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7 The physiology and genetics of CO₂ concentrating mechanisms in model
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Introduction

Diatoms are a diverse group of unicellular algae that live in a wide range of environments including the ocean, lakes, streams, and sea ice. They are especially prominent in highly productive marine waters, where they are typically the dominant autotrophs, and so are responsible for a significant fraction of total photosynthesis in the ocean [1]. While it is typically the availability of nutrients such as nitrogen and phosphorus or light that ultimately limits the growth of diatoms, CO₂ is often scarce in the environments where diatoms thrive. For example, in the ocean CO₂ concentrations are ~10-25 μM, much less than the >50 μM required to saturate diatom Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), the CO₂-fixing enzyme in the Calvin-Benson cycle. Saturating RubisCO with CO₂ is necessary to prevent the competing oxygenase reaction and to compensate for its slow maximal turnover rate (only a few times per second) [2]. To overcome CO₂ scarcity and the deficiencies of RubisCO, diatoms have developed a CO₂ concentrating mechanism (CCM) that raises the CO₂ concentration around RubisCO.

In this respect diatoms are similar to better studied organisms such as cyanobacteria and the green alga *Chlamydomonas reinhardtii*, which also possess CCMs [3,4]. However, the CCMs of these organisms provide only partial insight into the CCM of diatoms. The CCMs of prokaryotes and eukaryotes are not homologous, and even within the eukaryotes it appears that CCMs evolved independently in the major algal lineages after their diversification. For much of earth's history atmospheric CO₂ has been upwards of 20-fold higher than present day and CCMs were likely not required; large declines in atmospheric CO₂ ~300 million years ago and during the Cenozoic era (65 million years ago to present) likely prompted the independent development of CCMs [5-7].

Some basic characterization of diatom CCMs was carried out prior to the rapid development of molecular knowledge that accompanied the sequencing of diatom genomes [8,9] and the development of genetic manipulation tools [10-14]. This early work showed that, in a broad sense, the CCMs of diatoms have many similarities to those of other algae. Like many algae, diatoms take up both CO₂ and HCO₃⁻ from the environment [15-17], using membrane-embedded transporters to acquire HCO₃⁻, but relying on diffusive fluxes to import CO₂. Once imported, a pool of HCO₃⁻ is built up intracellularly [18,19], and this HCO₃⁻ is then equilibrated

with CO₂ in the vicinity of RubisCO, raising the CO₂ concentration. Like many eukaryotes, diatoms accumulate RubisCO in pyrenoid bodies within the chloroplast and CO₂ is ideally only elevated within this compartment to minimize the diffusive loss of CO₂ that plagues CCMs [2,20]. Carbonic anhydrases are key components of well-studied algal CCMs and diatom CCMs are no exception. Diatoms have long been known to possess both extracellular and intracellular carbonic anhydrase [15,21,22], and the molecular identity of some diatom CAs were determined [23,24].

With the new availability of molecular tools for diatoms, components of diatom CCMs have been identified much more rapidly in recent years and there have been accompanying advances in our understanding of CCM physiology and biochemistry. Consequently, this review will focus primarily on advances over the past ~5 years in the molecular biology and physiology of CCMs in model diatoms (*Phaeodactylum tricornutum*, *Thalassiosira pseudonana*), highlighting areas of agreement and some areas of disagreement between the molecular and physiological perspectives. For earlier reviews on related topics see [25-29].

Physiology of the diatom CCM

Aspects of diatom CCM physiology had been worked out well before the availability of molecular information as discussed above, but there have been some recent advances in purely physiological aspects of the CCM in model diatoms. Recent work has tried to shed light on details of the intracellular fluxes of carbon associated with the CCM, which are difficult to study but form a critical part of the CCM in eukaryotic algae. Fluxes of carbon associated with the *P. tricornutum* CCM were inferred from data on CO₂ uptake and ¹⁸O-isotope dynamics, which showed that transport of carbon into the chloroplast is the major CCM flux [30]. The *P. tricornutum* CCM was shown to be moderately efficient with about one-third of the carbon transported into the chloroplast being fixed by RubisCO and the remaining two-thirds leaking back out as CO₂ into the cytoplasm due to the high permeability of diatom membranes to CO₂. Measurement of these fluxes led to the proposal of a novel ‘chloroplast-pump’ mechanism for the diatom CCM (Figure 1). The primary novelty of this model is the suggestion that the HCO₃⁻ flux into the chloroplast greatly exceeds the net CO₂ and HCO₃⁻ fluxes across the plasmalemma, which results in both: 1) the build up of a large inorganic carbon pool in the chloroplast to raise CO₂ concentrations around RubisCO and 2) a depletion of inorganic carbon from the cytoplasm

leading to the diffusive influx of CO₂ into the cytoplasm from the environment [31]. The CCMs of other eukaryotic algae may function similarly, but internal fluxes have not been fully elucidated in these algae. The possible role of C₄-organic intermediates has been a topic of much research recently and is discussed in more detail below.

Bicarbonate transporters

In support of physiological data indicating that diatoms take up HCO₃⁻, putative bicarbonate transporters with homology to solute carrier 4 (SLC4) and solute carrier 26 (SLC26)-type bicarbonate transporters have been identified and characterized in model diatoms. Candidate SLC4 and SLC26 bicarbonate transporter genes were first identified in the genomes of *P. tricornutum* and *T. pseudonana*[32], but because these transporters have mainly been studied in humans and other mammals [33,34] and the SLC26 family has a broad substrate range [35] they could not be definitively identified as bicarbonate transporters. Consequently, the finding by Nakajima et al. [36] that an SLC4 homolog in *P. tricornutum* (known as PtSLC4-2 or protein ID 1677) does indeed transport inorganic carbon represented a significant advance in our understanding of the genetics of diatom CCMs (Figure 1). This sodium-dependent HCO₃⁻ transporter was localized to the plasmalemma showing that it is involved in the initial import of HCO₃⁻ into *P. tricornutum*[36].

The ‘chloroplast-pump’ model for the *P. tricornutum* CCM requires additional HCO₃⁻ transporters in the membranes surrounding the chloroplast. Recent localization of other SLC4 homologs via fluorescent-protein tagging has shown that some of these proteins are embedded in chloroplast membranes in both *P. tricornutum* and *T. pseudonana*, though full characterization of these proteins is still ongoing (C. Dupont; Y Matsuda, unpublished data). These results suggest that sequential transport steps by different SLC4 transporters move HCO₃⁻ from the external environment and into the chloroplast stroma where a HCO₃⁻ pool is accumulated. The functionally characterized SLC4 protein from *Phaeodactylum* belongs to a phylogenetic clade of SLC4 proteins found exclusively in phytoplankton genomes. However, other SLC4 proteins found in phytoplankton genomes form additional phylogenetic clades, which might reflect neofunctionalization (Figure 2).

Carbonic anhydrases

Carbonic anhydrases (CAs) interconvert CO_2 and HCO_3^- and play multiple, critical roles in CCMs as the intrinsic equilibration rate of $\text{CO}_2/\text{HCO}_3^-$ is slow. Diatoms possess numerous CAs from common (α , β , γ) and unusual (δ , ζ) families. Both the δ -CA and ζ -CA families were initially discovered in diatoms [23,24], but δ -CAs have since been found in other algal lineages. The δ -CA family has some structural and functional similarities to α -CAs [37,38], while the ζ -CA family is similar to the β -CAs, but is notable for its ability to use cadmium in the active site in addition to the typical zinc ion [39].

Functional studies of the diatom CCM have suggested that CAs have at least three critical roles in diatoms: 1) converting HCO_3^- to CO_2 at the cell surface to facilitate diffusive CO_2 uptake, 2) conversion of imported CO_2 to HCO_3^- in the cytosol, preventing diffusion out of the cell and facilitating transport to the chloroplast, and 3) conversion of HCO_3^- accumulated in the chloroplast stroma to CO_2 in the vicinity of RubisCO [28,30,40,41]. These suggestions from physiological studies have largely been supported by recent molecular localization and functional studies, but in some cases they have been challenged.

P. tricornutum and *T. pseudonana* have a broadly similar complement of CAs in terms of number and family identity (Figure 3), but the CAs are deployed in dramatically different ways in the two species. In *T. pseudonana*, two extracellular CAs (δ CA1, ζ CA1) are localized to the periplasmic space outside the cell serving to convert HCO_3^- to CO_2 at the cell surface for CO_2 uptake (Figure 3, [42]). In contrast, the commonly studied strains of *P. tricornutum*, including the genome-sequenced strain, lack extracellular CA activity, which is unusual for diatoms [43,44]. Moving inward, *T. pseudonana* has a single CA in the cytoplasm (γ CA2), which likely functions to convert imported CO_2 to HCO_3^- as hypothesized from physiological studies. However, *P. tricornutum* lacks any cytoplasmic CA. Instead CAs in the chloroplastic endoplasmic reticulum (CAII, CAVI, CAVII) or periplastidal space (CAI, CAII) may function in this role, as has recently been illustrated in a modeling study [31]. These CAs surrounding the chloroplast are likely also used to recover CO_2 leaking out of the chloroplast, with this additional role perhaps explaining the large number of CAs in the chloroplast membranes of *P. tricornutum*, in contrast to the single isoform in *T. pseudonana* (δ CA3), which may only be involved in CO_2 recovery.

The distribution of CAs inside the chloroplast is critical for CCM function and efficiency. *P. tricornutum* has no CA in the chloroplast stroma allowing a HCO_3^- pool to build up that can

then diffuse into the pyrenoid where HCO_3^- is dehydrated to CO_2 by a pair of CAs (PtCA1, PtCA2) [45]. The confinement of CAs to the pyrenoid, where RubisCO is localized, helps to keep the volume in which CO_2 is elevated small thus limiting diffusive CO_2 loss. This arrangement of CAs in the *P. tricornutum* chloroplast is consistent with expectations from physiological work. PtCA1 and PtCA2 are redox sensitive being activated under reducing conditions, such as occur in illuminated chloroplasts, suggesting that redox signaling is used to control inorganic carbon flows in the pyrenoid[46]. In contrast, *T. pseudonana* unexpectedly has the opposite distribution of CAs in its chloroplast, with a single α -CA in the chloroplast stroma (α CA1) and no CA in the pyrenoid. Such an arrangement would be expected to result in severe CO_2 leakage and dissipation of any HCO_3^- pool, yet the CCM of *T. pseudonana* is functional [47,48]. The possible use of a C_4 organic compound (see The controversial C_4 pathway in diatoms) as an intermediate rather than HCO_3^- may help explain this unexpected CA distribution.

In the mitochondria, *T. pseudonana* has a δ -CA (δ CA2), which potentially serves to convert respired CO_2 into HCO_3^- for use in anaplerotic reactions within the mitochondria or for export and use in photosynthesis. Both diatoms also have multiple mitochondrial proteins with sequence homology to γ -CAs (*P. tricornutum*: CAVIII, CAIX; *T. pseudonana*: γ CA1, γ CA3, γ CA4). In higher plants, mitochondrial proteins with homology to γ -CAs are associated with respiratory complex I and are involved in photorespiration[49-51]. Although CA activity has not been demonstrated in any of these proteins, it has been suggested that they might convert CO_2 produced through photorespiration to HCO_3^- . Another intriguing possibility is an interaction with the urea cycle, a metabolic entity found in both diatoms and higher metazoans but not in plants [8]. Recent genetic manipulations and physiology experiments in *P. tricornutum* have shown that the urea cycle is critical to diatom growth [52]. In humans, genetic deficiencies in mitochondrial CA5A results in an impaired urea cycle and ureagenesis, resulting in hyperammonemia[53], and potentially the mitochondrial CAs found in diatom serve a similar role.

The controversial C_4 pathway in diatoms

Ever since the first report of a C_4 photosynthetic pathway in the marine diatom *Thalassiosira weissflogii*[54], there has been excitement and controversy surrounding its existence, role, and ubiquity among diatoms. The C_4 pathway is essentially a CCM that involves

a four carbon organic intermediate that is accumulated and transported within the cell, rather than the more typical bicarbonate intermediate. While C₄ pathways are common among land-plants and multicellular aquatic plants, it was long thought to be absent from unicellular algae. As reviewed in [25] work up to that time largely supported a C₄-pathway in *T. weissfloggi*, but in other diatom species there was mixed or no support for a C₄-pathway. Since then, RNA-interference was used to reduce the activity of a key C₄ enzyme, pyruvate-orhtophosphatedikinase (PPDK), in *P. tricornutum* and no significant effect on inorganic carbon acquisition was found [55]. In combination with previous findings [25,32], these results demonstrate that *P. tricornutum* does not have a conventional C₄-CCM. The genes needed for conventional C₄ photosynthesis are widely distributed and diatoms likewise possess these genes [32]. However, localization of these genes is critical to a functional C₄ pathway with the key feature being the segregation of carboxylation and decarboxylation genes. Both bioinformatic and localization studies in diatoms indicate that the carboxylation genes are in plausible location for C₄ photosynthesis but the decarboxylation genes are not positioned to create a functional C₄ pathway [32,56].

Recently Kustka et al. [57] have argued that *T. pseudonana* may have an unconventional C₄ pathway that employs a pyruvate carboxylase working in reverse to produce HCO₃⁻ from oxaloacetate in the chloroplast (Figure 4). As they argue, given that the C₄ pathway is apparently fairly flexible having evolved multiple times in many distinct varieties within the land plant, it is not unreasonable to think that a novel C₄ variant would evolve in the diatoms. Such a C₄ pathway could help explain the unusual distribution of CA throughout the chloroplast stroma. The pyruvate carboxylase may be localized near RubisCO such that HCO₃⁻ is produced, and subsequently converted to CO₂ by the stromal CA only in a restricted region near RubisCO. However, subsequent work showing that the activity of PEPC and PPDK, key C₄ enzymes, declines at low CO₂ further complicates proposals for a C₄ pathway in *T. pseudonana*. The prevalence of C₄-CCMs among diatoms is still a matter of debate, but most evidence suggests they are the exception rather than the rule. It is worth noting that in all of the above studies, only the C₄ variation of an organic CCM has been explored. It is conceivable that such a process might use an organic intermediate with 2, 3, 5, or more carbon atoms.

Conclusions

For the model diatom *P. tricornutum*, there is good agreement between molecular and physiological perspectives on the CCM. The system is organized around the active transport of bicarbonate via SLC4 or SLC26 transporters into the chloroplast stroma. Bicarbonate accumulates in the chloroplast stroma and then diffuses into the pyrenoid where a pair of carbonic anhydrases convert it to CO₂, raising the CO₂ concentration around RubisCO. Some details remain to be worked out including how bicarbonate is transported through the multiple membranes surrounding the chloroplast and the exact roles of the multiple carbonic anhydrases in these layers, but the system is generally well-defined. In contrast, there are some discrepancies between the physiological and molecular data in *T. pseudonana*. In particular the presence of a carbonic anhydrase in the chloroplast stroma would be expected to prevent accumulation of a bicarbonate pool. The recent proposal of a modified C₄ pathway may provide a solution to this dilemma, but this system still remains to be validated.

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Reference Annotations

- Haimovich-Dayana et al. 2013 RNAi is used to silence a key gene in the traditional C₄ pathway, pyruvate-orthophosphate dikinase (PPDK). Silencing this gene in *Phaeodactylum tricornutum* did not alter cellular affinity for CO₂, demonstrating that a traditional C₄ pathway is not operating in this diatom.
- Hopkinson et al. 2011. Intracellular inorganic carbon (C_i) fluxes in *Phaeodactylum tricornutum* are inferred from isotopic exchange rates. The C_i fluxes showed that active transport of carbon from the cytoplasm to the chloroplast is the main driver of the CCM. However, the CCM is only moderately efficient because cellular membranes are highly permeable to CO₂ leading to diffusive efflux of CO₂ from the chloroplast.
- Kutska et al. 2014. Through analysis of transcript and protein responses to changes in CO₂ availability a novel C₄ pathway is proposed for *Thalassiosira pseudonana*. The most atypical aspect of the pathway is the proposal that pyruvate carboxylase (PYC) acts as the decarboxylase producing bicarbonate the chloroplast, rather than its typical role as a carboxylase.
- Nakajima et al. 2013. This study shows that an SLC4 homolog in the diatom *Phaeodactylum tricornutum* is an inorganic carbon transporter. SLC4 proteins are well-characterized bicarbonate transporters in mammals, and through homologs had been identified in diatoms and other

microorganisms, their function had not been demonstrated. The *P. tricornutum* SLC4 was shown to be Na⁺-dependent and inhibited by typical anion-exchange inhibitors.

Reinfelder 2011. This comprehensive review on CCMs details differences in CCM physiology among the major groups of marine phytoplankton, and explains how ocean chemistry drives the need for CCMs. It explores the ways in which evolution and ecology have led to differences in CCMs among the major phytoplankton lineages, and how in turn CCM physiology may alter marine ecology and biogeochemistry.

Figure Captions

Figure 1. Chloroplast-pump model for the CCM of *P. tricornutum* (similar to that presented in Hopkinson 2014). Active transport fluxes are indicated with light blue arrows, passive diffusion with wavy black arrows, carbonic anhydrase-catalyzed reactions with dark blue arrows, CO₂ and HCO₃⁻ concentrations are in red, and fluxes (in $\times 10^{-17}$ mol cell⁻¹ s⁻¹) are in grey. The active transport of bicarbonate into the chloroplast builds up a high concentration of bicarbonate in the chloroplast stroma and depletes bicarbonate in the cytoplasm. The SLC4 homolog PtSLC4-2 has been characterized as a plasmalemma-based bicarbonate transporter [36]. While the remaining bicarbonate transporters needed for this model have not yet been fully characterized, SLC4 homologs have recently been localized to membranes surrounding the chloroplast (C. Dupont, Y. Matsuda, unpublished data). The stromal bicarbonate pool passively diffuses into the pyrenoid where a carbonic anhydrase converts it to CO₂, elevating the concentration around RubisCO. The low concentration of bicarbonate in the chloroplast endoplasmic reticulum (CER) and periplastidal compartment (PPC) and the presence of CA there results in the net conversion of CO₂ to HCO₃⁻, which acts to draw CO₂ in from the extracellular environment and recover CO₂ leaking out of the chloroplast. The chloroplasts of diatoms are derived from a secondary endosymbiotic event in which a nonphotosynthetic eukaryote engulfed a red alga. As a consequence of this history, diatom chloroplasts are surrounded a complicated set of membrane and compartments [59]. The outermost compartment, the CER, is likely derived from the phagosomal membrane of the host and the plasmalemma of the engulfed symbiont. Whereas, the PPC is the remnant cytoplasm of the symbiont. Note that the concentrations and fluxes are derived from the model described in Hopkinson 2014. Because we have little information about the relative roles of the CER and PPC in the CCM, the compartments are merged in the model.

Figure 2. Maximum likelihood phylogeny of the SLC4 protein sequences found in *P. tricornutum* (protein IDs only or bd prefix), *T. pseudonana* (protein IDs with Tp prefix, and other select phytoplankton (*Emiliania huxleyi* protein IDs have Eh prefix). Functionally characterized SLC4 bicarbonate transporters from metazoans are included as an outgroup. Bootstrap support (100 resamplings) for the phylogeny is indicated at the branch nodes. The analysis identified four major clades in marine phytoplankton (Phyto1-4). Most clades have representatives from diverse algal groups including diatoms, haptophytes, brown algae, and green algae, suggesting they are

ancient clades. Some sequences from autotrophic bacteria (AutoBac) are also included for reference. The red star indicates the protein identified as a sodium dependent bicarbonate transporter by Nakajima et al. 2013. Amino acid sequences were retrieved from the NCBI non-redundant protein database (NR) after identification as homologs by blastP. Sequences were aligned using MUSCLE (Edgar) and phylogenetic inference was conducted in CLC.

Figure 3. Distribution of putative carbonic anhydrases (CAs) in *P. tricornutum* and *T. pseudonana* (from Samukawa et al. 2014, reproduced with permission from the publisher). Although both diatoms have a similar number of CAs from the various CA families, the CAs have contrasting distributions between the species. PPS = periplasmic space; Cyt = cytoplasm, mit = mitochondria, CER = chloroplast endoplasmic reticulum, PPC = periplastidal compartment, CEV = chloroplast envelope, Str = chloroplast stroma, Pyr = pyrenoid.

Figure 4. Modified C₄ pathway in *T. pseudonana* proposed by Kustka et al. 2014. In the proposed pathway, phosphoenolpyruvate (PEP) is combined with bicarbonate to form oxaloacetate (OAA) in the periplastidal compartment (PPC). OAA is then transported into the chloroplast and decarboxylated by pyruvate carboxylase (PYC). The bicarbonate released is converted to CO₂ by a carbonic anhydrase and fixed (generating phosphoglycerate – PGA) in the pyrenoid (pyr). Pyruvate (PYR) is converted back to PEP in a complex series of reactions in multiple compartments, which for simplicity here is shown in the cytoplasm with the dashed arrow indicating that additional proposed steps are not shown.

Figure_1

Figure_2

A

P. tricornutum

B

T. pseudonana

Figure_4