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MINIREVIEWS

Proposed Carbon Dioxide Concentrating Mechanism in *Chlamydomonas reinhardtii*[▽]

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Aquatic photosynthetic microorganisms account for almost 50% of the world's photosynthesis (19). These organisms face several challenges in acquiring CO_2 from the environment. The first challenge is presented by the properties of ribulose bisphosphate carboxylase-oxygenase (Rubisco). Rubisco is an unusually slow enzyme with a low affinity for CO_2. At atmospheric levels of CO_2, Rubisco can function at only about 25% of its catalytic capacity because the concentration of dissolved CO_2 is less than the $K_m(CO_2)$ of Rubisco and due to the relatively high concentration of O_2 which competes with CO_2. A second challenge these organisms face is that the diffusion of CO_2 in an aqueous solution is 10,000 times slower than the diffusion of CO_2 in air. Thus, the ability to scavenge CO_2 as quickly as it becomes available is highly advantageous to aquatic photosynthetic organisms. Third, algae often experience significant fluctuations in inorganic carbon ($C_i = CO_2 + HCO_3^-$) levels and pH, which change the availability of CO_2 and HCO_3^- for photosynthesis. At an acidic pH, the vast majority of C_i is in the form of CO_2, while at an alkaline pH, C_i is mostly in the form of HCO_3^-, with CO_2 making up only a small fraction of the available C_i (8, 25).

Algae have adapted to these challenges through the development of a CO_2 concentrating mechanism (CCM). The CCM is a biological adaptation to low carbon dioxide concentrations in the environment. It is a mechanism which augments photosynthetic productivity in algal cells by increasing levels of inorganic carbon many times over the environmental concentration of carbon dioxide. In this minireview, we aim to provide an update on the CCM and present a model on how the green alga *Chlamydomonas reinhardtii* concentrates CO_2.

TYPES OF CCMs

CCMs can be based on biochemical mechanisms such as C_4 photosynthesis and crassulaceous acid metabolism (CAM), on active transport of C_i across membranes, or on processes involving localized enhancement of the CO_2 concentration by acidification of a particular cellular compartment (28). The role of the CCM is to increase the concentration of CO_2 for Rubisco, the enzyme responsible for the initial fixation of CO_2. While three different mechanisms are discussed below, it is likely that aquatic photosynthetic organisms display a variety of ways to concentrate CO_2. Algae comprise a very diverse group of organisms and have been adapting to the slow diffusion of inorganic carbon in the water for a long time.

C_4 mechanism. C_4 photosynthesis and CAM in terrestrial higher plants were the first photosynthetic CCMs to be described in detail. They involve a spatial (C_4) or temporal (CAM) separation of the fixation of CO_2 by phosphoenolpyruvate (PEP) carboxylase to produce a four-carbon dicarboxylic acid which is transported and decarboxylated, increasing the CO_2 available to Rubisco (44, 74). In higher plants, the CCM is dependent on a specialized operation and the interaction of leaf mesophyll and bundle sheath photosynthetic cells. The primary CO_2 capture mechanism is through PEP carboxylase located in the cytosol of the mesophyll cells. PEP carboxylase uses HCO_3^- as its primary substrate for fixation of CO_2 into oxaloacetate, so CO_2 entering from the external environment must be hydrated rapidly by a carbonic anhydrase (CA) and converted to HCO_3^-. Thus, in C_4 plants, the predominant CA activity is found in the mesophyll cell cytosol in order to make this HCO_3^-, in contrast to C_3 plants, where the highest levels of CA activity are associated with the stroma of the mesophyll cell chloroplasts (6, 13, 40, 59). C_4 carboxylic acids such as malate or aspartate formed in the mesophyll cell cytosol serve as the intermediate CO_2 pool.

The presence of C_4- or CAM-like metabolism has been observed in submerged aquatic plants and algae. Examples include *Isoetes howellii* and *Sagittaria subulata* (39), the green ulvophycean benthic macroalga *Udotea flabelum* (79, 80), and the planktonic diatom *Thalassiosira weissflogii* (77, 78) grown under inorganic CO_2-limited conditions. Evidence of a CAM-like mechanism has also been proposed for brown macroalgae, where high levels of PEP carboxykinase and diel fluctuations in titratable acidity and malate have been observed (33, 72).

Active transport of inorganic carbon. Examples of active transport of HCO_3^- come primarily from studies using cyanobacteria. Cyanobacteria have a sophisticated CCM which involves a variety of active CO_2 and HCO_3^- uptake systems and an internal microcompartment, the carboxysome (7, 63). At least five distinct C_i transport systems are known for cyanobacteria (Fig. 1). An interesting feature of the cyanobacterial CCM is the induction of multiple transporters under C_i limitation. Cyanobacteria appear to utilize pairs of C_i transporters with complementary kinetics for the same C_i species. For example, two complementary HCO_3^- transporters are present in *Synechococcus* PCC7002. The BicA transporter has

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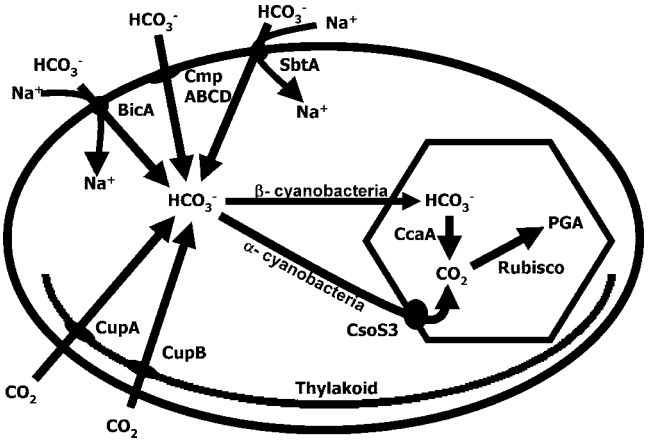

FIG. 1. Model for inorganic carbon acquisition by cyanobacteria. This model is based on the article by Woodger et al. (102). Most cyanobacteria do not contain all of the transporters depicted. In addition, any given cyanobacteria would have only one type of carboxysome (alpha or beta), although both pathways are shown in this figure.

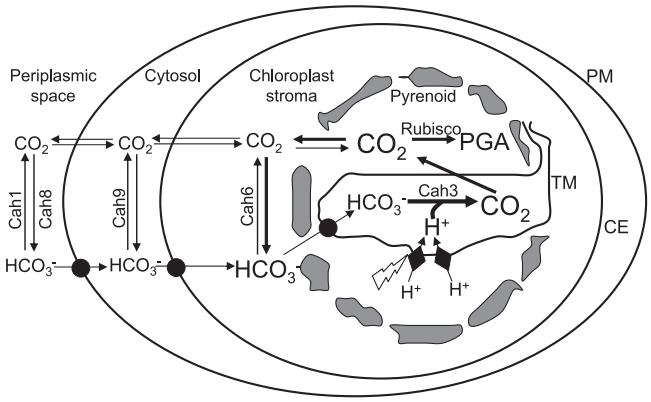

FIG. 2. Model of the CCM of *Chlamydomonas reinhardtii*. The figure depicts an algal cell with a single chloroplast containing a single pyrenoid. As indicated by the size of the lettering, the concentrations of bicarbonate and carbon dioxide within the chloroplast and pyrenoid are higher than those in the external environment. CAH1, CAH3, CAH6, CAH8, and CAH9 stand for specific CA isoforms. PGA, 3-phosphoglyceric acid; PM, plasma membrane; CE, chloroplast envelope; TM, thylakoid membrane. The filled circles indicate possible bicarbonate (or C_i) transporters, and the closed diamonds indicate the photosynthetic electron transport chain.

a relatively low transport affinity of around 38 μM but is able to support a high flux rate. It pairs with the SbtA transporter, which has a high transport affinity of 2 μM but possesses a lower flux rate (65). This strategy of employing a high-flux/low-affinity transporter with a low-flux/high-affinity transporter appears to be a common theme in freshwater and estuarine cyanobacteria (7).

In cyanobacteria, the carboxysome is the specialized compartment in which accumulated HCO_3^- is converted to CO_2 through the action of specific carboxysomal CAs (21, 106). Two types of carboxysomes are recognized in cyanobacteria. In α-type carboxysomes, bicarbonate is converted to CO_2 via the action of the CA CcaA (21, 85, 106). In β-type carboxysomes, a component of the carboxysome shell, CsoS3, is responsible for the dehydration of bicarbonate (84, 102). CO_2 is elevated due to diffusion restrictions on efflux by the carboxysome protein shell structure (35, 36, 63). Thus, the overall mechanism elevates HCO_3^- in the cytosol of the cell and converts this accumulated C_i back to CO_2 in the carboxysome, the location of Rubisco (102).

CO_2 concentration following acidification in a compartment adjacent to Rubisco. A third type of CCM found in eukaryotic algae relies on the pH gradient set up across the chloroplast thylakoid membrane in the light. In the light, a large change in pH is established across the thylakoid membrane; the chloroplast stroma has a pH of close to 8.0, and the thylakoid lumen has a pH of between 4 and 5. This pH differential is significant because the pK_a of the bicarbonate-to-CO_2 interconversion is about 6.3 ($HCO_3^- + H^+ \leftrightarrow H_2CO_3 \leftrightarrow CO_2 + H_2O$). Under these conditions, HCO_3^- is the predominant species of C_i in the chloroplast stroma while CO_2 is the most abundant form of C_i in the thylakoid lumen. Any bicarbonate transported into the thylakoid lumen would be converted to CO_2, thus elevating the CO_2 concentration above ambient levels. This mechanism, first suggested by Semenenko, Pronina, and colleagues, requires a CA in the acidic thylakoid lumen to rapidly convert the entering HCO_3^- to CO_2 (66, 68). In addition, since HCO_3^- cannot rapidly cross biological membranes (29), there must be a transport protein or complex that allows HCO_3^- to enter the thylakoid lumen. This model predicts that CO_2 accumulation would not occur in the dark, as light-driven photosynthetic electron transport is required to set up these pH gradients. As discussed below, evidence for this type of CCM comes primarily from work using the model eukaryotic green alga *Chlamydomonas reinhardtii*.

CHLAMYDOMONAS REINHARDTII CCM

The *C. reinhardtii* CCM. A proposed model for concentrating CO_2 in *C. reinhardtii* is shown in Fig. 2. In this model, the CCM can be divided into two phases. The first phase involves acquiring inorganic carbon from the environment and delivering CO_2 and HCO_3^- to the chloroplast. The components of this part of the CCM would include CAs in the periplasmic space (CAH1 and possibly CAH8) and a CA in the cytoplasm (CAH9) as well as HCO_3^- transporters and CO_2 channels on both the plasma membrane and the chloroplast envelope. The second part of the proposed model entails the generation of elevated levels of HCO_3^- in the chloroplast stroma, utilizing the pH gradient across the thylakoid membrane. This part of the CCM includes the CA located in the chloroplast stroma (CAH6) and the CA located within the thylakoid lumen (CAH3) as well as a proposed but still hypothetical HCO_3^- transporter on the thylakoid membrane.

It should be emphasized that *C. reinhardtii* has a strictly C_3 biochemistry, since unlike the C_4 pathway, wherein transported carbon is stored as organic C_4, *C. reinhardtii* accumulates inorganic carbon, specifically HCO_3^-, in the chloroplast stroma. In addition, while experiments indicate that the marine diatom *Thalassiosira weisflogii* has a C_4-like pathway, the same researchers concluded that a C_4-like pathway is unlikely to operate in green algae (78). Although *C. reinhardtii* has two PEP carboxylase genes, CR*PPC1* and CR*PPC2* (45), the PEP carboxylase activity in *C. reinhardtii* is never higher than 20%

TABLE 1. *C. reinhardtii* CCM mutants and other mutants that grow poorly on low CO_2

Mutant	Gene affected	Protein affected	Phenotype	Reference(s)
ca-1 cia3	*CAH3*	Thylakoid lumen CA	Very poor growth on low CO_2	24, 58, 90
ca1	*CAH1*	Periplasmic CA	Only minor growth differences	96
ycf10	*YCF10*	Hydrophobic protein	High light sensitive, decreased inorganic carbon uptake	81
pmp-1 ad-1	*LCIB*	Novel protein	Transport of C_i affected	91, 100
cia5 ccm1	*CIA5*	Transcription factor	Many CCM genes not expressed	22, 57
lcr1	*LCR1*	Transcription factor	Some CCM genes not expressed	105
pgp1	*PGP1*	Phosphoglycolate phosphatase	Photorespiration affected	94
hcr89	*GDH*	Glycolate dehydrogenase	Overaccumulated glycolate excreted	104
rca1	*RCA1*	Rubisco activase	Reduced photosynthesis at low CO_2	62
rh1	*RH1*	Transport protein	Reduced growth on high CO_2	87
had1	*HAD1*	Novel dehydrogenase	Poor growth on low CO_2	1
cia6	*CIA6*	SET domain protein	Reduced affinity for CO_2	Pollock et al., unpublished observations
cia7	*CIA7*	Probable metal-binding protein	Reduced affinity for CO_2	Ynalvez and Moroney, unpublished observations

of the Rubisco activity or the maximal rate of CO_2 fixation (9). Mamedov et al. concluded that CR*PPC1* and CR*PPC2* have an anaplerotic, nonphotosynthetic role in *C. reinhardtii* (45).

Physiological evidence for C_i uptake in *C. reinhardtii*. The physiological evidence that *C. reinhardtii* can accumulate C_i and enhance CO_2 fixation is twofold. First, *C. reinhardtii* has the ability to efficiently fix CO_2 even when the external CO_2 concentration is well below the $K_m(CO_2)$ for Rubisco (4, 55, 91). For example, whole-cell photosynthesis rates are saturated at about 2 to 3 μM CO_2, while the $K_m(CO_2)$ of *C. reinhardtii* Rubisco is about 20 μM (34). In addition, C_i uptake has been measured directly in a number of laboratories (3, 4, 6, 55, 92, 93), and the C_i concentration inside the cell is higher than can be accounted for by diffusion alone.

Further evidence for the existence of a CCM in *C. reinhardtii* comes from mutant studies. In these studies, mutagenized cells were screened for growth on high (5% CO_2 in air) and low (air levels of CO_2 or lower) CO_2. Mutant strains that grew well on elevated CO_2 but poorly on low CO_2 were then selected for further studies. This approach has yielded mutants in C_i uptake (91, 100), CA activity (24, 58, 90), the photorespiratory chain (94, 104), and novel proteins. Another interesting class of mutants are those that fail to respond fully to changes in the CO_2 environment (22, 57, 105). In particular, the *CIA5* (*CCM1*) gene appears to encode a protein required for the induction of the CCM (22, 57). A listing of these mutant strains is shown in Table 1.

In this model for C_i uptake (Fig. 2), it is predicted that the pH gradient across the thylakoid membrane is an essential part of the CCM. Since light-driven electron transport is required to set up the pH gradient, C_i uptake should occur only in the light. To date, inorganic carbon concentration in *C. reinhardtii* has been observed only in cells or chloroplasts exposed to light. The strongest evidence in support of the light requirement comes from the pioneering work of Spalding and Ogren (88), who showed that electron transport inhibitors as well as mutants in the electron transport chain also inhibited the CCM in *C. reinhardtii*. While this work does not prove that a pH gradient across the thylakoid membrane is required for the CCM to operate, their data are fully consistent with this model.

Another requirement of this model is a CA in the thylakoid lumen to rapidly convert the bicarbonate entering the lumen to CO_2. In *C. reinhardtii*, CAH3 has been localized to the thylakoid lumen, and mutations in the *CAH3* gene result in cells with a nonfunctional CCM (60). An additional requirement of this model is that the CO_2 generated in the thylakoid lumen becomes available to Rubisco before being converted back to HCO_3^- in the basic environment of the chloroplast stroma. The pyrenoid of the chloroplast might serve to separate Rubisco from the CA in the stroma of the chloroplast. The pyrenoid is a proteinaceous structure where most of the Rubisco is located. The pyrenoid undergoes a dramatic morphological change when cells are switched from high- to low-CO_2 conditions (76) (Fig. 3). When the CCM is functional, a starch sheath appears around the pyrenoid and 90% of Rubisco is present in the pyrenoid (11, 52, 70).

Notably, most eukaryotic photosynthetic algae have pyrenoids (10), while pyrenoids are almost absent from the chloroplasts of terrestrial higher plants. Exceptions include some strains of *Chloromonas* which exhibit a CCM but are demonstrated to lack pyrenoids (50). However, pyrenoid-less CCM-containing strains of *Chloromonas* were demonstrated to have small C_i pools, of 24 to 31 μM, in comparison with the large C_i pools, of 231 to 252 μM, in algae exhibiting typical (dense with a high concentration of Rubisco) pyrenoids. It has been speculated that the formation of a large intracellular C_i pool in

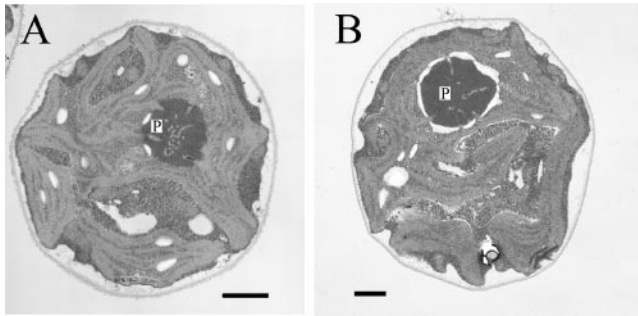

FIG. 3. Representative pyrenoids (P) from *C. reinhardtii* cells acclimated to high CO_2 (A) or low CO_2 (B). The white areas in the micrographs are starch. Bars, 1 μm.

TABLE 2. CAs in *C. reinhardtii*

CA	Gene family	Location
CAH1	α	Periplasm
CAH2	α	Periplasm
CAH3	α	Thylakoids
CAH4	β	Mitochondria
CAH5	β	Mitochondria
CAH6	β	Chloroplast stroma
CAH7	β	Chloroplast
CAH8	β	Periplasm
CAH9	β	Cytosol?

algae with a CCM is correlated with the presence of typical pyrenoids exhibiting a high concentration of Rubisco molecules. Algae lacking pyrenoids, such as *Chloromonas*, are often found in harsh environments, and perhaps the presence of a pyrenoid is not necessary because other factors besides CO_2 fixation are limiting growth (51).

Finally, a CA located in the chloroplast stroma would also be required for the operation of this type of CCM. This stromal CA would serve two functions: first, to convert CO_2 entering the chloroplast to HCO_3^- in the basic environment of the chloroplast stroma, and second, to recapture the CO_2 coming from the thylakoid lumen before it diffuses from the chloroplast. In *C. reinhardtii*, most of the required features of this type of CCM have been identified. There are CA isoforms in the thylakoid lumen (37) and the chloroplast stroma (48). What has not been established is how bicarbonate is transported across the thylakoid membrane. However, a number of proteins have been identified as potential HCO_3^- transporters, and this issue is discussed later in this article.

CAs. The acclimation of algae, including *C. reinhardtii*, to limiting CO_2 has been correlated with increased levels of CAs (2, 4, 16, 23, 90). In this minireview, we focus only on the α- and β-type CAs found in *C. reinhardtii*. These two forms of CAs are very different in structure and amino acid sequence. For example, the α-CAs share homology with the mammalian CAs and have three His residues coordinating the Zn ion. α-CAs are usually monomeric. In contrast, β-CAs have one His and two Cys residues coordinating the Zn (12) and are often multimeric. The *C. reinhardtii* β-CAs are quite similar to higher plant chloroplast CAs and bacterial CAs. As of this time, nine different α- and β-CA genes have been identified in the *Chlamydomonas* genome (Table 2). This plethora of CA genes has led to questions about what roles are played by these CAs and which ones are critical to the functioning of the CCM. A number of these proteins are implicated to have possible roles in the CCM.

The role of the periplasmic α-CA CAH1 is to facilitate entry of carbon dioxide into the algal cell. At pHs above 6.3, HCO_3^- is the predominant inorganic carbon species. This form of C_i, being an anion, cannot readily cross the plasma membrane (29, 53). CAH1, one of the first α-CAs reported for a photosynthetic organism, converts HCO_3^- to CO_2. Two lines of evidence have been presented for this physiological role of CAH1. First, membrane-impermeant CA inhibitors have a strong inhibitory effect on photosynthetic CO_2 fixation at high pHs, where HCO_3^- predominates, but a less pronounced effect at lower pHs, where most of the inorganic carbon is already in the form of carbon dioxide and the activity of periplasmic CA is no longer required (56). This view of the role of CAH1 was challenged by Van and Spalding, who found no evidence of growth inhibition in a mutant missing CAH1 (96). However, the presence of other CA isoforms in the periplasmic space, namely, CAH2 (75, 95) and possibly CAH8, makes the interpretation of these results more complicated. CAH1 biosynthesis is strongly regulated by changes in environmental CO_2 concentration as well as light. *CAH1* is very strongly induced under limiting CO_2 conditions, where the CCM is operational (23).

CAH1 has been shown to be controlled by two regulatory regions, namely, a silencer region, which represses transcription under high-CO_2 conditions or in the dark, and an enhancer region, which activates it under low-CO_2 conditions in the light (42). These sites may be important *cis*-acting elements that constitutively bind one or more proteins that assist in the regulated transcription of *CAH1* (43). *LCR1* has also been identified as a regulatory gene of *CAH1*. LCR1 is a Myb transcription factor that functions in amplification and maintenance of *CAH1* mRNA levels in response to limiting CO_2 (105).

CAH2 is also a periplasmic α-CA but is not thought to have an important role in the CCM. CAH2 is an active CA (75, 95) but is poorly expressed. In fact, *CAH2* expression is downregulated under limiting CO_2 conditions, the growth conditions under which the CCM is operational (75). *CAH2* is only 1.4 kb away from the *CAH1* gene (20) and may be the result of a recent gene duplication.

CAH3, the third CA gene described for *C. reinhardtii*, codes for an α-CA that has a leader sequence consistent with targeting CAH3 to the thylakoid lumen (24, 38). Immunoblot studies using antibodies raised against CAH3 demonstrated that CAH3 is associated with the thylakoid membrane (37). More specifically, immunolocalization studies indicated that CAH3 is localized on the luminal side of the thylakoids and inside the pyrenoid tubules (47). The evidence that CAH3 plays an essential role in the CCM is persuasive. *C. reinhardtii* strains defective in *CAH3* cannot grow in air levels of CO_2 even though they grow normally on elevated levels of CO_2 (37, 58, 67, 90). Putting the wild-type *CAH3* gene back into these strains restores normal photosynthesis (24, 37). Strains defective in *CAH3* also accumulate large pools of C_i but are unable to use C_i efficiently for photosynthesis (58, 90). Therefore, CAH3 appears to convert accumulated HCO_3^- to CO_2, the form of C_i that Rubisco can use. Its location suggests that CAH3 catalyzes the formation of CO_2 from HCO_3^- in the acidic lumen of thylakoids and that this CO_2 diffuses through the thylakoid membrane to the pyrenoid, where the CO_2 will be fixed by Rubisco (5, 53, 54, 60, 71). *CAH3* is expressed under both high- and low-CO_2 growth conditions, although there is a twofold increase in message abundance under low-CO_2 conditions.

CAH3 has also been proposed to be associated with photosystem II (PSII) and to help to stabilize the PSII manganese cluster and the catalytic function of PSII reaction centers (60, 99). This hypothesis is reinforced by the evidence that at low C_i concentrations, the *cah3* mutant, *cia3*, is impaired in maintaining high rates of electron transport and/or coupling the residual electron transport to ATP formation (31). However, sub-

sequent studies with the *Chlamydomonas cah3* mutant have shown that as CO_2 becomes limiting, the chloroplast ribulose 1,5-bisphosphate pool is increased compared with that in the wild type, which indicates a CO_2 supply limitation rather than a PSII energy supply defect (30).

C. reinhardtii contains identical mitochondrial β-CAs (mtCAs), CAH4 and CAH5, that exhibit a pattern of expression which correlates with the expression of the CCM. The genes encoding CAH4 and CAH5 are adjacent to each other in the *C. reinhardtii* genome (18). They are highly induced at both the transcriptional and translational levels under low-CO_2 conditions (17, 18, 26, 49) and may have an important role in the acclimation of *C. reinhardtii* to low-CO_2 conditions. However, the exact role of these CAs is still not clear. One suggested function of mtCAs is to buffer the mitochondrial matrix, since prior to the complete induction of the CCM, photorespiratory glycine decarboxylation produces equivalent amounts of NH_3 and CO_2. The mtCA might serve to catalyze the hydration of CO_2, producing H^+, which would prevent alkalinization in the mitochondrial matrix as a result of the generation of NH_3 by glycine decarboxylation (18). Alternatively, the mtCAs have been proposed to play a role in converting the CO_2 generated by respiration and photorespiration to HCO_3^-. This would effectively "recapture" the CO_2 generated by the photorespiratory pathway (73). More recently, it has been shown that even under low-CO_2 conditions, but with increasing NH_4^+ concentrations in the growth medium, the expression of mtCAs decreases at both the transcriptional and translational levels. Thus, it has been proposed that mtCAs are involved in supplying HCO_3^- to PEP carboxylase for NH_4^+ assimilation under certain conditions (27). As of this writing, there are no mutants of *C. reinhardtii* missing these mtCAs.

CAH6 is a constitutively expressed β-CA in the chloroplast stroma (47, 48). This CA might be involved in recapturing CO_2 as it effluxes from the thylakoid lumen and in helping to maintain a high concentration of inorganic carbon in the stroma. Likewise, it might be another CA responsible for supplying CO_2 for Rubisco. It might shuttle HCO_3^- to CO_2 in the stroma as CO_2 is depleted by the action of Rubisco. This is the same role proposed for chloroplast CAs of higher plants. The generation of mutants of *CAH6* could help to confirm the physiological role of CAH6 in photosynthesis and the CCM.

Two additional β-CAs, designated CAH7 and CAH8, are closely related CAs with 63% similarity. They are constitutively expressed at moderate levels in *C. reinhardtii*. An interesting feature of these two CAs is the presence of long, relatively hydrophobic C-terminal extensions that are unusual among β-CAs described to date. CAH7 has been localized to the chloroplast, while CAH8 has been localized to the periplasmic space close to the cell membrane (R. A. Ynalvez et al., unpublished data). The location of CAH8 at or near the plasma membrane suggests that it might help to facilitate C_i entry into the cell. The most recently discovered CA is CAH9. This β-CA has no leader sequence and has tentatively been assigned to be localized to the cytoplasm. An interesting point is that the sequence of this β-CA aligns more closely with bacterial CAs than with the other *C. reinhardtii* β-CAs (D. Deb and J. V. Moroney, unpublished results). The role, if any, of CAH9 in CO_2 acquisition remains to be determined.

Putative transporters. While a number of CAs have been shown to be part of the CCM in *C. reinhardtii*, no transporter has been linked definitively to the CCM. However, some promising candidate genes and proteins have been identified, and it is likely that one or more of the following proteins may participate in C_i uptake in *C. reinhardtii*. The candidate proteins are CCP1, CCP2, LCI1, NAR1.2 (LCIA), LCIB, HLA3, RH1, and YCF10. All of these proteins are encoded in the nucleus, with the exception of YCF10, which is encoded by the chloroplast genome. Most of these proteins, or the corresponding genes, were first identified because the protein or mRNA dramatically increases in abundance when *C. reinhardtii* is grown under limiting CO_2 growth conditions. For example, *CCP1*, *CCP2*, *LCI1*, *NAR1.2*, *LCIB*, and *HLA3* are all strongly induced when *C. reinhardtii* is making a functional CCM. In addition, mutations in the putative transcription factor CIA5/CCM1 (22, 103) reduce the expression of many of these proteins (15, 46, 49, 57).

Very few mutants that affect the expression of the genes encoding putative C_i transport proteins have been found. However, the *pmp1* mutant does have a mutation in *LCIB*, and this mutant is defective in C_i transport (91, 97). Recently, the allelic mutant *ad1*, air dier 1, was also described, and this strain also cannot grow in low CO_2 (350 ppm) but can grow either in high CO_2 (5% CO_2) or in very low CO_2 (200 ppm). The fact that the *pmp1*/*ad1* mutant fails to grow on air levels of CO_2 but manages to survive on very low levels of CO_2 has been interpreted as indicative of the existence of multiple C_i transport systems in *C. reinhardtii* corresponding to multiple CO_2 level-dependent acclimation states (89, 98, 100). This would be similar to the multiple C_i uptake systems seen in cyanobacteria. *pmp1*/*ad1* was found to be identical to the previously identified CO_2-responsive gene *LCIB* (49). LCIB does not have any significant homology to proteins from other organisms, but its predicted amino acid sequence has similarity with the predicted amino acid sequence of three genes, *LCIC*, *LCID*, and *LCIE*, in the *C. reinhardtii* genome. *LCIC* and *LCID* are also upregulated under low-CO_2 conditions. While these observations point to a role for LCIB in the adaptation to low CO_2, it is unlikely that LCIB is a transport protein by itself, as it lacks any hydrophobic transmembrane domains. Therefore, LCIB more likely has a regulatory role or might be part of a complex that transports C_i (97).

Another promising candidate protein to be a C_i transporter is LCI1. The *LCI1* gene was first identified as being very highly expressed in cells growing under low-CO_2 conditions (14). LCI1 contains four predicted transmembrane helices and also shows very little homology to any other protein in the NCBI database. Recent work with strains showing reduced expression of *LCI1* due to the presence of an *LCI1*-RNA interference (*LCI1*-RNAi) insert showed reduced growth on low CO_2 (Mason and Moroney, unpublished observations), but the physiological role of LCI1 remains to be determined.

Two other genes encoding putative C_i transport proteins are *CCP1* and *CCP2*. These genes encode the low-CO_2-inducible proteins LIP-36 G1 and LIP-36 G2 (26). These two proteins are 96% identical, have six transmembrane domains, are localized in the chloroplast envelope (54, 69), and have a high degree of similarity to the mitochondrial carrier family of proteins (15). When the abundance of *CCP1* and *CCP2* messages

was reduced using RNAi, the resultant strains grew poorly with low CO_2 levels but normally with elevated levels of CO_2 (62). However, C_i uptake was normal in these strains (61). This might indicate that CCP1 and CCP2 are transporters of metabolic intermediates of photorespiration or transporters of other metabolic intermediates (61) or that these proteins are part of a redundant system of C_i transport, as seen in cyanobacteria.

Another putative C_i transporter, LCIA, was also first discovered using expression analysis (49). LCIA is also called NAR1.2. LCIA/NAR1.2 was first annotated as a nitrite transporter and has strong similarity to the bacterial nitrite/formate family of transporters. *NAR1.2* belongs to a gene family consisting of six *NAR* genes in *C. reinhardtii*, and surprisingly, these genes have no obvious homolog in *Arabidopsis*. The expression of *NAR1.2* is induced under low-CO_2 conditions and is partially under the control of CIA5, a transcription factor that is required for the expression of other CCM genes (49). NAR1.2 is predicted to be localized to the chloroplast thylakoid or chloroplast envelope and has six transmembrane domains. The functional expression of NAR1.2 in *Xenopus* oocytes has shown that the presence of NAR1.2 increases the bicarbonate entry into oocytes twofold compared to that of the control (46). These features suggest that NAR1.2 is an attractive candidate to be a bicarbonate transporter.

Three other proteins suggested to be part of the C_i uptake system include HLA3 (32), RH1 (86), and YCF10 (81). *HLA3* was first identified as a gene showing expression when *C. reinhardtii* cells were exposed to high light. Subsequent work showed that *HLA3* expression is also controlled by the CO_2 concentration. HLA3 has strong sequence similarity to an ABC transporter and was first predicted to be localized to the chloroplast membrane (32). However, more recent versions of the prediction servers give much less clear predictions as to the location of HLA3. HLA3 might be a potential transporter in the acclimation of cells to low CO_2 or might be involved in redox control and only indirectly involved in the control of CCM expression (32). Another chloroplast envelope protein that has been implicated in C_i uptake is the product of the *ycf10* gene. It can form two or three transmembrane domains and has been localized in the inner chloroplast envelope membrane (82). Disruption of the open reading frame affected the uptake of inorganic carbon (81). These observations raise the possibility that this protein is a C_i transporter. However, subsequent experiments provided evidence that YCF10 may not be involved directly in C_i uptake but rather may regulate the C_i transport system. It could be associated with a system in the chloroplast envelope involved in HCO_3^- and/or CO_2 uptake (81).

RH1 has been implicated in CO_2 transport because it is very similar to bacterial proteins shown to be ammonia and/or CO_2 channels (86). However, the expression of this protein is not consistent with it being part of the CCM, as RH1 is expressed at high levels of CO_2 when cells are grown on elevated CO_2 and not when cells are grown on low CO_2. In addition, when RH1 expression is reduced by mutation, *C. reinhardtii* can still grow on low levels of CO_2 but shows reduced growth on elevated levels of CO_2 (87). Likewise, RH1 is not regulated by CIA5 (101). The possible physiological role of this protein is to facilitate CO_2 entry into the cell when the CO_2 level is high. The role of RH1 in CO_2 transport remains a very interesting question in this field.

CHALLENGES AND FUTURE DIRECTIONS

The biggest challenge facing researchers studying the CCMs in eukaryotic algae is identifying the transport components involved in inorganic carbon accumulation. This is especially true for the proposed thylakoid HCO_3^- transporter. In the case of the thylakoid, experiments need to be done to demonstrate whether HCO_3^- can cross the membrane at all, as the only report on HCO_3^- transport was negative (31). In an effort to identify additional components of the CCM in *C. reinhardtii*, a number of insertional mutants have been generated (Table 1). While a number of candidate transport proteins have been identified in *C. reinhardtii*, none of these proteins has been proven conclusively to be an essential part of the CCM. One issue that may be hampering these efforts is that there may be a number of transporter proteins and eliminating only one through mutation may not lead to an obvious growth phenotype. This is the case for cyanobacteria. One frustrating point has been that none of the transport proteins identified in cyanobacteria aligns well with an annotated gene product in *C. reinhardtii*. This lack of homology underscores both the evolutionary distance between green algae and cyanobacteria and the possibility that the CCM may have evolved independently in these different lineages.

In contrast, the number and location of the CA isoforms are becoming clearer. While mutants exist for only two of the CA genes (*CAH1* and *CAH3*), RNAi studies should help to clarify the physiological roles of the other isoforms. It will be interesting to see if the mtCAs are important to the CCM. The two mitochondrial proteins, CAH4 and CAH5, dramatically increase in abundance when *C. reinhardtii* is in a low-CO_2 environment. This induction implies that CAH4 and CAH5 are important to the cells' acclimation to limiting CO_2 conditions. However, whether these mitochondrial proteins are important in CO_2 recapture, the photorespiratory pathway, or some other anaplerotic function remains to be established.

The role of the pyrenoid remains another important topic of research. In *C. reinhardtii*, there is a dramatic rearrangement of starch granules when the cells are shifted from high- to low-CO_2 growth conditions (Fig. 3). When the cells experience high CO_2, the starch granules are evenly distributed throughout the chloroplast stroma. When they are switched to low CO_2, the starch strongly associates with the pyrenoid, forming a "shell" or "sheath" around the pyrenoid (11). Since almost all of the Rubisco is contained within the pyrenoid, that means that all of the Rubisco is encased in this carbohydrate shell (41, 52). This observation has evoked the speculation that the starch sheath might be an important acclimation to low-CO_2 growth conditions. However, when mutants unable to make starch were tested for growth with low CO_2, they were still able to grow at a rate indistinguishable from that of wild-type cells (Mason and Moroney, unpublished observations). For cyanobacteria, mutations that disrupt the carboxysome or cause Rubisco not to package in the carboxysome (64, 83) cause the bacteria to grow slowly on low levels of CO_2. To date, no mutations that disrupt the pyrenoid structure in *C. reinhardtii*

are known, except for mutations in *rbcL* itself, which eliminate Rubisco and also eliminate the pyrenoid altogether (76).

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