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Extracellular Carbonic Anhydrase Facilitates Carbon Dioxide Availability for Photosynthesis in the Marine Dinoflagellate *Prorocentrum micans*

Nabil A. Nimer*, Colin Brownlee, and Michael J. Merrett

School of Biological Sciences, University of Wales, Swansea SA2 8PP, United Kingdom (N.A.N., M.J.M.); and Marine Biological Association of the United Kingdom, Plymouth PL22PB, United Kingdom (C.B.)

This study investigated inorganic carbon accumulation in relation to photosynthesis in the marine dinoflagellate *Prorocentrum micans*. Measurement of the internal inorganic carbon pool showed a 10-fold accumulation in relation to external dissolved inorganic carbon (DIC). Dextran-bound sulfonamide (DBS), which inhibited extracellular carbonic anhydrase, caused more than 95% inhibition of DIC accumulation and photosynthesis. We used real-time imaging of living cells with confocal laser scanning microscopy and a fluorescent pH indicator dye to measure transient pH changes in relation to inorganic carbon availability. When steady-state photosynthesizing cells were DIC limited, the chloroplast pH decreased from 8.3 to 6.9 and cytosolic pH decreased from 7.7 to 7.1. Re-addition of HCO_3^- led to a rapid re-establishment of the steady-state pH values abolished by DBS. The addition of DBS to photosynthesizing cells under steady-state conditions resulted in a transient increase in intracellular pH, with photosynthesis maintained for 6 s, the amount of time needed for depletion of the intracellular inorganic carbon pool. These results demonstrate the key role of extracellular carbonic anhydrase in facilitating the availability of CO_2 at the exofacial surface of the plasma membrane necessary to maintain the photosynthetic rate. The need for a CO_2-concentrating mechanism at ambient CO_2 concentrations may reflect the difference in the specificity factor of ribulose-1,5 bisphosphate carboxylase/oxygenase in dinoflagellates compared with other algal phyla.

Marine phytoplankton species are the major dominant fixers of inorganic carbon in the oceans (Raven, 1994; 1997a), removing about 35 Pg of inorganic carbon per year from the ecosphere (Raven, 1997a). The key enzyme of photosynthetic CO_2 fixation, Rubisco (EC 4.1.1.39) (Raven, 1995), catalyzes the initial assimilation reaction of CO_2 and also the oxygenation of ribulose bisphosphate, initiating the photorespiratory pathway (Lorimer et al., 1973). The ratio of these reactions determines the specificity factor (τ) of the enzyme, which can indicate enzyme type. The diverse universal type I enzyme found in oxygenic phototrophs has a substantially higher specificity for CO_2 than for oxygen (Jordan and Ogren, 1981) compared with type II, the more oxygen-sensitive, homomeric form of the enzyme found in heterotrophic anaerobic proteobacteria and cyanobacteria (Delgado et al., 1995; Tabita, 1995; Raven, 1997a). Recently, among the dinoflagellates, a major component of marine phytoplankton, the species tested were found to possess the oxygen-sensitive homomeric type-II form of the enzyme (Morse et al., 1995; Whitney and Yellowlees, 1995; Rowan et al., 1996; Whitney and Andrews, 1998).

Dinoflagellates are morphologically and physiologically diverse, abundant in the marine ecosystem, and ecologically important; they make a major contribution to the global biological carbon pump (Raven and Johnston, 1991). Relatively little, however, is known about their mechanism of inorganic carbon acquisition. In the dinoflagellate species investigated, the specificity factor was approximately 2-fold greater than other homomeric Rubiscos but was still very unlikely to support photosynthetic rates at ambient CO_2 levels (Raven and Johnston, 1991; Whitney and Andrews, 1998). This explains the need for a CCM to elevate the CO_2 concentration around the active center of Rubisco, suppressing glycolate formation and enhancing carboxylation (Coleman, 1991; Badger and Price, 1994). An essential component of a CCM is an active influx of inorganic carbon across a membrane (Raven, 1995).

In some algal species, extracellular CA (EC 4.2.1.1) is a major component of the CCM (Badger et al., 1980; Spalding et al., 1983; Aizawa and Miyachi, 1986). Extracellular CA was shown to be important in inorganic carbon transport when HCO_3^- is available but CO_2 is limited external to the plasma membrane (Tsuzuki and Miyachi, 1989). Under these conditions the catalytic dehydration of HCO_3 by extracellular CA provides CO_2 external to the plasma membrane. Overall, the mechanism of inorganic carbon acquisition in marine phytoplankton is species dependent; some species rapidly acclimate to changes in CO_2 and/or HCO_3^- concentrations (Coleman, 1991; Nimer and Merrett, 1996; Nimer et al., 1997). In the dinoflagellate *Prorocentrum micans*, the activity of constitutive extracellular CA (Nimer et al., 1997) is modulated by environmental conditions, increasing under conditions of inorganic carbon limitation (Nimer et al., 1997).

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* Corresponding author; e-mail n.nimer@swansea.ac.uk; fax 44–1792–295–447.

Abbreviations: CA, carbonic anhydrase; CCM, CO_2-concentrating mechanism; DBS, dextran-bound sulfonamide; DIC, dissolved inorganic carbon; PFD, photon flux density.

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In the present study we investigated the potential role of extracellular CA in facilitating CO_2 entry and sustaining the photosynthetic rate in *P. micans* by monitoring transient changes in intracellular pH in relation to internal inorganic carbon concentration and photosynthetic CO_2 fixation.

MATERIALS AND METHODS

Growth of Cells

Axenic cultures of the marine dinoflagellate *Prorocentrum micans* (strain CCAP 1136/8 from the Provasoli-Guillard Centre for Culture of Marine Phytoplanktonoban, Oban, UK) were grown on f/2 medium (Guillard and Ryther, 1962) modified as described previously (Nimer et al., 1997). Cultures were grown at 15° ± 1°C with a PFD of 100 μmol m^{-2} s^{-1} at the culture surface provided by cool-white fluorescent tubes. Cells were grown in inorganic carbon-replete conditions (2.0 mM total DIC, pH 8.3) and, when required, cells were resuspended in 1.0 mM total DIC at pH 8.3 or 2.0 mM total DIC at pH 9.0 (i.e. inorganic carbon or CO_2-limited conditions).

Measurement of Alkalinity, Total DIC, and the Calculation of Free CO_2

We followed procedures described previously (Parsons et al., 1989; Merrett et al., 1996) to measure alkalinity and we measured pH with a digital pH meter (Hanna Instruments, Woonsockett, RI). Total DIC was calculated from carbonate alkalinity and pH (McConnaughey, 1991), and the free CO_2 concentration was calculated as described previously (Dong et al., 1993).

Measurement of Extracellular CA Activity

Intact cells were harvested by centrifugation at 1500*g*, washed once, and resuspended in 25 mM barbitol buffer. Intact cells were assayed for extracellular CA by an electrometric method described previously (Dixon et al., 1987; Nimer et al., 1997).

Inorganic Carbon-Dependent Photosynthetic Oxygen Evolution

Inorganic carbon-dependent, photosynthetic oxygen evolution was measured using a Clark-type oxygen electrode (Hansatech, King's Lynn, UK) as described previously (Nimer and Merrett, 1992, 1996). Intact cells were resuspended in 300 mM sorbitol and 25 mM Hepes (pK_a 7.5) to pH 8.3, and Hepes was replaced by 25 mM citric acid/trisodium citrate buffer for pH 5.0. Cells were allowed to deplete all endogenous carbon sources (measured by the cessation of oxygen evolution). We measured the rate of oxygen evolution after the addition of various concentrations of $KHCO_3$.

Inorganic ^{14}C Uptake and Photosynthesis

Intact cells were harvested, washed twice, and resuspended in 300 mM sorbitol and 25 mM Hepes at pH 8.3. We placed the cells in an oxygen electrode chamber and allowed them to deplete all endogenous carbon sources. We measured inorganic carbon uptake and photosynthesis after the addition of the required $Na^{14}CO_3$ (Amersham) at a specific activity of 5.6 × 10^8 Bq/mol, as described previously (Badger et al., 1980; Nimer et al., 1992; Nimer and Merrett, 1996). We used 5 μM [^{14}C]inulin and ^3H_2O to estimate the intracellular water space and the free water space taken down with the cells through the silicone oil. Separate incubation times for each were 20 s (time needed for equilibration). We determined the ^{14}C and the ^3H in the pellet by liquid scintillation counting (Beckman) and the intracellular space (inulin impermeable) as total water minus the inulin-permeable space volume.

Measurement of Intracellular pH

Cells were loaded for 30 min with the fluorescence indicator SNARF (10 μM carboxy-SNARF-1-acetomethylester, Molecular Probes, Eugene, OR). The fluorescence properties of this dye are such that the ratio of fluorescence emission at 630 and 590 nm is pH dependent (Seksek et al., 1991). Cells were settled on a coverslip pretreated with poly-L-Lys (Anning et al., 1996). Single cells were observed with a confocal laser-scanning microscope (model MRC 1024, Bio-Rad). Fluorescence emission was recorded following excitation at 488 nm. We obtained ratio images (630/590) of dye-loaded cells using time-course image-analysis software (Bio-Rad). The fluorescence intensities of the cytosol and the chloroplast from the ratio images were measured and compared with fluorescence ratio images of an "in vitro" calibration, which we obtained from buffered media containing 10 μM SNARF-free acid at the required pH. A good agreement between "in vivo" and "in vitro" calibrations was established previously (Anning et al., 1996). We located the position of the chloroplast by monitoring its autofluorescence at 515 nm emission. A 50-W ellipsoid halogen-reflector bulb (Philips, Eindhoven, The Netherlands) provided light during the imaging of the cells.

We used an improved Neubauer hemocytometer (Weber Scientific International, Cambridge, UK) to determine cell number. A stock solution of DBS (Synthelic, Lund, Sweden) was prepared in double-distilled water and used at a final concentration of 200 μM. DCMU (Sigma) was dissolved in 96% ethanol to give a stock solution of 200 mM and used at a final concentration of 50 μM.

RESULTS

Photosynthetic Rate and Intracellular DIC Accumulation

The rate of inorganic carbon-dependent photosynthetic oxygen evolution was measured at different DIC concentrations, and the affinity of the cells for DIC was determined by calculating the concentration of DIC required to give the half-maximal rate of photosynthetic oxygen evolution, $K_{0.5}$[DIC]. The maximum rate of photosynthetic oxygen evolution was unaffected by external pH (Fig. 1, A and B). At pH 8.3 the $K_{0.5}$[DIC] was 750 μM, whereas at pH

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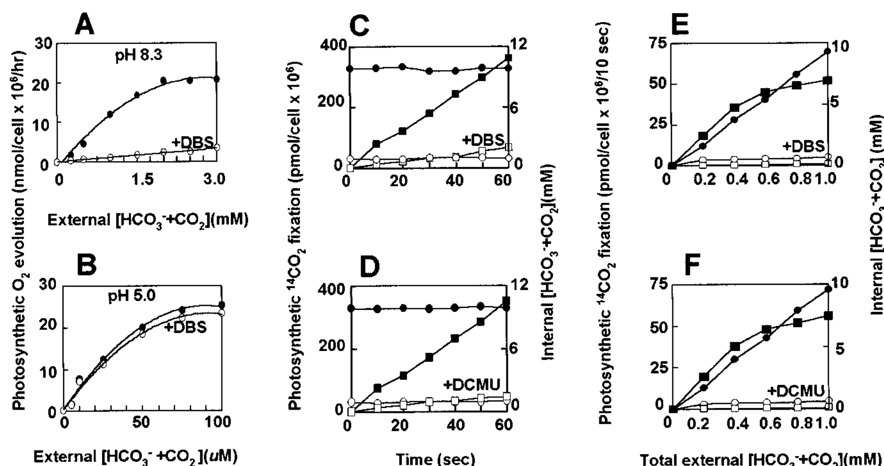

Figure 1. A and B, Rates of photosynthetic oxygen evolution in response to external DIC concentration at pH 8.3 (A) and pH 5.0 (B) in the absence (●) and presence (○) of DBS. C to F, Internal inorganic carbon concentration by *P. micans* in the absence (●) and presence (○) of DBS (C and E) and in the absence (●) and presence (○) of DCMU (D and F) and photosynthetic $^{14}CO_2$ fixation in the absence (■) and presence (□) of DBS (C and E) and in the absence (■) and presence (□) of DCMU (D and F). The final cell concentration was 2.5×10^6 cells mL^{-1}. In C and D, the intracellular water space was 0.87 μL and the total water space was 1.75 μL; in E and F, the intracellular water space was 0.53 μL and the total water space was 1.08 μL (n = 3). In A and B, the temperature was 15°C ± 1°C, and the PFD was 500 μmol m^{-2} s^{-1}; in C and D, the pH was 8.3, the temperature was 15°C ± 1°C, and the PFD was 500 μmol m^{-2} s^{-1}; in E and F, the temperature was 15°C ± 1°C and the PFD was 500 μmol m^{-2} s^{-1}.

5.0 it was 25 μM. These results are consistent with the belief that CO_2 is the species of inorganic carbon that crosses the plasma membrane. We used DBS, a membrane-impermeable inhibitor of CA, to investigate the role of extracellular CA in DIC utilization. At pH 8.3, DIC-dependent photosynthetic oxygen evolution was 90% inhibited by DBS, even at a high external DIC concentration (i.e. 3.0 mM). At pH 5.0 photosynthetic oxygen evolution was unaffected by the presence of DBS.

These results suggest a major role for extracellular CA at an alkaline pH when the available free CO_2 concentration is lower, simulating the conditions in the marine environment. This role of extracellular CA was confirmed by measuring the extracellular CA and the photosynthetic rate at pH 8.3 (2.0 mM total DIC and 9.0 μM free CO_2) and pH 9.0 (2.0 mM total DIC and 1.0 μM free CO_2) (Table I). Cells of *P. micans* maintained an almost constant photosynthetic rate at pH 8.3 and 9.0 (Table I), although the free CO_2 concentration was much lower at pH 9.0 (Table I). At pH 8.3 and 9.0, DBS effectively inhibited extracellular CA activity and DIC-dependent photosynthetic oxygen evolution (Table I). At pH 9.0 extracellular CA activity was more than 60% greater than at pH 8.3 (Table I).

Table I. *Photosynthetic oxygen evolution in relation to extracellular CA activity in P. micans grown at 2.0 mM DIC, pH 8.3, and resuspended under the conditions indicated at a PFD of 500 μmol m^{-2} s^{-1} at 15°C*

Values are ±SE; n = 4.

Condition	Photosynthetic Oxygen Evolution	External CA
	nmol 10^6 $cells^{-1}$ h^{-1}	*EU (10^6 $cells^{-1}$)*
pH 8.3		
2.0 mM total DIC	20.47 ± 0.92	0.25 ± 0.01
2.0 mM total DIC plus DBS	2.31 ± 0.20	0.03 ± 0.00
pH 9.0		
2.0 mM total DIC	19.78 ± 0.53	0.41 ± 0.03
2.0 mM total DIC plus DBS	2.11 ± 0.13	0.05 ± 0.00

Effect of DBS on Photosynthetic Rate and Inorganic Carbon Uptake

Measurement of DIC uptake at pH 8.3 by the silicone oil centrifugation technique showed that a rapid steady state was achieved between the internal inorganic carbon pool of the cells and DIC in the external medium (Fig. 1, C and D). Over the same period, photosynthetically fixed $^{14}CO_2$ showed a linear increase with time (Fig. 1, C and D). The inhibitors DBS (Moroney et al., 1985; Nimer and Merrett, 1996; Nimer et al., 1998) and DCMU were equally effective in blocking the transport of inorganic carbon into the cell and the photosynthetic fixation of $^{14}CO_2$ (Fig. 1, C and D).

The rate of photosynthetic $^{14}CO_2$ fixation increased over a range of external DIC concentrations until nearby inorganic carbon saturation of photosynthesis was observed at 1.0 mM external DIC (Fig. 1, E and F). DBS and DCMU were equally effective in inhibiting the photosynthetic rate and the accumulation of inorganic carbon within the cell and over a range of external DIC concentrations (Fig. 1, E and F). At all of the external DIC concentrations used, the average intracellular inorganic carbon concentration was 10-fold greater than the DIC concentration outside the cells (Fig. 1, E and F).

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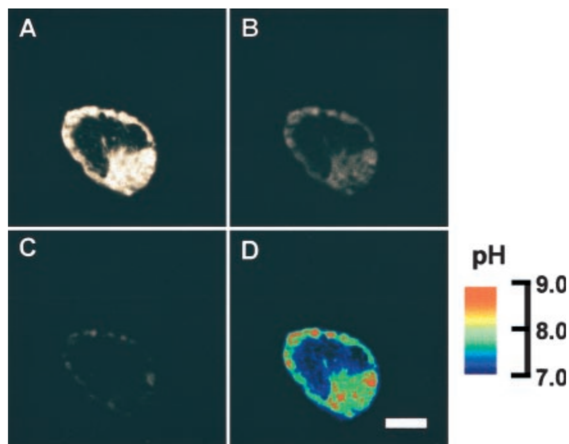

Figure 2. Confocal microscope image of SNARF-loaded *P. micans* cell displaying the distribution of the dye in subcellular compartments. A, Emission at 630 nm; B, emission at 590 nm; C, chloroplast autofluoresence at 515 nm; and D, 630/590 florescence ratio. Bar = 10 μm.

Intracellular pH

Confocal images of SNARF-loaded *P. micans* cells show the distribution of the pH-sensitive dye in subcellular compartments (Fig. 2). The position of the chloroplast was determined by autofluoresence (Fig. 2C). The chloroplast autofluoresence and the distribution of SNARF fluorescence (Fig. 2) were comparable to the chloroplast multilobular arrangement and position observed in transmission electron microscope images of *P. micans* (Vesk and Jeffrey, 1977; Dodge and Greuet, 1987). Both the chloroplast and the cytosol were successfully loaded with SNARF, but the dye was excluded from the vacuoles. The ratio image (Fig. 2D) shows the ability to determine pH in the chloroplast and the cytosol. In the steady-state cells under inorganic carbon-replete conditions (2.0 mM total DIC and external pH 8.3), the average chloroplast and cytosol pH were 8.3 ± 0.13 and 7.7 ± 0.11, respectively (Fig. 2D).

Inorganic Carbon Concentration and Intracellular Homeostasis

When cells photosynthesizing under steady state were DIC limited (1.0 mM total DIC, pH 8.3), the average cytosolic and chloroplast pH decreased to 6.9 ± 0.10 and 7.14 ± 0.12, respectively (Fig. 3A). The re-addition of HCO_3^- (2.0 mM DIC final concentration) to these cells led to the rapid re-establishment of the steady-state pH values in the cytosol and chloroplast (Fig. 3, B–D). This response was abolished in the presence of DBS (Fig. 3, E–H). Preincubating the cells with DCMU significantly lowered the pH of the cytosol and chloroplast (Fig. 3I). In the presence of DCMU, the addition of HCO_3^- to inorganic carbon-limited cells did not result in the recovery of the chloroplast and cytosol pH (Fig. 3, J–L).

Intracellular pH, Internal Inorganic Carbon Pool, and Photosynthesis

To observe the immediate response of intracellular pH to HCO_3^- concentration and inhibitors, ratio images were

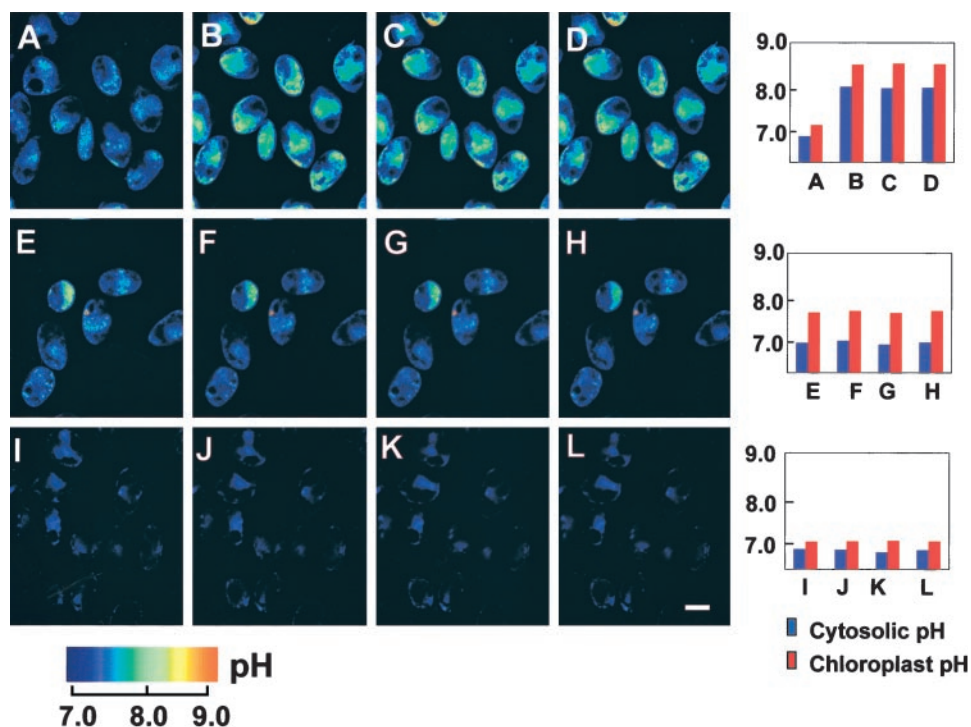

Figure 3. Cytosolic and chloroplast pH in relation to DIC uptake and photosynthesis in *P. micans*. A to D, Addition of DIC to inorganic carbon-limited cells; E to H, addition of DIC to inorganic carbon-limited cells in the presence of DBS; and I to L, addition of DIC to inorganic carbon-limited cells in the presence of DCMU. Bar = 10 μm.

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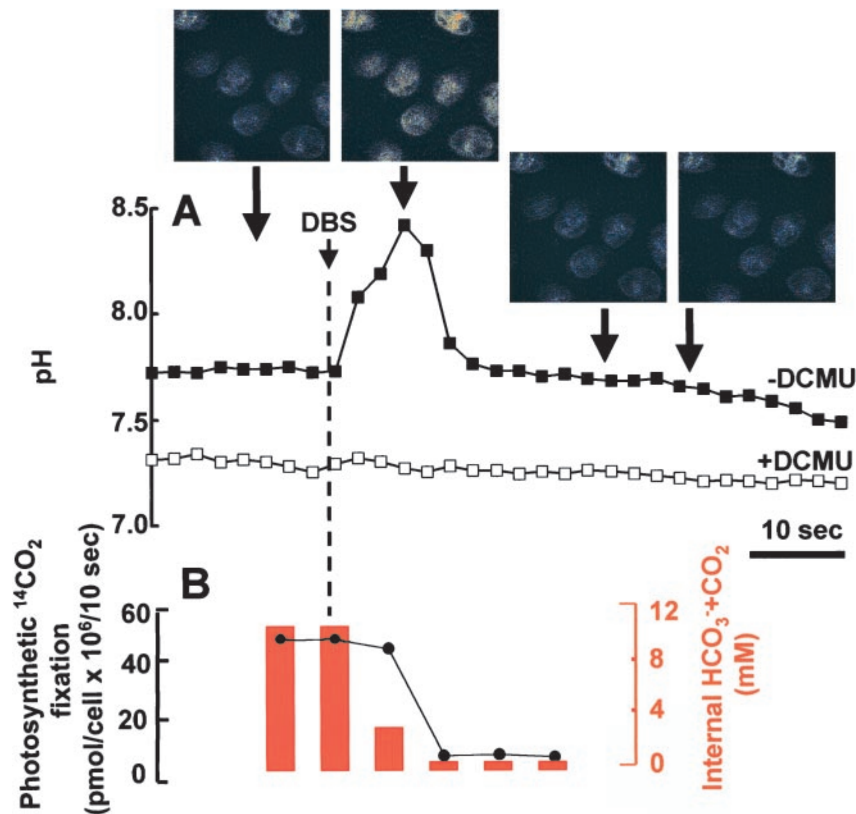

Figure 4. A, Immediate effect of DBS addition on transient intracellular pH shown by a time-course experiment with ratio images recorded every 2 s in the absence (■) and presence (□) of DCMU. B, Immediate effect of DBS addition on the internal inorganic carbon concentration (red bars) and photosynthetic $^{14}CO_2$ fixation (●). Bar = 10 s.

collected at 2-s intervals (Fig. 4). Direct images were recorded for the rapid measurement of transient intracellular pH (in seconds), because the filtering of images was not possible. The addition of DBS by a custom-made diffusion system to photosynthesizing cells resulted in an immediate transient increase of average intracellular pH from 7.7 to almost 8.5, followed by a steady decline (Fig. 4A). We did not observe this transient change in intracellular pH in the presence of DCMU (Fig. 4A). The intracellular inorganic carbon concentration and the photosynthetic $^{14}CO_2$ were measured in parallel experiments (Fig. 4B). After the addition of DBS, a decrease in the intracellular inorganic carbon pool was measured within 4 s. The photosynthetic $^{14}CO_2$-fixation rate was maintained for 4 s but had declined to near zero by 8 s (Fig. 4B). The time course for transient intracellular pH during DBS inhibition closely paralleled the time course for DIC depletion, whereas the photosynthetic rate was only briefly maintained.

DISCUSSION

Marine phytoplankton species acquire inorganic carbon for photosynthesis from the DIC of seawater. Within the pH range of seawater (8.0–8.3), the bulk of total DIC is HCO_3^-, with CO_2 being less than 1% of the total (Skirrow, 1975). Although physical parameters (particularly pH, temperature, and salinity) in the marine environment affect the free CO_2 concentration (Raven, 1995), it is always well below the $K_m[CO_2]$ of Rubisco in those algal species for whichRubisco has been characterized (Colman and Rotatore, 1995; Raven, 1997b). This suggests that, in marine phytoplankton species that rely solely on the diffusive entry of CO_2 from the bulk phase of the medium to Rubisco in the cell, ambient CO_2 concentrations may be limiting for photosynthesis (Talling, 1976; Raven and Geider, 1988; Reibesell et al., 1993). This problem is circumvented in many phytoplankton species that have evolved strategies of inorganic carbon acquisition that require either the direct uptake of HCO_3^- across the plasma membrane (Merrett et al., 1996; Nimer et al., 1997; Tortell et al., 1997) and/or the indirect use of HCO_3^- through the catalytic production of CO_2 by exofacial CA (Nimer et al., 1997, 1998). The inhibition of extracellular CA by DBS and the concomitant reduction of the photosynthetic rate in *P. micans* (Table I) provides the most unequivocal evidence to date that indirect HCO_3^- use can provide the bulk of CO_2 fixed in photosynthesis by a marine phytoplankton species.

Of the marine phytoplankton species investigated thus far, most possess a CCM (Raven, 1991), the presence of which is characterized by two distinctive features: (a) the intracellular accumulation of DIC being several times that of the external medium during photosynthesis (Badger et al., 1980; Burns and Beardal 1987; Colman and Rotatore, 1995) and (b) the $K_{0.5}[CO_2]$ of the cells being substantially lower than the $K_m[CO_2]$ of Rubisco (Raven and Johnston, 1991). The intracellular accumulation of inorganic carbon in a dinoflagellate relative to the external medium, shown in this study for the first time to our knowledge (Fig. 1), provides convincing evidence for the presence of a CCM in *P. micans*. The $K_{0.5}[CO_2]$ at pH 5.0 for *P. micans* (Fig. 1) is

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substantially below the $K_m[CO_2]$ that would be expected if the cells possessed a type-II Rubisco (Whitney and Andrews, 1998). The increase in average intracellular pH after the addition of HCO_3^- to inorganic carbon-limited cells could arise in response to the stimulation of photosynthesis caused by increased CO_2 availability or by direct HCO_3^- influx and accumulation; however, both processes would result in an alkaline pH, a prerequisite for the substantial accumulation of DIC.

The measurement of intracellular pH (Fig. 4) suggests that in *P. micans* the accumulation of inorganic carbon probably occurs mainly in the chloroplast, as shown for *Chlamydomonas reinhardtii* (Moroney and Mason, 1991); this accumulation can arise via CO_2 uptake with "alkaline trapping," as HCO_3^- in the plastid. The presence of DBS sharply decreases the accumulation of intracellular inorganic carbon, the photosynthetic rate (Fig. 1), and the ability of cells to re-establish steady-state intracellular pH values (Fig. 3), reinforcing the major role of extracellular CA in facilitating the availability of CO_2 at the exofacial surface of the plasma membrane.

The transient increase in intracellular pH after the addition of DBS may result from the use of the intracellular inorganic carbon pool in photosynthesis, causing alkalization of the cytosol and the chloroplast. In the presence of DBS, another contributory factor affecting cytosolic pH is the absence of CO_2 transport and conversion to HCO_3^- in the cytosol (Volokita et al., 1984). When the photosynthetic rate decreases due to depletion of the intracellular inorganic carbon pool, a decrease in intracellular pH may result from excess reducing equivalents in the cell under conditions of inorganic carbon limitation in the chloroplast. Marine phytoplankton species (Moroney et al., 1985; Jones and Morel, 1988) and other cells (Rubinstein and Luster, 1993) possess a plasma membrane redox chain. The rate of transfer of reducing equivalents to the exterior surface of the plasma membrane is dependent on the inorganic carbon status of the photosynthetic cell (Moroney et al., 1985). The plasma membrane redox system may fuel a transmembrane proton pump (Jones and Morel, 1988), providing an essential component of a CCM through the maintainance of extracellular CA in the form needed to catalyze the dehydration of HCO_3^- (Coleman, 1984; Nimer et al., 1998), although active transport at the chloroplast envelope remains a possibility (Moroney and Mason, 1991).

Extracellular CA is constitutive in most of the dinoflagellates tested, including *P. micans* (Nimer et al., 1997), whereas in other marine phytoplankton species, the development of extracellular CA activity is a response to very low concentrations of CO_2 (Iglesias and Merrett, 1997; Nimer at al., 1997). This suggests that in a dinoflagellate such as *P. micans* there is a need for a CCM even at ambient CO_2 concentrations. This may reflect a difference in the specificity factor of Rubisco in some dinoflagellates (Whitney and Andrews, 1998) compared with other algal phyla (Delgado et al., 1995; Raven, 1997a). The development of dinoflagellate blooms occurs under conditions of low turbulence and high temperature (Holligan, 1985; Steindinger and Vargo, 1988), which results in low ambient free CO_2 concentrations compared with the open-ocean conditions under which a CCM may be a prerequisite. Therefore, the CCM may be one of several factors that contribute to the existence of near-monospecific dinoflagellate blooms over thousands of miles of coastal waters lasting from weeks to months under conditions of CO_2 limitation (Steindinger and Vargo, 1988).

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