An External δ-Carbonic Anhydrase in a Free-Living Marine Dinoflagellate May Circumvent Diffusion-Limited Carbon Acquisition\textsuperscript{1}[W]

Mathieu Lapointe\textsuperscript{2,3}, Tyler D.B. MacKenzie\textsuperscript{2,4}, and David Morse\textsuperscript{*}

Institut de Recherche en Biologie Végétale, Département de Sciences Biologiques, Université de Montréal, Montreal, Quebec, Canada H1X 2B2

The oceans globally constitute an important sink for carbon dioxide (CO\textsubscript{2}) due to phytoplankton photosynthesis. However, the marine environment imposes serious restraints to carbon fixation. First, the equilibrium between CO\textsubscript{2} and bicarbonate (HCO\textsubscript{3}\textsuperscript{−}) is pH dependent, and, in normal, slightly alkaline seawater, [CO\textsubscript{3}] is typically low (approximately 10 μM). Second, the rate of CO\textsubscript{2} diffusion in seawater is slow, so, for any cells unable to take up bicarbonate efficiently, photosynthesis could become carbon limited due to depletion of CO\textsubscript{2} from their immediate vicinity. This may be especially problematic for those dinoflagellates using a form II Rubisco because this form is less oxygen tolerant than the usually found form I enzyme. We have identified a carbonic anhydrase (CA) from the free-living marine dinoflagellate Lingulodinium polyedrum that appears to play a role in carbon acquisition. This CA shares 60% sequence identity with δ-class CAs, isoforms so far found only in marine algae. Immuno-electron microscopy indicates that this enzyme is associated exclusively with the plasma membrane. Furthermore, this enzyme appears to be exposed to the external medium as determined by whole-cell CA assays and vectorial labeling of cell surface proteins with \textsuperscript{125}I. The fixation of 14CO\textsubscript{2} is strongly pH dependent, suggesting preferential uptake of CO\textsubscript{2} rather than HCO\textsubscript{3}−, and photosynthetic rates decrease in the presence of 1 μM acetazolamide, a non-membrane-permeable CA inhibitor. This constitutes the first CA identified in the dinoflagellates, and, taken together, our results suggest that this enzyme may help to increase CO\textsubscript{2} availability at the cell surface.

Oceanic phytoplankton contribute roughly one-half of the total global photosynthetic carbon fixation (Behrenfeld et al., 2006), despite the fact that availability of dissolved CO\textsubscript{2} (the direct substrate of Rubisco) in the marine environment is generally low (approximately 10 μM). Indeed, most of the dissolved inorganic carbon (DIC) in the ocean is bicarbonate (approximately 2 mM HCO\textsubscript{3}−), and thus phytoplankton have had to develop a panoply of mechanisms to cope with the critical step of acquiring carbon from the environment (Giordano et al., 2005). The situation is particularly difficult for phytoplankton that lack the capacity to transport HCO\textsubscript{3}− because dehydration of HCO\textsubscript{3}− to CO\textsubscript{2} is generally considered to be slow (uncatalyzed half-life approximately 1 min) and the diffusion of CO\textsubscript{2} in aqueous solution is much slower than in air. Rapid photosynthesis by these phytoplankton can potentially remove CO\textsubscript{2} from their immediate surroundings faster than it can be replenished, potentially limiting carbon fixation by carbon availability (Riebesell et al., 1993).

Dinoflagellates are a major constituent of the oceanic phytoplankton and, unlike all other eukaryotic phytoplankton, use a form II Rubisco as their primary carbon-fixing enzyme (Morse et al., 1995). Dinoflagellate form II Rubisco has a lower affinity and lower CO\textsubscript{2}/O\textsubscript{2} specificity than the more common form I Rubisco (Whitney and Andrews, 1998), suggesting net carbon fixation in dinoflagellates should be unfavorable at the low ambient CO\textsubscript{2} levels in the marine environment. This indicates these cells probably have sophisticated biophysical mechanisms to increase CO\textsubscript{2} concentration within the cell to assure an adequate carbon supply (Giordano et al., 2005).

The enzyme carbonic anhydrase (CA; EC 4.2.1.1), which mediates the rapid interconversion of CO\textsubscript{2} and HCO\textsubscript{3}−, is an important part of most mechanisms used to alleviate the restriction of CO\textsubscript{2} near Rubisco in marine phytoplankton. Most directly, CA within the chloroplast would act to maintain the equilibrium concentration of CO\textsubscript{2} at stromal pH and thus provide a source of CO\textsubscript{2} formation in the vicinity of Rubisco (Jacobson et al., 1975). Alternatively, many algae have developed some form of a CO\textsubscript{2}-concentrating mechanism (CCM) to transport inorganic carbon into the cell (Giordano et al., 2005). CCMs can take many forms, but often include CA to increase the rate of intercon-

\textsuperscript{1} This work was supported by the National Science and Engineering Research Council (NSERC) of Canada (grant no. 171382-03). T.D.B.M. was the beneficiary of an NSERC postdoctoral fellowship.
\textsuperscript{2} These authors contributed equally to the article.
\textsuperscript{3} Present address: Département de Biologie Cellulaire, Université de Montréal, Montreal, Quebec, Canada H1X 2B2.
\textsuperscript{4} Present address: Monroe Community College, Rochester, NY 14623.
\textsuperscript{*} Corresponding author; e-mail david.morse@umontreal.ca.

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\textsuperscript{[W]} The online version of this article contains Web-only data. www.plantphysiol.org/cgi/doi/10.1104/pp.108.117077
version of CO₂ and HCO₃⁻ in different intra- and extracellular spaces. For example, HCO₃⁻ transported into the acidic thylakoid lumen could be efficiently transformed by a lumenal CA to CO₂, which could then diffuse freely out into the stroma (Raven, 1997). In addition, intracellular CAs could trap CO₂ within compartments by converting it into membrane-impermeable HCO₃⁻ (Rumeau et al., 1996). Last, an extracellular CA (eCA) located on the outer surface of the plasma membrane could accelerate the conversion of the abundant HCO₃⁻ in seawater, in the immediate vicinity of the cell, to CO₂, which could freely diffuse into the cell. CA activity is so important for carbon metabolism that most algae likely employ several different CA isoforms acting in concert to ensure an adequate supply of CO₂ to Rubisco (Sülttemeyer, 1998).

CA activity is distributed in phytoplankton (Moroney et al., 2001) and has been found within all studied species of dinoflagellates, including symbiotic species (Leggat et al., 2002) and those species free living in marine (Nimer et al., 1997, 1999; Leggat et al., 1999; Dason et al., 2004; Rost et al., 2006; Ratti et al., 2007) or freshwater (Berman-Frank et al., 1994, 1995) environments. eCA activity is less common, but has been reported in several species of phytoplankton from diverse groups, including some species of dinoflagellates (Berman-Frank et al., 1995; Nimer et al., 1997, 1999). These eCA activities are generally observed only under carbon-limited conditions, although it has been reported that several marine dinoflagellates displaying eCA activity when carbon limited also had a low constitutive eCA activity in carbon-replete conditions (Nimer et al., 1997). The requirement for this constitutive eCA was ascribed to the presence of form II Rubisco.

All CA enzymes have remarkably high catalytic rates and are able to greatly accelerate the interconversion of HCO₃⁻ ions and CO₂. There are four main, yet evolutionarily unrelated, families of CA (α-, β-, γ-, δ-types) distributed among bacteria, animals, and plants (Tripp et al., 2001). All are obligate metalloenzymes because removal of the normally bound Zn²⁺ by dialysis leads to inactivity. Interestingly, the δ-CA has so far been reported only in marine algae (Roberts et al., 1997; Tripp et al., 2001; Soto et al., 2006). A new family, termed ζ-CA, has recently been described that binds Cd²⁺ instead of Zn²⁺ (Lane et al., 2005) and also lacks sequence homology to other CA types. This latter isoform has also been documented in cyanobacteria (So et al., 2004).

We report here the identification and characterization of a dinoflagellate δ-CA. This enzyme is located on the external face of the plasma membrane and CA activity in whole cells is susceptible to the poorly membrane-permeable CA inhibitor acetzolamide (AZ). Carbon fixation rates are also sensitive to AZ, suggesting that the δ-CA may generate membrane-permeable CO₂ from the more abundant HCO₃⁻ at the cell periphery to support an increased photosynthetic rate. This constitutes the first CA identified from the dinoflagellates.

RESULTS

Lingulodinium Expresses a δ-CA Isoform

Two different CA cDNA sequences (LpCA1 [1.03 kb] and LpCA2 [1.18 kb]) were isolated from a Lingulodinium polyedrum cDNA library by colony hybridization using a PCR product with sequence homology to a δ-CA from the diatom Thalassiosira weissflogii (TWCA1). Western blot performed with an antibody raised against TWCA1 (Roberts et al., 1997) and affinity purified against the LpCA2 expressed in bacteria showed only a single band of 31 kD in extracts from Lingulodinium (Fig. 1A, lane 1). Because the LpCA2 sequence detects a polyadenylated RNA of roughly 1,600 nucleotides in Lingulodinium RNA (Fig. 1B), the original clones were not complete. To attempt to recover the remaining 5' sequence, we performed nested PCR using internal sequences from either LpCA1 or LpCA2 cDNA and the 5'-trans-sliced leader common to all dinoflagellate mRNA (Zhang et al., 2007). Although this procedure was not successful for LpCA1, the complete 5' end was recovered for LpCA2. The LpCA1 and LpCA2 shared 80% amino acid sequence identity in the region of overlap and between 58% and 62% amino acid sequence identity with TWCA1 (Supplemental Fig. S1).

Both LpCA1 and LpCA2 have homologs with incomplete sequences in EST banks derived from the dinoflagellates Karlodinium micrum, Karenia brevis, Alexandrium tamarense, and L. polyedrum. Interestingly, no sequences were recovered from the dinoflagellate Amphidinium carterae, in agreement with the observation that no δ-CA was detected in extracts from A. carterae using the affinity-purified antibody (Fig. 1A, lane 2).

Figure 1. Lingulodinium expresses a δ-CA similar to that of haptophytes. A, Affinity-purified antibody raised against Thalassiosira δ-CA reacts with a 31-kD protein from Lingulodinium on western-blot analysis. B, The Lingulodinium δ-CA is expressed as a roughly 1.6-kb RNA by northern-blot analysis. C, Phylogenetic analysis places the two Lingulodinium δ-CAs close to those of haptophytes (Emiliania huxleyi and Isochrysis galbana) and stramenopiles (T. weissflogii) and further from that of a green alga (Micromonas sp.).
exhaustive search of the EST sequence databanks has also revealed homologous sequences in chlorophytes, haptophytes, and stramenopiles. To address the evolutionary origin of LpCA, phylogenetic reconstructions were performed with all the full-length sequences obtained. Unlike nuclear gene phylogenies, which place the stramenopiles closest to the dinoflagellates among the species illustrated (Harper et al., 2005), the δ-CA phylogeny places the dinoflagellate CA closest to that found in haptophytes (Fig. 1C).

Lp-δ-CA Is Associated with the Plasma Membrane

The deduced amino acid sequence of LpCA2 contains an N-terminal signal peptide followed by a signal peptidase site (Fig. 2A), and SignalP and Phobius both predict the protein will be directed to the secretory pathway. The location of the δ-CA was tested experimentally by first comparing the distribution of LpCA immunoreactivity in cell extracts separated into soluble and particulate fractions by low-speed centrifugation. In contrast to the distribution of the plastid stromal enzyme Rubisco, which appears soluble due to breakage of the plastids during extraction (Wang et al., 2005b), the affinity-purified anti-δ-CA reacted only with the particulate fraction (Fig. 2B). This particulate fraction contains primarily cell wall and cell membrane fragments, as well as contaminating plastid thylakoid membranes.

Interestingly, whereas the bulk of the CA activity is obtained in the soluble fraction, activity can also be measured in intact cells. The activity in intact cells represents approximately 15% of the activity found in the soluble fraction and is thus unlikely to result from contamination by the soluble fraction because <1% of the cells are broken during the assay as assessed by microscopic examination of the sample. The activity found in the particulate fraction (approximately one-third of the CA activity found in intact cells; Fig. 2C) is also unlikely to result from contamination by the soluble fraction because the particulate fraction contains <5% of the anti-Rubisco signal observed in the soluble fraction (Fig. 2B).

To directly visualize the subcellular distribution of the δ-CA, the affinity-purified antibody was used for immunoelectron microscopy. The cellulose cell wall is found in membranous sacs, called alveolae, immediately below the plasma membrane. However, antibody reaction was detected only with the plasma membrane (Fig. 2D, black arrows) rather than the membrane of the alveolae (white arrow). No significant label is observed over any other compartments, including the chloroplast.

Lp-δ-CA Is Exposed to the External Medium

To confirm that the Lp-δ-CA was exposed at the external face of the plasma membrane, intact cells were labeled with ^{125}I. Because previous labeling experiments using an 8 M urea extraction revealed only a single radiolabeled protein of 43 kD (Bertomeu et al., 2003), for these experiments additional extraction pro-

![Figure 2](image-url)

**Figure 2.** Lp-δ-CA is associated with the plasma membrane. A, A 136-amino acid N-terminal extension shows a predicted signal peptide (underlined) followed by a signal peptidase site (asterisk). The sequence is rich in acidic (−) and hydroxylated (α) amino acids. B, Fractionation of intact extracts (I) into a 12,000g pellet (P) and supernatant (S) shows a reaction with an antibody raised against δ-CA (α-CA) only in the insoluble fraction, in contrast to the immunoreactivity observed with an antibody raised against form II Rubisco (α-Rubisco). C, CA activity in whole cells, supernatant, and pellet fractions. D, Immunocytochemical labeling with affinity-purified anti-CA and a 20-nm gold-labeled secondary antibody. Label is associated with the plasma membrane (arrowheads) rather than the alveolar membrane (white arrow) surrounding the cellulosic wall plates (w), the cytoplasm (c), the plastid (p), or the vacuole (v). Scale bar = 1 μm. No label is present when the primary antibody is omitted from the procedure.
tocols were tested. We found that extraction of the labeled cell wall fraction using 2% SDS revealed several additional radiolabeled proteins, including one comigrating with the anti-δ-CAT signal (Fig. 3A). The same protein sample, prepared for two-dimensional PAGE (2D-PAGE) by a CHCl₃-methanol precipitation, also showed comigration of a radiolabeled protein for each of the four δ-CAT isoforms resolved by 2D electrophoresis (Fig. 3B). The relative staining intensity between the four different CA isoforms detected by the antibody differs when compared to the radiolabel incorporation, presumably due to the distribution of Tyr residues available for iodination on each protein. Clearly, the δ-CAT on the cell surface is exposed to the external environment, although these results cannot distinguish between LpCA1 and LpCA2.

To test the possibility that the active site of the δ-CAT was exposed to the external medium, the amount of immunoreactive protein was compared with intact cell CA activities for cultures grown at two different pHs. Algae grown at alkaline pH often have increased external CA activity, presumably due to a decrease in the ambient [CO₂] (Williams and Colman, 1996; Elzenga et al., 2000), and this is also the case for Lingulodinium (Fig. 3D, gray bars). When the amount of immunoreactive δ-CAT was measured by western blots (Fig. 3, C and D, white bars), Lingulodinium grown at pH 9 was found to contain twice the immunoreactive δ-CAT than cells grown at pH 8.2. The increase in intact cell activity is again not due to differential cell breakage because both samples show <1% broken cells.

The non-membrane-permeable CA inhibitor AZ (Moroney et al., 1985; Colman et al., 2002) is often used to test for the presence of an external CA. We thus anticipated, based on the exposure of the δ-CAT to the external medium, that AZ might inhibit CA activity in intact cells. Indeed, AZ greatly reduced the CA activity in intact cells (Fig. 3D, black bar).

### External CA Activity Is Required for Efficient CO₂ Acquisition at [CO₂] Near the Kₘ

Cells that take up CO₂ from their environment would be predicted to exploit an external CA to increase carbon acquisition by accelerating the formation of CO₂ from HCO₃⁻ in the surrounding seawater, thereby regenerating external [CO₂] as it is used by the photosynthetic machinery. To discriminate between HCO₃⁻ and CO₂ uptake, whole-cell photosynthetic carbon fixation rates were measured as a function of the pH, which alters the equilibrium concentrations of the HCO₃⁻ and CO₂ components of DIC (Schulz et al., 2006). At typical seawater pH, HCO₃⁻ is the most abundant component of DIC and the [CO₂] is relatively low; the [CO₂] increases markedly as the pH decreases. Our assays, which consist of 30-min incubations in different pH buffers, expose the cells to a dramatic range of [CO₂] without appreciable change in the relatively large [HCO₃⁻]. The equilibrium to a new [CO₂] when cell cultures are added to concentrated buffer at the desired pH is expected to occur on a time scale of tens of seconds (Zeebe et al., 1999; Schulz et al., 2006).

In algae, which preferentially use CO₂ for carbon uptake (Elzenga et al., 2000), photosynthetic activity is expected to decrease at the low [CO₂] found at alkaline pH; extrapolation to zero [CO₂] should show very low carbon fixation rates. To test this for Lingulodinium, an initial series of assays was performed using centrifugation both to concentrate cell cultures roughly 10-fold and to replace the culture medium with buffer containing known concentrations of DIC. By using a range of DIC concentrations at three different pHs, cells were exposed not only to ranges of [HCO₃⁻] and pH, but also to a wide range in [CO₂], which was determined from the combinations of DIC and pH. This approach thus allows effects of DIC and pH from effects of [CO₂]...
on photosynthetic rate to be distinguished. At each DIC concentration, carbon fixation rates were indeed lower at alkaline pH (Fig. 4A). More importantly, when [CO₂] is calculated for each experimental condition and the data replotted on an axis of [CO₂], we observed that the photosynthetic rate tends toward zero when [CO₂] approaches zero (Fig. 4B), despite the large reservoir of DIC in the form of HCO₃⁻. This suggests that CO₂ is the preferred substrate for carbon fixation. In addition, we observed that carbon fixation rates using different combinations of pH and DIC only follow a single curve when plotted against calculated [CO₂], indicating that [CO₂] rather than DIC or pH alone constrains carbon fixation rates and that the kinetic parameters of carbon fixation are unlikely to be affected by pH independent from the known effect of pH on [CO₂].

In assays using centrifuged cells, however, the cells rapidly fall to the bottom of the microtiter plate wells, raising the concern that carbon acquisition by the cells might be reduced by an unusual crowding of the cells and a resulting decrease in cell surface area exposed to the medium. This potential problem would be exacerbated by the use of cells concentrated far beyond their normal culture conditions, which could potentially remove CO₂ from their surroundings more rapidly. To most accurately model [CO₂] near the cell and the effect of inhibiting eCA, we therefore decided to measure carbon fixation rates using larger volumes of uncentrifuged cells (i.e. in the original culture medium). While this does not allow replacement of the medium or manipulation of [DIC], because the cells are still swimming, they are surrounded on all sides by the culture medium without crowding, in conditions very similar to their larger parent cultures. These samples recapitulate the essential features observed with centrifuged cells, although, as predicted, generally higher per cell photosynthetic rates are observed consistent with potentially higher CO₂ availability (Fig. 5A). Again, when [CO₂] is calculated for each pH and the data replotted, saturation with CO₂ and inhibition by AZ can both be observed (Fig. 5B). These curves approximate Michaelis-Menton saturation kinetics and illustrate an increase in the apparent Kᵐ for CO₂ in cultures exposed to the CA inhibitor (Fig. 4B). The predicted Vₘₚₓ is unchanged in the inhibited culture, however, suggesting that sufficiently high [CO₂] would still be able to saturate the photosynthetic machinery.

The inhibition of photosynthesis by AZ is consistent with carbon limitation, brought about when photosynthetic rates decrease [CO₂] at the cell periphery faster than they can be replenished by diffusion from the bulk medium or the slow uncatalyzed conversion of HCO₃⁻ to CO₂ near the cell. To test this, we used photosynthetic rates measured in the presence of AZ (black circles) to calculate the [CO₂] expected at the cell surface (Fig. 5C, dotted line). As expected, these [CO₂] are lower than what would be expected for control cultures able to use an external CA to regenerate equilibrium [CO₂] at the cell surface (white circles).

Interestingly, the predicted reduction of [CO₂] at the cell surface compared to that in the bulk medium is not constant, but rather is a function of the [CO₂]ₘₑₜ (Fig. 5D, black circles). The predicted reduction in cell surface CO₂ concentration is less substantial where photosynthesis rates are low (i.e. at low [CO₂]) and where photosynthesis rates are very high (due to saturating [CO₂]), and greatest at intermediate [CO₂] more typical of normal growth conditions. These predicted cell surface [CO₂] agree well with the degree to which the photosynthetic rate itself is inhibited by AZ (Fig. 5D, white circles).

**DISCUSSION**

We report here the identification of a CA in a free-living marine dinoflagellate (Fig. 1A). This enzyme is a δ-CA isoform, similar to that expressed in a wide range of marine algae, including chlorophytes, haptophytes, and streptophytes. Despite the limited number
of species from which this sequence has been recovered, the mature protein sequence from the dinoflagellates appears more closely related to the haptophytes than to the streptophytes, the phytoplankton expected to be their closest relatives. It is possible that this gene may have arisen in some of the species by lateral gene transfer (Fig. 1C) because a growing number of examples suggest lateral gene transfer of dinoflagellate genes may be relatively frequent (Fagan et al., 1998; Keeling and Inagaki, 2004; Waller et al., 2006). In any event, it is noteworthy that, to date, the δ-CA family appears restricted to marine algae.

It also is important to note that the sequence similarity observed with the mature protein sequence does not extend to the N-terminal leader sequence. Unlike the diatom sequence (Roberts et al., 1997), the N terminus of the Lingulodinium δ-CA contains a predicted signal peptide consistent with secretion of the protein (Fig. 2A). This predicted secretion of the protein is in agreement with the results of vectorial labeling using membrane-impermeable 125I, confirming that at least part of the δ-CA is exposed to the external environment. Furthermore, both immunogold labeling showing an association with the plasma membrane (Fig. 2D) and association of the immunoreactive protein following low-speed subcellular fractionation are both consistent with this interpretation. It is curious that a secreted protein would remain associated with the particulate fraction after cell lysis and fractionation, and this suggests the δ-CA may be held at the membrane by association with other proteins or by posttranslational modifications. One possibility for such an anchor may be S-acylation because CSS-Palm predicts the presence of a high-confidence palmitoylation site in the primary sequence.

To address whether the enzyme’s active site could be exposed at the external face of the membrane, the amount of immunoreactive protein was compared with the amount of activity measured in intact cells (Fig. 3C). When cells grown at pH 8.2 were compared with cells grown at pH 9, a similar increase in the amount of immunoreactive protein and the amount of whole cell CA activity is observed. While we cannot exclude the possibility that a different CA, also present at the cell surface, increases in concert with the amount of the δ-CA, these results are certainly consistent with the view that the δ-CA has an active site on the external face of the cell.

The precise role played by external CA activity in inorganic carbon uptake is still controversial. For example, in Chlamydomonas reinhardtii, the inhibition of O₂ evolution by membrane-impermeable CA inhibitors at alkaline pH (where [CO₂] is low) was taken to support a role for the external CA in carbon acquisition (Moroney et al., 1985). However, mutants lacking the major periplasmic CA isoform CAH1 have no phenotype (Van and Spalding, 1999), indicating that this CA is not essential for growth. Possibly, the ability of C. reinhardtii to also use HCO₃⁻ (Williams and Turpin, 1987; Spalding, 2008) may contribute to the difficulty in ascribing a role to this external CA.

Interestingly, the Thalassiosira δ-CA is unlikely to be directed to the plasma membrane, and, although the protein is induced by CO₂, there is no direct evidence for an involvement of this enzyme in photosynthesis (Lane and Morel, 2000). The function of the large N-terminal extension (approximately 300 amino acids) of the Emiliania δ-CA is unknown because its size is more suggestive of a functional domain rather than a targeting signal. The different N-terminal extensions found in the δ-CAs of these three species may be a consequence of lateral gene transfer between organisms with subsequent selection of different targeting sequences to fulfill different requirements.
The targeting of a given CA to a particular subcellular location may provide clues to the role played by the enzyme in photosynthesis. For the external δ-CA in *L. polyedrum*, this role may involve sustaining the equilibrium concentration of CO₂ at the cell boundary to compensate for use of CO₂ in photosynthesis. This can be directly observed by the reduction in carbon fixation rates following addition of the poorly membrane-permeable CA inhibitor AZ (Fig. 5A). The simplest interpretation of this inhibition is that when the observed photosynthesis rates exceed the capacity of diffusion to restore the [CO₂] at the cell surface to equilibrium concentrations, the cells become carbon limited and the rate of carbon fixation decreases. There are several caveats to this interpretation, however. First, if AZ were able to pass the plasma membrane of *Lingulodinium*, this would support instead a role for internal CA isoforms in carbon acquisition. However, AZ is often used as a membrane-impermeable CA inhibitor for phytoplankton (Elzenga et al., 2000; Tortell and Morel, 2002; Morant-Manceau et al., 2007), suggesting any effect on internal CA activity may be small. We also note that our measured cell surface CA activity is almost completely abolished by AZ (Fig. 3C), indicating that any effect of AZ must take this into account. Second, it is possible that pH changes might have independent effects on the cells other than the rapid change in [CO₂]. For example, it has been reported that the pH can directly affect growth of dinoflagellates in a manner virtually independent of the effect on inorganic carbon (Hansen et al., 2007). However, these studies used algae grown for extended periods at different pHs, whereas our assays take place over only 30 min, unlikely to be sufficient time for significant physiological acclimation to a new pH. Furthermore, in our brief ¹⁴C uptake assays varying DIC at three different pHs (Fig. 4), carbon fixation rates at all pHs clearly aligned on an axis of calculated [CO₂]. Thus, while we cannot exclude the possibility that photosynthetic parameters may change as a complex function of pH and DIC, it seems more reasonable to assume that it is the [CO₂] as calculated from known chemical equations and rate constants, that constrains photosynthetic rate and that direct effects of pH are negligible.

A theoretical underpinning of carbon limitation by the rate of diffusion in aqueous solution has been previously proposed for diatoms that do not possess a CCM (Riebesell et al., 1993; Wolf-Gladrow and Riebesell, 1997). Our results suggest that the δ-CA may be the catalyst that fulfills this role in *L. polyedrum*. Strikingly, the proportional inhibition of photosynthesis by AZ is more marked at intermediate concentrations of CO₂, an observation recapitulated by the calculated [CO₂] at the cell boundary (Fig. 5D). At high CO₂ concentrations, inhibition of eCA had less effect than at intermediate concentrations of CO₂ because even a substantial demand for carbon is unable to reduce CO₂ below saturating levels. At the other end of the [CO₂] spectrum, internal recycling of respired CO₂ or photorespiration could become proportionately more important, causing an underestimate in photosynthetic rate as measured by uptake of ¹⁴C from the external medium. In this case, the demand for external CO₂ would be lower than expected, resulting in a proportionately smaller decrease in cell surface [CO₂] and proportionately smaller effect of eCA inhibition. Taken together, these results suggest not only that *L. polyedrum* uses CO₂ preferentially, as do several other dinoflagellates (Colman et al., 2002), but also that the external δ-CA may have a role in providing this CO₂.

The observation that CO₂ diffusion rates can limit photosynthesis in diatoms (Riebesell et al., 1993) initially seems at odds with the fact that the diatom δ-CA is not localized to the plasma membrane. However, there may well be other plasma membrane CAs in these organisms. Indeed, CA immunoreactivity has been observed over the cell membrane space in air-grown *Phaeodactylum* (Szabo and Colman, 2007). It has also been proposed that the acidic silica cell wall around diatoms may serve as a buffer to make conversion of HCO₃⁻ to CO₂ more efficient (Milligan and Morel, 2002). To date, the only characterized CA is from *Phaeodactylum tricornutum*, a β-CA called PtCA1 (Satoh et al., 2001) localized in the chloroplast stroma adjacent to the thylakoid membrane (Tanaka et al., 2005). Inhibition by the membrane-permeable CA inhibitor EZA was observed to increase the half-saturation constant (Kₘ) for photosynthesis (Satoh et al., 2001), although it cannot be concluded that this is the only target for the inhibitor.

It must be emphasized that the results reported here consider photosynthetic rate as a function of CO₂ concentration at the cell surface. We know neither the form nor the concentration of DIC within the cytoplasm, yet clearly, in the absence of active transport, uptake of CO₂ would require a concentration gradient between the external medium and Rubisco. It would be interesting to determine internal CO₂ concentrations to assess the possibility of active transport. It would also be of interest to trace the movement of CO₂ to the plastid following its entry into the cell.

We do not yet know whether the plasma membrane δ-CA described here will be a conserved feature of dinoflagellate carbon acquisition. EST sequences indicate the presence of a δ-CA in *Alexandrium*, *Karlodinium*, and *Karenia*, but *Amphidinium* has no immunoreactivity with the δ-CA antibody and no δ-CA ESTs can be identified from this species. Furthermore, physiological demonstrations of external CA in dinoflagellates are few and often contradictory. For example, different reports using *Prorocentrum minimum* suggest that it either has an external CA (Nimer et al., 1997) or lacks an external CA and uses HCO₃⁻ directly (Rost et al., 2006). The situation for *A. carterae* is similar, with some reports describing the presence (Nimer et al., 1997) and others the absence (Dason et al., 2004) of external CA activity. Some of these differences in external CA activities may reflect leakage of internal CA during the measurements because some dinoflagellates may be
less robust than the *Lingulodinium* used here. Alternatively, the difference may reflect different environmental conditions under which the cells were grown or the use of different isolates. Clearly, the identification of a δ-CA in dinoflagellates will provide important new tools to address these issues.

**MATERIALS AND METHODS**

**Cell Culture Conditions**

*Lingulodinium polyedrum* (CCMP 1936; formerly *Gonyaulax polyedra*) was obtained from the Provasoli-Guillard Culture Center for Marine Phytoplankton (Boothbay Harbor, Maine) and cultured as described (Wang et al., 2005a). Cultures were grown in 1/2 medium under 12 h light (40 μmol m−2 s−1 cool-white fluorescent light) and 12 h dark at a temperature of 18°C ± 1°C, and experimental samples were taken from late-log-phase cultures (approximately 0.5 μmol Chl a/mL) midway through the light phase. Total DIC ranged between 1.8 and 2.1 mmol/L as measured with a CO2 ISE (Analytical Sensors), and total alkalinity was approximately 2.1 meq/L as measured by acid titration, conditions that resemble those found in very dense coastal algal blooms (Hansen et al., 2007). Culture pH ranged daily between pH 8.4 and 8.9 as previously described (Eisensam and Roenneberg, 2004).

**Photosynthetic Carbon Fixation Measurements**

Rates of photosynthetic carbon fixation were determined by 14C incorporation into acid-insoluble material. For the assays, duplicate 1-mL samples were taken for cell counts and 70-mL samples of late-log-phase culture were removed to a darkened room. NaH14CO3 (ICN; 310 MBq/mmol) was added to bring the 70-mL culture sample (pH approximately 8.7, DIC = 2.0 mm, total alkalinity 2.1 meq/L) to a specific activity of 1.1 kBq/mmol. This 14C-enriched culture was gently distributed in 940-mL aliquots into wells of duplicate microtiter plates preloaded with 50 μL of 1 mL Tris buffer (pH 8.5, 8.7, 8.9, 9.2, or 9.5) and either 10 μL of dimethyl sulfoxide (DMSO) or 10 μL of 100 mM AZ in DMSO (final concentration 1 mM). The final pH in the microtiter plates was measured with unlabeled controls and found to range between pH 8.05 and pH 9.44. The [CO2] concentrations at the different pH values were calculated according to Schulz et al. (2006) and were not corrected for the slight changes to DIC caused by addition of the different pH buffers. DIC cannot be measured in Tris buffers, but if buffer stocks were at their maximal air-equilibrium values, the final [CO2] would differ by a maximum of 0.5 μmol/L (at pH 9.5). The interconversion of HCO3− and CO2 is reported to be measured on a time scale of tens of seconds (Schulz et al., 2006), so prepared microtiter plates were loaded, sealed from the atmosphere, and then held in the dark for 5 min to allow equilibration of HCO3− and CO2 at the new pH. The CO2 concentrations were also uncorrected for CO2 present in the sealed airspace (this approximately 1-mL volume would contribute <1% to the DIC pool in the samples) or produced by respiration (based on the amount of available DIC is fixed and assuming equal rates of respiration, we estimate <2% contamination of external DIC from respiratory CO2).

One plate from each duplicate pair was exposed to the light under culture conditions for 30 min, while the other was kept in the dark to quantify the small, but significant, nonphotosynthetic 14C fixation (roughly 10% of light fixation). During the incubation in the light, between 0.2% and 1.9% of available DIC was fixed, and the carbon fixation rates were approximately linear over the 10- to 30-min range. For each experimental condition, the reaction in quadruplicate samples was halted by the addition of 0.2 volumes each of 5 M HCl and methanol to stop uptake and drive off excess inorganic H2CO3. Additional samples were stopped with 5 M NaOH to trap all inorganic H2CO3 to confirm that the specific radioactivity was maintained throughout the incubation. Excess 14C in the acidified samples was allowed to escape from the sealed plates overnight, the samples transferred to scintillation vials with 3-mL ScintVerse cocktail (Fisher Scientific), and the radioactivity measured in a TriCarb 200TR scintillation counter (Perkin-Elmer). Photosynthesis rates are reported as light-induced C fixation minus dark C fixation.

For some experiments, cells were concentrated by gentle centrifugation (30 s at 1,000g), then resuspended in artificial seawater (0.46 mM NaCl, 0.01 mM KCl, 0.01 mM CaCl2, 0.05 mM MgCl2) containing known amounts of DIC as the only electron sink. These concentrated cultures were treated as above. However, whereas this protocol allows the culture medium to be replaced with medium of differing DIC concentrations, centrifugation causes the flagella to break, and cells rapidly sink to the bottom of the ELISA plate wells.

Last, in experiments varying DIC, the addition of 10 or 50 mM HCO3− to artificial seawater decreased the pH of despite the buffering action of the 50 mM Tris. For these samples, the pH measured in a separate aliquot of buffered artificial seawater containing the DIC was used to calculate CO2 concentrations.

**cDNA Cloning and Screening**

PCR fragments were amplified using DNA derived from a previously constructed cDNA library using degenerate primers prepared from conserved regions in sequence alignments of α-, β-, and γ-CA family members. The primer pair (5′-ATGATARARRARATGATG-3′ and 5′-CCGTRGCTNCGNAC-RCC-3′) amplified a 304-bp fragment with significant homology to β-CA from the diatom *Thalassiosira weissflogii*. This fragment was used as a probe to isolate longer clones from the library by colony hybridization. Eight sequences were recovered, and could be put into two groups termed LpCA1 and LpCA2 whose sequences were 80% identical (85% similar) at the amino acid level and shared between 58% and 62% sequence identity with a β-CA from the diatom *Thalassiosira weissflogii*. Although the DNA sequences were incomplete (approximately 1.1 and 1.2 kb, respectively), the full 5′ sequence of LpCA2 was recovered by PCR using an oligonucleotide containing the sequence of the trans-splice leader (5′-CCGATGACCATTGTTGGCCTAAG-3′) found in all dinoflagellate mRNAs (Zhang et al., 2007). LpCA1 and a complete 1.6-kb LpCA2 sequence were deposited in GenBank under accession numbers EU044833 and EU051497. Most sequence analyses, including phylogenetic reconstruction, was performed using MacVector software (Accelrys) using the CA sequence from the diatom *T. weissflogii* (AAV39532), the C-terminal end of a CA from the haptophyte *Emiliania huxleyi* (ABG37687), and CA sequences assembled from ESTs for the chlorophyte *Micromonas* (EC484383 and EC47708) and the haptophyte *Isochrysis galbana* (EC146202 and EC142695). The possible presence of a signal peptide was tested with SignalP (http://www.cbs.dtu.dk/services/SignalP), the presence of palmitoylation sites was checked with CSS-Palm (http://bioinformatics.lcd-ustc.org/css_palm), and the predicted sequence topology of the complete LpCA2 protein was evaluated using Phobius (http://phobius.cbg.ki.se).

**Antibody Purification and Electrophoretic Analyses**

Antibody raised against the δ-CA of *Thalassiosira* (Roberts et al., 1997) was generously provided by F.M.M. Morel (Princeton University). The antibody was affinity purified using the LpCA2 cDNA expressed in bacteria and transferred to nitrocellulose after SDS-PAGE (Claeyssens et al., 2006). For electrophoretic analyses on standard SDS-PAGE, protein samples were boiled for 5 min in five packed cell volumes 2% SDS, 0.7 mM β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, and 10% glycerol and electrophoresed at 150 V on 12% SDS-PAGE (2D-PAGE). For 2D-PAGE, samples were prepared by removing 1 mL of chloroform-methanol and the pellets dried and resuspended in 265 μL of 8 μL urea, 4% CHAPS, 20 mM dithiothreitol, and 0.5% pH 3 to 10 nonlinear (NL) immobilized pH gradient (IPG) buffer. This sample was used to rehydrate a 13-cm IPG strip, pH 3 to 10 NL, and was electrophoresed using an IPGphor isoelectric focusing system (GE Healthcare) for 20,000 Vh. For the second dimension, strips were incubated 20 min in SDS equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 μL urea, 30% glycerol, and 2% SDS) and then run on a 12% SDS-PAGE. For immunoblotting, proteins were transferred electrophoretically to polyvinylidene difluoride membranes for 20 min at 20 V using a semidry transfer cell (Bio-Rad) and stained with Ponceau red to confirm transfer. Membranes were blocked for 10 min in Tris-buffered saline plus Tween (5 mM Tris-HCl, pH 8.0, 157 mM NaCl, 2.5 mM KCl, and 0.05% Tween 20) containing 3% bovine serum albumin and incubated with antibodies overnight at 4°C diluted in fresh buffer.

Cell surface proteins were iodinated with Na121I as described (Bertomeu et al., 2003), except that the purified cell wall fractions were extracted with SDS sample buffer and precipitated with 3 volumes of chloroform-methanol before resuspension in sample buffer for 2D gels.

**Immunocytochemistry**

Cells were harvested from the culture by centrifugation, washed with 0.4 M phosphate-buffered saline (PBS), and fixed with 3% glutaraldehyde in 0.4 M PBS for 30 min without osmum to preserve antigenicity on the sections. The
fixed cells were washed three times in PBS and water, dehydrated with a standard ethanol series, and embedded in LR White resin polymerized for 24 h at 60°C in gelatin capsules. Thin sections were cut for transmission electron microscopy, poststained with uranyl acetate, and observed using a JEOL JEM 100S microscope operating at 80 kV. Immunolabeling with affinity-purified anti-δ-CA was performed essentially as described (Nassoury et al., 2001) using 1/100 dilution of the primary antibody and a 1/100 dilution of 20 nm gold labeled anti-rabbit IgG (Ted Pella) as a secondary antibody. No labeling of the cell sections was observed with the secondary antibody alone.

CA Assays

CA activity was assayed in whole cells by an electrometric method similar to Wilbur and Anderson (1948). Culture samples containing approximately 10^6 cells were pelleted by gentle centrifugation (<10 s) in an ICN clinical centrifuge and resuspended in 2.7 mL of CA assay buffer (300 mM sorbitol, 1 mM MgCl_2, 640 mM, 20 mM Tris, pH 8.8, at 0°C) on ice. The pH of the assay was logged by computer (PH-27A pH sensor; Vernier Software & Technology) after addition of 0.9 mL of CO_2-saturated water. The buffer was agitated by a magnetic stir-bar at the slowest rate possible to maintain accurate pH measurement, and, at this rate, cell breakage as determined by microscopic examination was below 1% even for bouts of stirring much longer than the 1 or 2 min required for the assay. After addition of CO_2-saturated water, pH of the assay buffer fell rapidly because of H^+ liberated by the hydration of CO_2. The times elapsed over intervals of particular pH levels were converted into absolute rates of CO_2 hydration to HCO_3^- by acid titration of the CA assay buffer to these pH levels in a method similar to Ratti et al. (2007). Absolute rates of spontaneous CO_2 hydration were used as controls and determined by addition of 0.9 mL of CO_2-saturated water to buffer alone, without intact cells or extracts. Activity attributable to cell surface CA was determined by subtracting the spontaneous rates from the faster rates of CO_2 hydration in samples containing intact cells and was confirmed to be due to CA by addition of the CA inhibitor AZ (1 mM), which rapidly reduced the CO_2 hydration rate to control levels.

For determining CA activity in cell extracts, cells were pelleted in a clinical centrifuge, resuspended in CA assay buffer with 1% Saponin (practical grade; ICN), and broken in a Wheaton glass homogenizer on ice. The homogenate was fractionated into a soluble supernatant and insoluble pellet fraction by centrifugation for 2 min at 12,000g in an Eppendorf centrifuge (5415D). Each fraction was then diluted to 2.7 mL in CA assay buffer and processed through the protocol as for whole cells above. The activity in each crude fraction would decline over time; thus, only one fraction was processed from alternate cell samples and the other fraction discarded to avoid bias due to the time required to assay the activity of each fraction.

Carbon Limitation Calculations

The [CO_2] as a function of the distance from the cell center (r) was calculated from the rate of photosynthesis (Q_a in mmol CO_2 fixed per cell per second) using the following equation:

\[ [\text{CO}_2] = [\text{CO}_2]_{\text{aq}} - \frac{1,000 \times Q_a}{4\pi r^2 \times D_{\text{CO}_2}} \times \left( \frac{1}{a_0} - \frac{1}{a_w} \right) \]

with \( D_{\text{CO}_2} \) the diffusion constant for CO_2 (1.5 x 10^{-5} cm²/s), a the radius of the cell (0.0017 cm; Nassoury et al., 2001), and \( a_w \) and \( a_0 \) the reacto-diffusive length (Riebesell et al., 1993). A unit conversion factor of 1,000 cm²/L is included to allow use of concentrations in μM. The equilibrium CO_2 concentrations in seawater were calculated based on pH and titrated alkalinity measurements using the formula:

\[ [\text{CO}_2] = \left( \frac{k_w \cdot [\text{H}^+] + k_w \cdot [\text{OH}^-]}{k_w \cdot [\text{H}^+] + k_w \cdot [\text{OH}^-]} \right) \]

using the rate constants \( k_w = 3.7 \times 10^{-12} \text{ s}^{-1}; k_w = 2.67 \times 10^4 \text{ kg mol}^{-1} \text{s}^{-1}; k_w = 2.23 \times 10^4 \text{ kg mol}^{-1} \text{s}^{-1}; k_w = 9.7 \times 10^3 \text{ s}^{-1} \) as described (Schultz et al., 2006).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EU044833 and EU044834.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Alignment Data S1. Sequence alignment of LpCA1 and LpCA2 with the diatom TWCA.

ACKNOWLEDGMENTS

We thank Dr. Haeswon Park and Dr. François M.M. Morel (Princeton University) for generously providing anti-δ-CA raised against the diatom enzyme. We also thank L. Pelletier (Université de Montréal) for technical assistance with the electron microscope.

Received January 29, 2008; accepted April 18, 2008; published May 8, 2008.

LITERATURE CITED


