEPCase inhibition with CO_2 supply in this diatom. In C_4 organic carbon-inorganic carbon competition experiments, the ^{12}C-labeled C_4 products of PEPCase, oxaloacetic acid and its reduced form malic acid suppressed the fixation of ^{14}C-labeled inorganic carbon by 40% to 50%, but had no effect on O_2 evolution in photosynthesizing diatoms. Oxaloacetic acid-dependent O_2 evolution in *T. weissflogii* was twice as high in cells acclimated to 10 μM rather than 22 μM CO_2, indicating that the use of C_4 compounds for photosynthesis is regulated over the range of CO_2 concentrations observed in marine surface waters. Short-term ^{14}C uptake (silicone oil centrifugation) and CO_2 release (membrane inlet mass spectrometry) experiments that employed a protein denaturing cell extraction solution containing the PEPCKase inhibitor mercaptopicolinic acid revealed that much of the carbon taken up by diatoms during photosynthesis is stored as organic carbon before being fixed in the Calvin cycle, as expected if the C_4 pathway functions as a CO_2 concentrating mechanism. Together these results demonstrate that the C_4 pathway is important in carbon accumulation and photosynthetic carbon fixation in diatoms at low (atmospheric) CO_2.

Diatoms are important marine photoautotrophic protists that account for up to 25% of the primary production on Earth (Falkowski and Raven, 1997). They are also important fractionators of stable carbon isotopes that are used to evaluate trophic relationships in marine food webs (Checkley and Entzeroth, 1985; Fry and Wainright, 1991) and marine carbon cycling in modern and ancient oceans (Francois et al., 1993; Hayes, 1993). As a result, the mechanisms of uptake and fixation of inorganic carbon (C_i) in these organisms have been the topics of extensive research (Korb et al., 1997; Tortell et al., 2000; Rost et al., 2003; Tchernov et al., 2003). In particular, the existence and role of a C_4 pathway for photosynthetic carbon fixation in marine diatoms has been the subject of research for over 25 years (Beardall et al., 1976; Mortain-Bertrand et al., 1987; Descolas-Gros and Oriol, 1992). Recently, Reinfelder et al. (2000) demonstrated short-term labeling of C_4 compounds and transfer of carbon to PGA and sugars in the model marine diatom *Thalassiosira weissflogii*. But, the designation of diatoms as unicellular C_4 photoautotrophs has not been readily accepted (Riebesell, 2000; Johnston et al., 2001); the C_4 pathway is seen as playing chiefly an anaplerotic role and its importance in photosynthesis has been questioned. Further a C_4 pathway to support photosynthesis at low CO_2 seems redundant with the known CO_2 concentrating mechanism (CCM) of marine diatoms. In addition, the presence and localization of the necessary C_4 enzymes have been questioned.

The question of the existence of enzymes necessary for a C_4 pathway in diatoms has now been largely resolved by the recently sequenced genome of *Thalassiosira pseudonana* (E.V. Armbrust, J.A. Berges, C. Bowler, B.R. Green, D. Martinez, N.H. Putnam, S. Zhou, A.E. Allen, K.E. Apt, M. Bechner, M. Brzezinski, B.K. Chaal, A. Chiovitti, A.K. Davis, M.S. Demarest, J.C. Detter, T. Glavina, D. Goodstein, M.Z. Hadi, U. Hellsten, M. Hildebrand, B.D. Jenkins, J. Jurka, V.V. Kapitonov, N. Kröger, W.W.Y. Lau, T.W. Lane, F.W. Larimer, J.C. Lippmeier, S. Lucas, M. Medina, A. Montsant, M. Obornik, M.S. Parker, B. Palenik, G.J. Pazour, P.M. Richardson, T.A. Rynearson, M.A. Saito, D.C. Schwartz, K. Thamatrakoln, K. Valentin, A. Vardi, F.P. Wilkerson, and D.S. Rokhsar, unpublished data). Genes coding for phosphoenolpyruvate carboxylase (PEPCase), phosphoenolpyruvate carboxykinase

[1] This work was supported by the NSF-EMSI program through the Center for Environmental Bioinorganic Chemistry (CEBIC) and by a Hatch/McIntyre-Stennis grant through the New Jersey Agricultural Experiment Station.

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.041319.

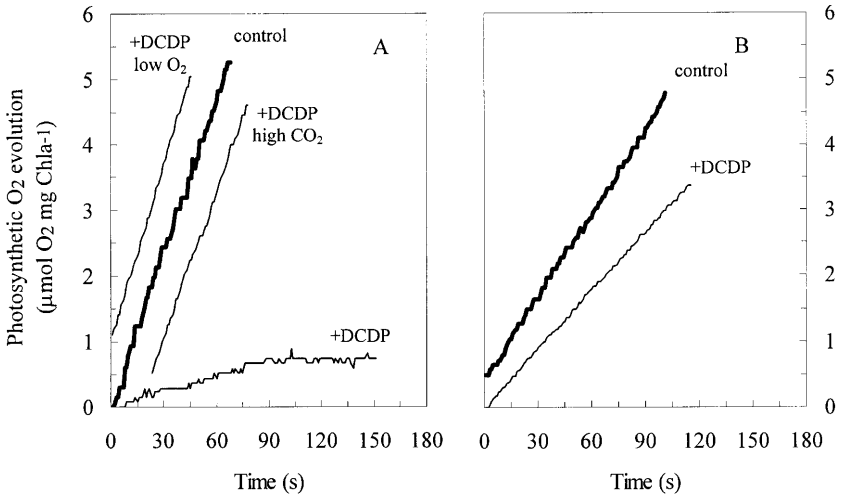

Figure 1. Effects of PEPCase inhibition on net photosynthetic O_2 evolution in (A) the marine diatom *T. weissflogii* and (B) the marine chlorophyte Chlamydomonas sp. Graphs depict whole cell O_2 evolution in control incubations (thick lines; 15–30 μM CO_2 and 300–400 μM O_2) or in the presence (thin lines) of the PEPCase inhibitor DCDP (750 μM). For *T. weissflogii,* photosynthesis rates of PEPCase-inhibited cells with 150 μM CO_2 plus 300 to 400 μM O_2 (+DCDP high CO_2) and 80 to 180 μM O_2 plus 15 to 30 μM CO_2 (+DCDP low O_2) are also shown.

(PEPCKase), and pyruvate orthophosphate dikinase (PPDK, which catalyzes the synthesis of PEP in many C_4 plants) have been identified in the genome of this diatom. The intracellular localizations of all of these enzymes in diatoms, which are critical to a complete understanding of carbon metabolism in these organisms, are uncertain. The absence of upstream targeting sequences adjacent to the genes for PEPCase and PPDK in *T. pseudonana* is consistent with cytoplasmic localizations. The localization of PEPCase in the cytoplasm provides the necessary intracellular compartmentalization for simultaneous carbon fixation by PEPCase and Rubisco in a single cell (Magnin et al., 1997; Voznesenskaya et al., 2002). PEPCKase localization cannot be evaluated with certainty from the gene targeting sequence of *T. pseudonana*, but its activity was found to follow that of Rubisco in crude cell fractions of *T. weissflogii* (Reinfelder et al., 2000) and PEPCKase protein was immunolocalized within the chloroplast of the marine diatom *Skeletonema costatum* (Cabello-Pasini et al., 2001).

In this study, we examine the importance of the C_4 pathway in diatom photosynthesis by measuring the effects of PEPCase inhibition and C_4 organic carbon-C_i competition on whole cell O_2 evolution and inorganic carbon fixation in *T. weissflogii.* We also examine the form of carbon concentrated during short-term ^{14}C uptake (silicone oil centrifugation) and CO_2 release (membrane inlet mass spectrometry) experiments to evaluate the role of the C_4 pathway in the diatom CCM. To provide a benchmark to differentiate C_4 and C_3 pathways, we conduct parallel experiments with the marine Chlorophyte Chlamydomonas sp.

RESULTS AND DISCUSSION

The Importance of C_4 Carbon Fixation in Diatom Photosynthesis

If the C_4 pathway is quantitatively important to photosynthesis in diatoms, then the inhibition of PEPCase should have a major effect on photosynthetic O_2 evolution. To test the role of PEPCase in diatom photosynthesis, we used the PEPCase-specific inhibitor 3,3-dichloro-2-dihydroxyphosphinoylmethyl-2-propenoate (DCDP), an analog of PEP that inhibits PEPCase from a range of C_4 and C_3 plants, but does not inhibit enzymes that catalyze other reactions in which PEP is a substrate (Jenkins et al., 1987). The addition of DCDP to suspensions of *T. weissflogii* cells grown in air-equilibrated medium caused a more than 90% decrease in photosynthetic O_2 evolution compared with uninhibited cells (Fig. 1). Thus, in diatoms acclimated to 10 μM CO_2, a major fraction of net carbon fixation depends on the synthesis of C_4 organic carbon by PEPCase. In Chlamydomonas sp. grown under identical conditions as *T. weissflogii*, DCDP had a small effect on whole cell photosynthetic O_2 evolution (Fig. 1). Chlamydomonas sp. are C_3 photoautotrophs in which PEPCase serves an anaplerotic role replacing TCA cycle carbohydrates used in amino acid synthesis (Huppe and

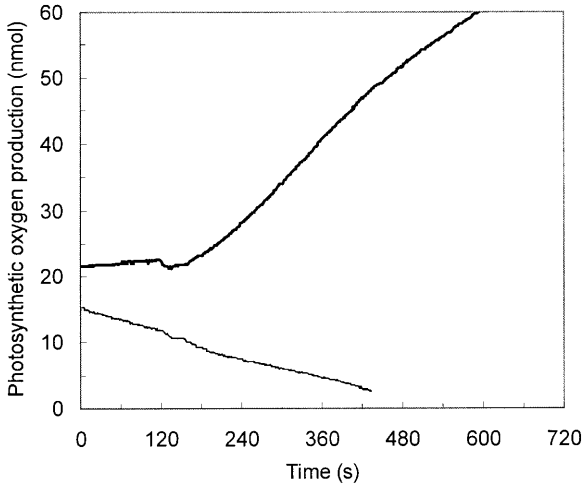

Figure 2. Oxaloacetic acid-dependent O_2 evolution (thick line) and dark O_2 consumption (thin line) in *T. weissflogii* cells at the C_i compensation point given 1 mM OAA at 120 s.

Turpin, 1994; Rivoal et al., 1998). The minimal effect in this organism provides confidence that the major depression of photosynthesis by DCDP observed in the diatom was not due to the inhibition of anaplerotic PEPCase activity. If the inhibition of PEPCase in *T. weissflogii* impairs the ability of the diatom cells to concentrate CO_2, but not carbon fixation, then the addition of a high concentration of CO_2 to the external medium should restore photosynthesis in DCDP-inhibited diatoms to the level of uninhibited cells. Indeed the addition of 150 μM CO_2 to DCDP-inhibited *T. weissflogii* cells restored photosynthesis to the level of the control (Fig. 1), demonstrating that DCDP did not interfere with the Calvin cycle and that the shutdown of photosynthesis resulting from PEPCase inhibition in *T. weissflogii* is linked to the supply of inorganic carbon to the cell. Thus DCDP-inhibited cells behave as C_3 autotrophs lacking a mechanism to actively concentrate inorganic carbon for photosynthesis. Such cells should be particularly susceptible to the negative effects of O_2 on photosynthesis as a consequence of photorespiration and show increased photosynthesis rates when the concentration of O_2 is lowered. Decreasing the O_2 concentration in the medium did in fact result in an increase in photosynthetic O_2 production of DCDP-inhibited diatom cells to the levels of the uninhibited control (Fig. 1). The complete recovery of photosynthesis in response to lowering the O_2 concentration in these experiments is likely due to the high carboxylation to oxygenation ratio of diatom Rubisco compared with that from other eukaryotic microalgae (Badger et al., 1998). This effect may have been pronounced due to the somewhat elevated CO_2 concentrations (15–30 μM) at the experimental pH.

A complementary test of the importance of C_4 organic carbon in diatom photosynthesis is provided by the measurement of the effects of C_4 compounds

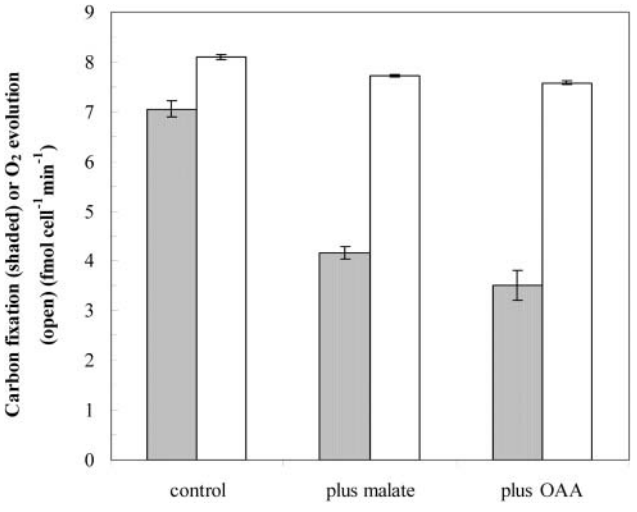

Figure 3. Effects of C_4 organic acids on carbon fixation (shaded bars) and O_2 evolution (open bars) in the marine diatom *T. weissflogii*. Measurements were made in the presence or absence of 2 mM malic acid or OAA. Error bars represent propagated ^{14}C counting errors for carbon fixation rates and SES for O_2 evolution rates.

Table I. *Carbon-dependent O_2 evolution rates in T. weissflogii cells acclimated to 10 or 22 μM CO_2 (360 or 700 μmol mol^{-1} CO_2, respectively)*

Photosynthesis was measured in cells given either 1 mM C_i (170 μM CO_2) or 1 mM OAA at pH 7. Values in parentheses are SES of the rate estimates.

Acclimation [CO_2]	$P_{1\ mM\ Ci}$	$P_{1\ mM\ OAA}$
μM	fmol cell^{-1} min^{-1}	fmol cell^{-1} min^{-1}
10	10.5 (0.11)	6.3 (0.03)
22	9.6 (0.03)	3.1 (0.03)

on photosynthetic O_2 evolution and inorganic carbon fixation. In diatoms at the inorganic carbon (C_i) compensation point, the C_4 compound oxaloacetic acid stimulated O_2 evolution, but not respiratory O_2 consumption (Fig. 2), indicating that the C_4 compound was decarboxylated by a nonrespiratory pathway to supply carbon-depleted cells with CO_2. In photosynthesizing *T. weissflogii* cells given sufficient inorganic carbon (1.2 mM) to maintain photosynthesis at its maximum carbon-saturated rate, the addition of 2 mM oxaloacetic acid (OAA) or malic acid suppressed inorganic carbon fixation by 40% to 50%, but had no effect on photosynthetic O_2 evolution (Fig. 3). This confirms that the C_4 compounds can provide a large fraction of the photosynthetically fixed carbon in this diatom.

If C_4 carbon fixation is the primary mechanism to concentrate CO_2 in diatoms, then the ability to use C_4 compounds for photosynthesis should be modulated by the concentration of CO_2 to which the diatoms have been acclimated. PEPCase activity in *T. weissflogii* was previously found to increase in cells acclimated to low CO_2 concentrations (Reinfelder et al., 2000). As an extension of this observation, we compared OAA-dependent O_2 evolution rates in cells acclimated to 10 and 22 μM CO_2. In diatoms brought to the C_i compensation point ($C_i < 2$ μM), the rate of OAA-dependent O_2 evolution was twice as high in diatoms acclimated to 10 μM CO_2 as in cells grown with 22 μM CO_2 (Table I). Since the concentration of CO_2 to which the diatoms were acclimated had no effect on C_i-saturated (1 mM C_i, 170 μM CO_2) photosynthesis rates (Table I), these results indicate that the capacity of the diatoms to decarboxylate C_4 organic acids and provide carbon for photosynthesis is greater in cells acclimated to low CO_2.

The Nature of CO_2 Concentration in Marine Diatoms

Unequivocal evidence that marine diatoms possess a CCM has been obtained by a number of researchers (Rotatore et al., 1995; Burkhardt et al., 2001; Tortell and Morel, 2002), but the biochemical mechanism of the diatom CCM is unknown. The production and decarboxylation of C_4 organic carbon to supply Rubisco with CO_2 represents a carbon storage and delivery mechanism that may function as a CCM to support diatom photosynthesis in low CO_2 surface waters. Thus, the CCM and the C_4 pathway in diatoms may be one and the same. This is supported indirectly by the

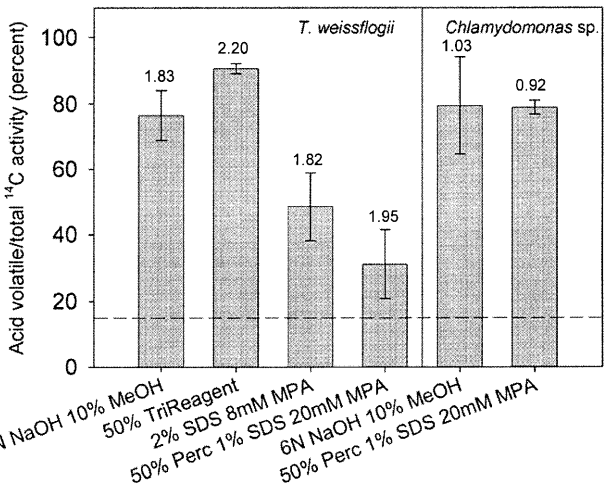

Figure 4. The fraction of short-term carbon accumulation measured as inorganic carbon (acid volatile ^{14}C activity) by the silicon oil centrifugation technique in the marine diatom *T. weissflogii* and the marine chlorophyte Chlamydomonas sp. Treatments correspond to various extraction-trapping solutions. Numbers above bars are total (organic plus inorganic) ^{14}C activities (kBq) collected after the cells were transferred to the trapping solutions by centrifugation. Error bars are SDS of means ($n = 3$). Note that in such experiments the entrainment of medium with the cells during centrifugation results in a background inorganic carbon in the trapping solution of up to 15% (dashed line) of the total (Tortell et al., 2000).

observation that C_4 carbon fixation (PEPCase activity) in *T. weissflogii* increases over the range of CO_2 concentrations (10 μM and lower; Reinfelder et al., 2000) where CCM activity increases in marine diatoms (Rotatore et al., 1995; Burkhardt et al., 2001; Tortell and Morel, 2002).

If the C_4 pathway serves as the CCM in diatoms, transported carbon should be stored as an organic (C_4) rather than inorganic (presumably HCO_3^-) compound before fixation in the Calvin cycle in these organisms. In this case, the inorganic carbon that has been measured as intracellular in previous studies of the CCM in diatoms should have resulted from rapid decarboxylation of a C_4 compound. We thus attempted to prevent such decarboxylation in the course of the same types of experiments—using the silicon oil centrifugation or the membrane inlet mass spectrometry (MIMS) methods—that have been used to demonstrate the existence of a CCM in diatoms.

In the silicone oil centrifugation method, inorganic carbon is measured in cells that have been spiked with inorganic ^{14}C and then collected within seconds by centrifugation into a lower layer of an organic extraction and inorganic carbon trapping solution containing methanol and NaOH. In an attempt to inactivate all intracellular enzymes after centrifugation, we tried various extraction-trapping solutions containing cell membrane and protein denaturants (TriReagent, SDS) at low osmotic strength. In experiments run with SDS in the trapping solution, the amount of intracellular ^{14}C measured as inorganic carbon (acid volatile) in *T. weissflogii* during short-term uptake was lower than that obtained with the normal trapping solution of methanol and NaOH (Fig. 4). The lowest amount of short-term carbon accumulation measured as inorganic carbon was observed with a low osmotic strength trapping solution containing 1% SDS and 20 mM mercaptopicolinic acid (MPA). MPA is an inhibitor of PEPCKase that catalyzes the decarboxylation of OAA in some C_4 plants (Rathnam and Edwards, 1977) and is hypothesized to catalyze C_4 decarboxylation in the chloroplast of the marine diatom *T. weissflogii* (Reinfelder et al., 2000). With the SDS-MPA metabolic inactivation solution, approximately 70% of the ^{14}C taken up by *T. weissflogii* in 10 s was present as acid-stable organic carbon compared to only 24% with the standard methanol-NaOH trapping solution (Fig. 4). Since total ^{14}C activities—organic plus inorganic—were the same (average residual within 6% of the mean) in all treatments with *T. weissflogii*, this result indicates that, in experiments with the standard trapping solution, a substantial portion of newly formed organic carbon was rapidly converted to inorganic carbon and that the trapping solution routinely used in the silicone oil centrifugation method does not immediately stop all metabolic activity after cells are removed from ^{14}C-C_i. The most potent trapping solution for *T. weissflogii* (SDS-MPA) had no effect on the amount of intracellular ^{14}C measured as inorganic carbon during short-term ^{14}C exposures in Chlamydomonas sp. (Fig. 4). Thus a large fraction of the carbon taken up in 10 s is stored as organic carbon prior to fixation in the Calvin cycle in the diatom *T. weissflogii*, but not in the C_3 microalga Chamydomonas sp.

In the MIMS method, the accumulation of carbon by microalgae is quantified by measuring the rate of

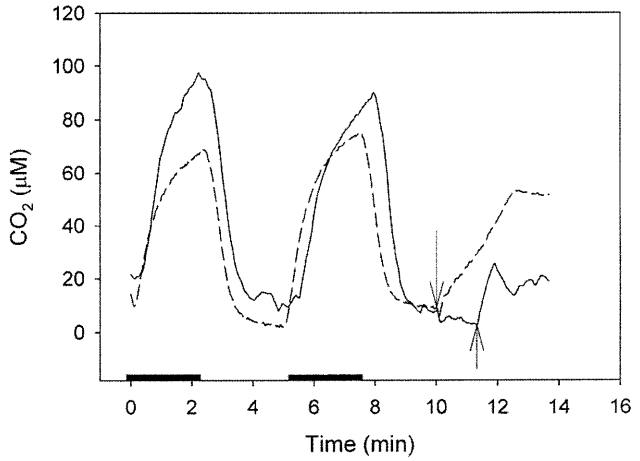

Figure 5. Uptake and release of CO_2 by *T. weissflogii* (solid line) and Chlamydomonas sp. (dashed line) during alternating periods of light and dark (bar on *x* axis) and following the addition (in the light) of 20 mM MPA in 1% SDS (arrows) at constant pH (7.5) recorded in a membrane inlet mass spectrometer. Note that in the presence of the carbonic anhydrase inhibitor acetazolamide, the production of CO_2 by the cells in these concentrated suspensions may lead to CO_2 concentrations that are higher than that at equilibrium with HCO_3^-.

increase in the concentration of CO_2 in cell suspensions in which photosynthesis is stopped by turning off all light (Rotatore et al., 1995; Burkhardt et al., 2001). Such an increase of CO_2 concentration (Fig. 5) results from the sum of three processes: (1) dehydration of HCO_3^-, (2) dark respiration, and (3) release of stored carbon by the cells (Burkhardt et al., 2001). Note that the hydration of CO_2 released by dense cell suspensions is slow in the presence of added inhibitors of carbonic anhydrase resulting in a CO_2 concentration in the medium that is higher than its equilibrium value. If the third process, which is often dominant, results from the rapid decarboxylation of a C_4 compound, we would expect the increase in extracellular CO_2 observed following the addition (in the light) of our protein denaturing extraction-trapping solution (SDS-MPA), which would stop both C_4 decarboxylation and respiration, to be markedly lower than that observed upon turning off the light. Indeed the amount of CO_2 released from *T. weissflogii* cells following the addition of SDS-MPA (Fig. 5, arrow on solid line) was nearly 90% lower than the amount of CO_2 released from diatom cells after the light was turned off (Fig. 5, dark periods beginning at 0 and 5.2 min), indicating the presence of a major pool of stored organic carbon. In Chlamydomonas sp. (Fig. 5, dashed line), the amount of CO_2 release after the addition of SDS-MPA was only 30% lower than the amount of CO_2 released from Chlamydomonas cells after turning off the light. For the marine diatom *T. weissflogii*, stopping all metabolic activity and inhibiting PEPCKase dramatically decreased CO_2 release, while for Chlamydomonas, metabolic inactivation resulted in a smaller decrease in CO_2 release, perhaps corresponding to that associated with respiration. The results of the MIMS experiments are thus wholly consistent with the silicone oil centrifugation experiments and with the storage of an organic carbon compound in the diatom and of inorganic carbon in Chlamydomonas sp. prior to ultimate fixation.

In conclusion, our results indicate that the C_4 pathway plays a central role in photosynthesis in diatoms acclimated to low (e.g. atmospheric) CO_2 concentrations. In addition, the carbon accumulated intracellularly by the CCM of these organisms is chiefly organic rather than inorganic, consistent with the formation of C_4 intermediates. As reflected in their high ^{13}C content (Fry and Wainright, 1991), diatoms have a photosynthetic mechanism that is clearly unlike that of green algae. On the basis of these and earlier results (Reinfelder et al., 2000), it appears that diatoms utilize a type of unicellular C_4 photosythesis under low ambient CO_2 that is analogous to that observed in some aquatic plants (Bowes et al., 2002).

MATERIALS AND METHODS

Phytoplankton Cultures

Axenic cultures of the marine diatom *Thalassiosira weissflogii* (CCMP 1336) and the marine chlorophyte Chlamydomonas sp. (CCMP 222) were maintained in air-equilibrated synthetic ocean water (Aquil; Price et al., 1988, 1989) in continuous light (200 μmol photon m^{-2} s^{-1}) at 18°C. Stock cultures were maintained in glass tubes and experimental cultures were grown in acid-soaked (1 N HCl) clear polycarbonate bottles. Cell growth was monitored by measuring in vivo fluorescence and microscope cell counts. Cells for all experiments were harvested in mid-exponential growth phase (μ = 1 d^{-1}).

PEPCase Inhibition Experiments

The inhibition of whole cell photosynthesis by DCDP, a specific, competitive (with PEP) inhibitor of PEPCase (Jenkins et al., 1987), was measured in *T. weissflogii* and Chlamydomonas sp. Photosynthesis was measured as oxygen evolution using an oxygen electrode cell (Hansatech, King's Lynn, UK) with 400 μmol photon m^{-2} s^{-1} photosynthetically active radiation at 22°C. For PEPCase inhibition treatments, DCDP was added to a final concentration of 750 μM from a 10-mM stock solution in 10 mM HCl. Cells were concentrated to 2×10^6 (*T. weissflogii*) and 5.6×10^6 (Chlamydomonas) cells mL^{-1} in seawater culture media with 2 mM bicine (pH 7.4), 15 to 30 μM CO_2, and 300 to 400 μM O_2. Photosynthesis rates of DCDP-inhibited cells with excess CO_2 (150 μM CO_2, 300–400 μM O_2) and low O_2 (80–180 μM O_2, 15–30 μM CO_2) were also measured. DCDP inhibition experiments were also tried at pH 8 to maintain lower CO_2, but DCDP was not taken up above pH 7.4.

C_4 Acid-Dependent Oxygen Evolution Experiments

The stimulation of O_2 evolution by C_4 compounds was studied in *T. weissflogii* cells brought to the C_i compensation point (photosynthesis is equal to respiration) in an oxygen electrode. Cells were concentrated to 10^6 cells mL^{-1} in buffer (25 mM HEPES, 350 mM sorbitol, pH 7) and incubated in the light until nearly all inorganic carbon was consumed and the concentration of O_2 remained constant. Based on the photosynthesis-C_i relationship and respiration rates of this diatom, the total C_i at the compensation point was estimated to be <2 μM. Once the cells were brought to the C_i compensation point, 1 mM OAA was added. Maximum OAA-dependent O_2 evolution rates were compared with maximum rates in cells resuspended in buffer with 1 mM C_i. Cells were grown as described above and for at least two transfers (9–10 generations) in media bubbled with either air (10 μM CO_2) or air containing 700 μmol mol^{-1} CO_2 (22 μM CO_2).

C_4-C_i Competition Experiments

The inhibition of inorganic carbon fixation by OAA or its reduced form, malic acid, was measured in photosynthesizing *T. weissflogii* cells concentrated to 2.5×10^5 cells mL^{-1} in buffer (25 mM HEPES, 350 mM sorbitol, pH 7.5) and incubated in the light (400 μmol photon m^{-2} s^{-1}) at 22°C with 1.2 mM ^{14}C-C_i. C_4 organic acids were added from freshly prepared stock solutions of the free acids (50 mM) to give final concentrations in the incubations of 2 mM OAA or malic acid. For the malic acid experiments, incubations were begun with the simultaneous addition of ^{14}C-C_i (pH 9.5) and C_4 acid dissolved in HEPES buffer (pH 7.5) to cells that had been photosynthesizing in the light for 5 min. In the OAA experiment, photosynthesizing cells were incubated with 2 mM OAA for 30 s prior to the addition of ^{14}C-C_i. At various times during the 5-min incubations, 1-mL subsamples were transferred to 2 mL methanol plus 50 μL 6 N HCl. The liquid was evaporated and the residues resuspended in a small volume (0.2–0.3 mL) of ultra-pure water. The acid-stable (organic) ^{14}C content of extracts was quantified by liquid scintillation counting. Carbon fixation rates were estimated from the slopes of the ^{14}C fixation curves and the specific activity of the radiocarbon. The spontaneous decarboxylation rate of oxaloacetic acid in the HEPES/sorbitol buffer was sufficiently slow (<1 nmol min^{-1} over 5 min) so as not to be a significant source of CO_2. The effects of OAA and malic acid on photosynthetic O_2 evolution in *T. weissflogii* were also measured.

Silicone Oil Centrifugation

Short-term ^{14}C carbon accumulation experiments were conducted using the silicone oil centrifugation technique (Badger et al., 1980; Tortell et al., 2000). Cells were resuspended in C_i-free 50 mM HEPES buffer with seawater salts (pH = 8). Concentrated cells (3.5×10^6 for *T. weissflogii* and 9×10^6 for Chlamydomonas sp. in 200 μL) were layered on top of silicon oil in 0.6-mL microcentrifuge tubes. Illumination was provided from the side at a photon

flux density of 500 μmol m^{-2} s^{-1} using a tungsten-halogen bulb from a slide projector. To start the incubation 1 mM $NaH^{14}CO_3$ was added and mixed with the pipette. Incubations (duration 10 s) were terminated by centrifugation through the silicon oil layer into a basic cell extraction and inorganic carbon trapping solution (6 N NaOH with 10% methanol). Other trapping solutions (all at pH 8) included 50% TriReagent (Sigma, St. Louis), 2% SDS and 8 mM MPA (Toronto Research Chemicals), and 50% Percol (to provide density at low ionic strength) with 1% SDS and 20 mM MPA. The resulting pellet was immediately frozen in liquid nitrogen. The base of the centrifuge tube was cut off and retained quantified using liquid scintillation counting. The ^{14}C counts (corrected for quench) for the unacidified samples were used to determine the total accumulated carbon (inorganic and organic) and those for the acidified samples were used to determine the amount of organic carbon fixed.

Membrane Inlet Mass Spectrometry

The short-term accumulation and release of carbon was followed using a membrane inlet system attached to a Prisma QMS-200 (Pfeiffer) quadrapole mass spectrometer with closed ion source recording at mass/charge (m/z) ratios of 40 and 44. The membrane inlet system was modified from a water-jacketed DW/2 oxygen electrode chamber (Hansatech Instruments) in which the electrode base plate was replaced by a stainless steel base plate with a gas port drilled through the center. The standard Teflon membrane (thickness 12.5 μm) supplied with the DW/2 system was used. Illumination was provided by a tungsten projector bulb at 300 μmol m^{-2} s^{-1}. Temperature was maintained at 20°C. The mass spectrometer was calibrated for CO_2 using buffer equilibrated with 100 and 750 μmol mol^{-1} CO_2. Cells were concentrated to 9.4×10^6 (*T. weissflogii*) and 3.6×10^6 (Chlamydomonas) cells mL^{-1} in buffer (25 mM HEPES, 350 mM sorbitol, pH 7.5) with 100 mM acetazolamide to suppress extracellular carbonic anhydrase activity and an initial C_i concentration of 100 μM. Release of CO_2 was measured in cells transferred to the dark and in cells treated with 1% SDS plus 20 mM MPA (pH = 7.5), an inhibitor of the C_4 PEPCKase.

ACKNOWLEDGMENT

We thank Katsura Izui (Kyoto University) for kindly providing the DCDP.

Received February 19, 2004; returned for revision April 27, 2004; accepted April 29, 2004.

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