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Abstract
Many aquatic photosynthetic microorganisms possess inducible CO2 concentrating mechanisms (CCMs) that allow them to optimize carbon acquisition in environments with frequently changing and often limiting CO2 concentrations. The CCMs function by accumulation of a large quantity of intracellular inorganic carbon (Ci) through concerted Ci uptake systems and enzymes catalyzing the interconversion between different species of Ci. In addition, an array of regulatory devices appears present to facilitate the sensing of CO2 availability and the regulation of metabolic pathways. Over the past several decades, significant advances have been made in understanding the CCM and its regulation. With the aid of mutant studies and the availability of several cyanobacterial and eukaryotic algal genomes, an integrated picture is emerging to reveal many of the molecular details in the microalgal CCMs. This review will focus on the recent advances in identifying and characterizing the major components involved in the CCM, including Ci uptake systems and regulatory pathways in eukaryotic microalgae, especially in the model organism, Chlamydomonas reinhardtii.

Keywords
acclimation, algae, carbonic anhydrase, Chlamydomonas reinhardtii, inorganic carbon, signal transduction pathway, transport systems

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CO₂ Concentrating Mechanisms in Eukaryotic Microalgae

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ABSTRACT

Many aquatic photosynthetic microorganisms possess inducible CO₂ concentrating mechanisms (CCMs) that allow them to optimize carbon acquisition in environments with frequently changing and often limiting CO₂ concentrations. The CCMs function by accumulation of a large quantity of intracellular inorganic carbon (Ci) through concerted Ci uptake systems and enzymes catalyzing the interconversion between different species of Ci. In addition, an array of regulatory devices appears present to facilitate the sensing of CO₂ availability and the regulation of metabolic pathways. Over the past several decades, significant advances have been made in understanding the CCM and its regulation. With the aid of mutant studies and the availability of several cyanobacterial and eukaryotic algal genomes, an integrated picture is emerging to reveal many of the molecular details in the microalgal CCMs. This review will focus on the recent advances in identifying and characterizing the major components involved in the CCM, including Ci uptake systems and regulatory pathways in eukaryotic microalgae, especially in the model organism, Chlamydomonas reinhardtii.

Keywords: acclimation, algae, carbonic anhydrase, Chlamydomonas reinhardtii, inorganic carbon, signal transduction pathway, transport systems

Abbreviations: CA, carbonic anhydrase; CCM, CO₂ concentrating mechanism; Ci, inorganic carbon

INTRODUCTION

As the primary machinery for carbon fixation on the earth, photosynthetic organisms constantly confront changing ambient CO₂ concentrations and must adjust themselves accordingly. Although rising levels of CO₂ in the atmosphere have lately been a serious concern, a shortage in CO₂ supply, paradoxically, is often a major stress for photosynthetic organisms, not only due to low ambient CO₂ concentrations for photosynthesis, but also due to the inherent inefficiency of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a key enzyme in carbon fixation. Several strategies have evolved in photosynthetic organisms to accommodate the CO₂ limitation by raising the CO₂ concentration at the site of Rubisco, including the well-known C4 and CAM photosynthetic pathways adopted in certain higher plants and a unique CO₂ concentration mechanism (CCM) adopted in many aquatic microalgae. While CO₂ enrichment in C4 or CAM pathways is achieved by spatial or temporal separation of ambient CO₂ fixation into C4 acids, and re-release of CO₂ into the C3 pathway at the site of Rubisco, the microalgal CCM relies on active inorganic carbon (Ci)
transport by which a large intracellular Ci pool is created. Because of its ubiquitous existence in photosynthetic microorganisms and its simple and efficient nature, the microalgal CCM has gained significant attentions since it was discovered. First, since microalgal photosynthesis accounts for a significant portion of the carbon capture and sequestration on the earth, the operation of microalgal CCMs has a profound influence on global atmospheric CO2 concentration. Understanding microalgal CCM has been of great interest because of their potential influences on global environments and biomass production. Moreover, as microalgal C4 transport systems exhibit a great efficiency in concentrating CO2, they appear to be excellent candidates for engineering many traditional C3 crop plants and other economically important C3 plants whose photosynthetic performances might be elevated by introducing a microalgal-like CCM. Last, but not the least, because the microalgal CCM represents an exquisite system by which an organism responds and interacts with environmental stresses, understanding the microalgal CCMs helps us to comprehend the acclimation of an organism to changing environments.

Since the discovery of the microalgal CCMs, significant effort has been devoted in understanding the mechanisms of their operation and regulation. Knowledge of microalgal CCMs is largely based on studies in some model organisms, including several cyanobacterial species and eukaryotic algal species. Considerable progress has been made thus far with regard to understanding cyanobacterial CCMs. With the aid of genetic and biochemical studies and availability of genome sequences from many cyanobacteria species, an integrated picture is merging, which reveals many fundamental aspects of cyanobacterial CCM functions, including diverse genes and proteins involved in Ci transport systems and carboxysome micro-compartments (Badger and Price 2003; Badger et al. 2006). For the CCM in eukaryotic algae, however, the molecular basis of the CCM still remains largely unknown. Chlamydomonas reinhardtii, a unicellular green alga with a photosynthetic apparatus similar to that of higher plants, is often used as a model system to study the microalgal CCM, and most of our knowledge of the eukaryotic algal CCM has been derived from physiological and biochemical studies on this organism. C. reinhardtii has a well-characterized genetic background and many unique physiological and biochemical characteristics which make it an attractive model system for studying the interaction of photosynthetic eukaryotes with their environments. The recent completion of the genome sequences of C. reinhardtii and several other eukaryotic microalgae, in combination with the increasingly available genetic and molecular tools, is facilitating research that will finally reveal many functional components involved in the eukaryotic CCM. This review will focus on the recent advances in our understanding of the eukaryotic microalgal CCMs, especially in C. reinhardtii.

**ACCLIMATION TO LIMITING CO2**

The CCM of microalgae was originally recognized as distinguishable physiological states induced by limiting CO2 conditions (Spalding 1998; Kaplan and Reinhold 1999). One significant acclimation to limiting CO2 appears as a much higher efficiency in photosynthetic carbon assimilation and the apparent suppression of photorespiration. As well demonstrated, such high photosynthetic efficiency in limiting CO2-acclimated cells can be attributed to their capacity for accumulating a large intracellular Ci pool, which is resulted from induction of the CCM. The high internal Ci level maintains a saturated or near saturated CO2 concentration at the site of Rubisco, favoring its carboxylation activity over its oxygenation activity and thus efficiently promoting photosynthesis and suppressing photorespiration. Transport systems for active Ci uptake and enzymatic systems catalyzing rapid interconversion between different Ci species are generally considered to be two essential components of the CCM. In a simplified working model, the CCM comprises at least the following elements: active Ci transport and Ci accumulation, interconversion among different Ci species, and final dehydration of accumulated bicarbonate (HCO3-) to release CO2 at the sites of Rubisco (Kaplan and Reinhold 1999; Badger and Spalding 2000; Giordano et al. 2005).

**Ci CONCENTRATING SYSTEMS**

**Diversity in Ci transport systems**

Active Ci transport plays a vital role and exhibits a great diversity in microalgal CCMs. Five different Ci transport modes have so far been identified in various cyanobacteria species (Badger et al. 2006). Eukaryotic Ci transport systems are expected to be of greater complexity than those in cyanobacteria, not only due to the highly compartmented structures in eukaryotic cells, but also due to the heterogeneity of Ci species being transported and the existence of multiple Ci-level-dependent acclimation states in these organisms.

In eukaryotic microalgae, active uptake of Ci appears to take place in at least two locations: chloroplast envelope and plastid. C. reinhardtii, Ci species must penetrate these two barriers to reach the pyrenoid, a specialized protein structure in chloroplasts where Rubisco is localized. It has been demonstrated that the induction of an active Ci transport system occurs at both the plasma membrane and the chloroplast envelope (Goyal and Tolbert 1989; Sümeyer et al. 1989, 1991; Palmqvist et al. 1994). There is still some debate as to whether the plasma membrane or plastid envelope is the primary site for Ci transport. However, given the fact that all photosynthetic enzymes are located in the chloroplast, it is commonly accepted that chloroplasts play an irreplaceable role in Ci concentrating (Moroney et al. 1987; Amoroso et al. 1998; Spalding 1989; Kaplan and Reinhold 1999). The C. reinhardtii pmp1 mutant was identified as conditional lethal in air levels of CO2 and was demonstrated to be deficient in Ci transport (Spalding et al. 1983a). A plastid localization of the PMP1 gene product has been predicted (Miura et al. 2004; Wang and Spalding 2006), and been recently confirmed by immunolocalization (Wang and Spalding, unpublished). The lethal phenotype caused by a single lesion in pmp1 supports the essentiality of plastid Ci transport. However, this does not necessarily exclude a requirement for any potential plasma membrane transporters. If the plasma membrane Ci transport functions concomitantly with the plastid transport system, plastid transport might be unnecessary. If the phenotype of pmp1 implies no functional redundancy between the plastid transport system and the one on plasma membrane. Nevertheless, it supports a central role played by the chloroplast in active Ci transport in C. reinhardtii.

Heterogeneity of Ci species is another possible reason for the requirement of diverse Ci transport systems. HCO3- and CO2 are the two principal Ci species over the physiological pH range, although HCO3- and other Ci species can not be excluded as potential substrates. The distribution of the individual Ci species vary depending on the pH of the extra- and intracellular environments or the intracellular compartments. Different Ci transporters may display distinct substrate specificity. HCO3- has been postulated as the primary substrate for chloroplast Ci uptake (Moroney et al. 1987; Moroney and Mason 1991), and CO2 as the species predominantly transported across the plasma membrane, possibly by passive diffusion (Spalding 1998; Kaplan and Reinhold 1999). Mass-spectrometric disequilibrium analyses have apparently demonstrated the active transport of CO2 by both cells and isolated chloroplasts of C. reinhardtii (Amoroso et al. 1998). Uptake of both HCO3- and CO2 proceeded at similar rates in isolated chloroplasts from C. reinhardtii, although HCO3- was the dominant Ci species transported in Dunaliella tertiolecta. Similar patterns of active Ci uptake by chloroplasts also were demonstrated in Tetraselmis minimum and C. noc.
CCMs of eukaryotic microalgae. Wang and Spalding

tigama (van Hunnik et al. 2002). It is not clear whether an individual transport system always displays preferences for certain Ci species, but it appears that existence of substrate specificity is common for many Ci transport systems (Gior-dano et al. 2005).

Diversity of Ci transport systems also might be associated with multiple acclimation states. In nature, the ambient CO2 concentrations for photosynthetic organisms can vary according to the magnitude and duration of habitats and seasonal or daily changes in environmental CO2. Accordingly, distinct acclimation states are induced to cope with various levels of Ci supply. Natural habitats for Chla-mydomonas species include metabolite-rich environments (such as sewage or rich soil) where the levels of Ci or acetate can be considerably high and freshwater aquatic systems (such as freshwater pond) where CO2 levels are low and diffusion of Ci species are highly restricted. Moreover, extreme low levels of CO2 or depleted Ci can be resulted in a rapidly growing population with active photosynthesis. In the past, two physiological states corresponding to enriched CO2 concentrations (1-5% CO2 in air, high CO2) and low CO2 concentrations (air, -0.03% CO2) have been well documented. Another physiological state corresponding to very low CO2 (<0.02% CO2), perhaps neglected previously, was recently recognized based on an unusual growth phenotype of pmp1 (Van et al. 2001; Spalding et al. 2002; Wang and Spalding 2006). The pmp1 mutant, as well as its allelic mutant air dier 1 (ad1), displays an “air-dieing” phenotype: it grows well in either high or very low CO2, but dies in low (air-level) CO2. This conspicuous phenotype indicates that at least three distinct CO2 acclimation states exist in C. reinhardtii, associated with high, low, or very low CO2, respectively. The three distinct CO2 acclimation states were confirmed by comparisons of physiological and photosynthetic characteristics among high CO2-, low CO2- and very low CO2-acclimated cells (Vance and Spalding 2005). The cells acclimated to very low CO2 exhibited a much lower Vmax, a longer cell-doubling time and a smaller cell size when compared with those acclimated to low CO2 and high CO2.

Despite the common assumption that the CCM is induced only in limiting CO2, there is evidence indicating that active uptake of Ci may take place also in high CO2 grown cells. Active transport of HCO3- has been reported in high CO2 grown cells from C. reinhardtii and D. tertiolecta, although with a much lower apparent affinity than that of low CO2 cells (Palmyquist et al. 1994; Amoroso et al. 1998). The main reported differences between the transporters from high CO2 grown cells and low CO2 grown cells appear to be their substrate affinities (Amoroso et al. 1989), since no significant differences in maximal Ci uptake activities were observed. This would be consistent with the observation that high CO2 cells and low CO2 cells displayed similar photosynthetic Vmax (Vance and Spalding 2005).

As discussed in a recent paper, requirement for transport systems with different Ci affinities and transport capacities in C. reinhardtii may represent a common survival strategy in eukaryotic microalgae (Wang and Spalding 2006). When grown in very low CO2, transporters with high Ci affinities may favor uptake of Ci present at a very low concentration, and the low capacities for Ci influx may allow C. reinhardtii to maintain some growth without quickly depleting all available Ci. On the other hand, in low CO2 (air level), a system with low Ci affinity would be sufficient to accommodate the available Ci concentration, and the higher transport capacity would maintain a growth rate comparable to that of high CO2 cells. The ability to acclimate to multiple CO2 levels in eukaryotes apparently requires a network of transport systems which is elaborately orchestrated.

Candidate Ci transporter genes in Chlamydomonas

Although there is substantial evidence demonstrating the existence of active transport systems in eukaryotic algae, only very few candidate Ci transport genes have been identified, and the functions of their gene products in the CCM are far from being fully characterized.

**PMP1/AD1/LCIB and LCIB family**

The pmp1 mutant is one of only a small number of mutants with a lesion directly affecting the CCM, and it has been touted as demonstrating a requirement for active Ci transport in the CCM (Spalding et al. 1983a). Recently the gene defective in pmp1 was identified by genetic characterization of its allelic insertional mutant, ad1 (Wang and Spalding 2006). PMP1/AD1 protein is encoded by LCIB, a gene previously identified as a CO2 responsive gene (Miura et al. 2004). Blast searches and domain searches with LCIB revealed no significant sequence similarity except for three additional genes in the C. reinhardtii genome, LCIC, LCID and LCIE (Wang and Spalding 2006). LCIB and LCIC show similar expression patterns: very low expression in high CO2 (5%) and substantial increased expression induced by a wide range of limiting CO2 (0.15%-0.01%). LCID shows a similar CO2 expression pattern but at a much lower apparent mRNA abundance, while expression of LCIE so far has been confirmed only by its existence in a cDNA library.

Although physiological and biochemical characterization of pmp1 and ad1 suggests that LCIB is involved in active Ci transport, LCIB seems unlikely to perform as a Ci transporter by itself, because it is predicted to be a soluble protein lacking any obvious transmembrane regions. It is possible that LCIB is an integral component of a multi-subunit transport complex and that its Ci transport function relies on its interaction with other proteins. Plastid localization was predicted for both LCIB and LCIC. There is evidence indicating a possible physical interaction between LCIB and LCIC. There is evidence that the LCIB associated Ci transport system is essential for the low CO2 (air) acclimation state in C. reinhardtii. Even though LCIB also is expressed in very low CO2 and high CO2, obviously it is not essential under such conditions, if it functions at all at those CO2 concentrations.

**YCF10 (Cem A) dependent system**

Disruption of the plastid ycf10 open reading frame was found to cause light sensitivity and decreased Ci uptake in C. reinhardtii (Rolland et al. 1997). The product of the chloroplast ycf10 gene is localized in the inner chloroplast envelope and displays sequence homology with the chloroplast CemA protein from plants and cyanobacterial CotA product. Ycf10, like CemA and CotA, is a membrane protein with four transmembrane domains. The cyanobacteria cotA mutants exhibited a slow growth in limiting CO2 and decreased CO2 transport, but it was suggested that the decreased CO2 transport in the mutants was a result from the impaired proton extrusion (Katoh et al. 1996a, 1996b). Even though the ycf10 mutants display defective CO2 uptake, their sensitivity to high light in both high CO2 and low CO2 argues against the direct involvement of ycf10 gene product in CO2 uptake (Kaplan and Reinhold 1999). Whether and how ycf10 participates in Ci uptake still remains to be determined.

**CCP1 and CCP2**

CCP1 and CCP2 (Lip36) were first identified as two low CO2 induced polypeptides in C. reinhardtii (Spalding and Jeffery 1989; Ramazanov et al. 1993). These proteins are associated with chloroplast envelope and have been suggested as candidates for the involvement in chloroplast Ci transport. CCP1 and CCP2 were demonstrated to encode two similar proteins sharing 95.7% identity or amino acid sequence (Chen et al. 1996). CCP1 and CCP2 are predicted
to be membrane proteins with six transmembrane domains, and are similar to the mitochondrial carrier protein superfamily. Mitochondrial carrier proteins are small proteins catalyzing the translocation of metabolites across the mitochondrial inner membrane, which include ATP/ADP translocators, uncoupling proteins (proton carriers), phosphate carriers and carriers for a variety of metabolites in the mitochondrial carbon/energy metabolism (Kuan and Saier Jr. 1993; Palmieri 1994). It was reported that strains with silenced CCP1/2 expression by RNA interference (RNAi) grew slower in low CO2, but exhibited photosynthetic kinetics and Ci affinity similar to those in wild type cells (Pollock et al. 2005). The lack of a clear phenotypic link between the CCP1/2 RNAi knockdowns and CCM function raises questions regarding any essential role for CCP1 and CCP2 in the CCM, although it is possible that their functional importance was masked by the compensating function of other Ci transport systems under the conditions employed, as has been observed for Ci transport in cyanobacteria (Shibata et al. 2002).

**LCIA/NAR1.2**

LCIA, also named as NAR1.2, was identified as a low-CO2 inducible gene that encodes a protein belonging to the NAR1 multigene family from *C. reinhardtii* (Galvan et al. 2002; Miura et al. 2004; Mariscal et al. 2006). The members of NAR1 family are related to the formate/nitrite transporter (FNT) family (Miura et al. 2004; Mariscal et al. 2006). Proteins in FNT family have been found in various bacteria and some eukaryotic organisms such as fungi, yeast, algae and protozoa, but not in plants (Galvan et al. 2002; Mariscal et al. 2006). LCIA is a polypeptide of 336 amino acids with a predicted chloroplast transit peptide and 6 transmembrane domains. LCIA has been postulated as a Ci transporter because the expression of LCIA is regulated by CO2, irrespective of the nitrogen sources (Miura et al. 2004). LCIA also has recently been reported to exhibit transport activities for both bicarbonate and nitrite when expressed in *Xenopus* oocytes (Mariscal et al. 2006). However, in this research, LCIA exhibited a rather low affinity for HCO3-, but a high affinity for nitrite, leaving the question of its importance as a major Ci transporter open.

**HLA3/MRP1**

Im and Grossman (2001) identified a limiting CO2-induced gene, HLA3, encoding a putative ATP-binding-cassette (ABC)-transporter. HLA3 also has been named MRP1, and its gene product is suggested as a Ci-transporter (Miura et al. 2004). ABC transporters are ubiquitous membrane proteins that transport a large variety of molecules across membranes, and represent one of the largest protein families (Rea 2007). In cyanobacteria, the best-known Ci transporter belonging to the ABC transporter family is BCT1, an inducible high affinity HCO3- transporter encoded by the bct1 operon (Badger and Price 2003; Badger et al. 2006). Unlike BCT1, which is a bacterial type, multimeric, four subunit complex, HLA3 is a whole-molecule ABC transporter with significant homology to members of the multidrug-resistance-related protein (MRP) subfamily of ABC transporters (Im and Grossman 2001). MRP-related transport is often inhibited by vanadate, an inhibitor of the plasma membrane bound ATPase, because ABC-dependent transport is energized by ATPase (Rea 2007). The inhibitor of vanadate was reported (Palmqvist et al. 1988), which may reflect the sensitivity of HLA3 associated Ci transport to the inhibitor. Although HLA3 was first suggested as being a plastid-localized protein, based on protein targeting prediction (TargetP, http://www.cbs.dtu.dk/services/TargetP/; or PSORT, http://psort.nibb.ac.jp/), the HLA3 gene product was predicted to enter the endomembrane system, and thus may be targeted to the plasma membrane. HLA3/MRP1 is the only clear candidate for a plasma membrane Ci transporter identified so far in eukaryotic systems.

**LCI1**

LCI1 was originally identified as a limiting CO2 inducible gene encoding a putative protein of 192 amino acids (Burrow et al. 1996). The expression of LCI1 was regulated by CIA5, a master regulator in the CCM (Im et al. 2003; Miura et al. 2004; and by LCR1, a transcriptional factor associated with CO2 signal transduction pathways (Yoshioka et al. 2004). LCI1 appears to be a membrane protein with three predicted transmembrane domains and a signal peptide (TMHMM, http://www.cbs.dtu.dk/services/TMHMM/; TargetP and PSORT), suggesting that LCI1 is perhaps targeted to the plasma membrane. No information is currently available with regard to the possible function of LCI1 in the CCM.

**RHP1**

*C. reinhardtii* has two Rhesus (Rh) proteins (RHP1 and RHP2 or Rh1 and Rh2) which are similar to the Rh proteins in the human red blood membrane (Soupene et al. 2002). Both RHP1 and RHP2 are predicted to have 12 transmembrane domains. RHP1 has a potential chloroplast transit sequence, and may be associated with the chloroplast membranes (Soupene et al. 2002; Kustu and Inwood 2006). The expression of RHP1 is highly inducible by high CO2 (3%) with very low expression in low CO2 (air). RNAi mutants that had no RHP1 expression exhibited a growth defect in high CO2, but normal growth in air (Soupene et al. 2004). RHP1 apparently functions as bidirectional CO2 gas channels, which allow quick equilibration of CO2 under high CO2 conditions.

**Carbonic anhydrase in the CCM**

Carbonic anhydrase, being present in three independent evolutionary lines (α-, β- and γ-type), is ubiquitously present over a wide spectrum of living organisms (Hewett-Emmett and Tashian 1996). In the CCM, CA plays a critical role by catalyzing the interconversion between different Ci species. Diverse forms of CAs appear to be involved in various fundamental aspects of the eukaryotic CCM (Spalding 1998; Moroney and Somanchi 1999; Mitra et al. 2004; Giordano et al. 2005; Mitra et al. 2005). In cyanobacteria, one prominent feature regarding the function of CA is the co-localization of CA with Rubisco in the carboxysomes, which is thought to be necessary for dehydrating accumulated intracellular HCO3- and generating an elevated CO2 concentration in close proximity to Rubisco. No Rubisco associated CA in pyrenoid has been demonstrated in eukaryotic algae, but the similar role has been proposed for a thylakoid luminal CA in *C. reinhardtii* (CAH1, CAH2, and CAH3) and three β-CAs (CAH4, CAH5 and CAH6).

**CAH3**

Despite multiple CAs present in *C. reinhardtii*, CAH3 is so far the only one that has been confirmed as essential for limiting CO2 acclimation. Mutants defective in CAH3 (cah1-12-1C, cah2-18-6A, cal-3-18-7C, and cia3) cannot survive under limiting CO2 conditions (Spreitzer and Mets 1981; Spalding et al. 1983b; Moroney et al. 1986; Suzuki and Spalding 1989; Karlsson et al. 1995; Funke et al. 1997; Karlsson et al. 1998). Even though over-accumulation of internal Ci has been demonstrated in mutant cells with defects in CAH3, photosynthesis is still limited by CO2 in these mutants (Spalding et al. 1983b; Hanson et al. 2003). CAH3 is targeted to the thylakoid lumen and may be asso-
CAH5

The uptake of CO2 at the plasma membrane (Moroney et al. 1997). This model requires a low CA activity in the stroma, where the alkaline pH would favor formation of HCO3\(^-\), unless the CO2 released from lumen can directly reach Rubisco in the pyrenoid without traversing much stromal space. In any case, it requires the absence of CA activity in the pyrenoid. Some researchers also have proposed that CAH3 is involved in PSII function, or in the formation of a proton gradient across the thylakoid membranes (Villarejo et al. 2002; van Hummik and Sültemeyer 2002). However, a recent re-examination of CAH3 still supports the role of CAH3 as providing CO2 to Rubisco, because PSI11 electron transport was not affected by the cia3 mutation (Hanson et al. 2003).

CAH1 and CAH2

Under limiting CO2 conditions, the majority of the CA activity in C. reinhardtii is found in the periplasmic space. The products of two genes, CAH1 and CAH2, were identified as α-type CA isoforms associated with the periplasmic CA activity (Fujisawa et al. 1990; Fukuzawa et al. 1990; Rawat and Moroney 1991). The regulation of CAH1 and CAH2 expression by Ci availability shows opposite trends: whereas the less abundant CAH2 gene product (pCA2) is expressed at high Ci concentrations and repressed by low Ci concentrations, the CAH1 gene product (pCA1), which accounts for the majority of the extracellular CA activity, is expressed only in limiting Ci concentrations. The regulation of the CAH1 expression has been closely scrutinized, and different roles for the periplasmic CA in the CCM have been proposed (Spalding 1998; Giordano et al. 2005). It appears that the periplasmic CA activity could facilitate the uptake of CO2 at the plasma membrane (Moroney et al. 1985; Spalding 1998; Giordano et al. 2005). However, a null-mutant with a disrupted CAH1 gene did not show any discernable phenotype differing from the wild type cells, questioning the essentiality of pCA1 in the CCM (Van and Spalding 1999).

CAH4 and CAH5

Two mitochondrial CAs encoded by very similar genes, CAH4 and CAH5 (βCa1 and βCa2), were identified as members of the β-type CA family (Eriksson et al. 1996). The expression of CAH4 and CAH5 is regulated by CO2 at the transcriptional level, with the abundant CAH4 and CAH5 transcripts induced by low CO2 (Eriksson et al. 1998). Although CAH4 and CAH5 are among the most abundant low CO2 inducible proteins, their functions in the CCM have not been determined. It has been demonstrated that they are required for buffering pH in mitochondria to neutralize ammonia released during photosynthesis (Eriksson et al. 1996). Giordano et al. (2003) proposed that CAH4 and CAH5 might be involved in anaerobic carbon recycling in C. reinhardtii to balance carbon and nitrogen assimilation. β-type CAs have an ancient evolutionary origin and are ubiquitously distributed in almost all living organisms (Smith et al. 1999). A study has showed that a β-type CA is essential for the growth of Corynebacterium glutamicum under atmospheric conditions (Mitsuhashi et al. 2004), as demonstrated by a high CO2 requiring phenotype caused by a deficiency in the CA. It is possible that the functions of CAH4 and CAH5 are related to mitochondrial carbon metabolism, but not directly related to the CCM.

CAH6

CAH6 is another β-type CA recently identified as a probable chloroplast stromal CA (Mitra et al. 2004). The presence of CA activity in the stroma indicates that the CCM in eukaryotic microalgae must be different from the cyanobacterial CCMs, which are largely dependent on the absence of CA activity in the cytosol. As demonstrated in the cyanobacterium Synechocystis PCC7002, heterologous expression of human CA, HCAIII, in the cyano bacteria cytosol caused dramatic decreases in photosynthetic QA affinity and Ci accumulation, and resulted in a high CO2 requiring phenotype (Price and Badger 1989). As discussed earlier, a stromal CA does not favor the entry of stromal CO2 into pyrenoids. It would require very close contact between the thylakoid membranes and the pyrenoid so that luminal CO2 can be directly released into the pyrenoid. Indeed, it has been reported that CAH3, although distributed throughout the chloroplast, is enriched in the thylakoids around the pyrenoid, and also is present in the thylakoid tubules penetrating the pyrenoid (Mitra et al. 2005). The function of CAH6 has been proposed to be trapping of CO2 that diffuses out from the pyrenoid as the more easily confined HCO3\(^-\), the formation of which would be facilitated by the alkaline stromal pH (Mitra et al. 2004, 2005).

Other CAs in C. reinhardtii

The recent completion of the C. reinhardtii genome sequence has revealed a large number of CAs. Besides of the six CAs mentioned above, at least three additional putative β-type CAs (CAG7, CAG8, CAG9) are present in the C. reinhardtii genome, although none of these has yet been fully characterized (Mitra et al. 2005; http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). Moreover, a γ-CA-like gene, CAG3 (GLP1), also was identified in the genome, but no CA activity was detected in CAG3 when over-expressed (Mitra et al. 2005). Except that CAH8 was confirmed to exhibit CA activity, no information is available with regard to the biochemical characteristics or intracellular locations of these CAs or the CA-like protein. Villarejo et al. (2001) have demonstrated a CA activity associated with the chloroplast envelope, but it is not clear if there is any relationship between this envelope CA and CAH6 or any other identified putative CAs. Further investigation of the CA distribution in chloroplasts is critical to elucidate the CCM model in eukaryotic algae.

REGULATION OF THE CCM AND SIGNAL TRANSDUCTION

Like many other acclimation responses, the CCM is highly regulated to allow the cells to respond the variability of CO2 supply in an efficient and economical way. During acclimation, rapid changes in gene expression and biochemical events occur, which are believed to be regulated by an array of regulatory devices apparently present to facilitate the sensing of CO2 availability and monitor carbon metabolism. Many questions with regard to CO2 sensing and signaling are still unsolved, largely due to lack of knowledge of the molecular components associated with these functions.

Perception of CO2 and signals controlling the CCM expression

In cyanobacteria, either the external HCO3\(^-\) or the internal Ci pool has been postulated as the signal controlling the expression of the CCM (Mayo et al. 1986; Woodger et al. 2005). It appears that at least certain types of the eukaryotic microalgal CCMs are not controlled by the internal Ci pool. This can be demonstrated by the C. reinhardtii pmp1 mutant (Spalding et al. 1983a; Wang and Spalding 2006). The functional CCM associated with very low CO2 cannot be induced by low CO2 in pmp1, even though a very low inter-
nal Ci is expected under such conditions because of the defective LCIB. However, a very low external Ci is enough to induce the CCM in the same mutant, indicating that the induction of the CCM is not correlated with the internal Ci, at least not in the very low CO2 acclimation state. It is not clear whether different signals, or different levels of the same signal, triggers the different CO2-level-dependant acclimation states. In several Chlorophyta species, it has been proposed that external CO2 is the primary signal being directly perceived (Matsuda and Colman 1995; Bozzo and Colman 2000; Bozzo et al. 2000; Giordano et al. 2005). The conclusion of CO2 being the signal in these Chlorophyta is based on the absolute correlation between the induction of the CCM by low CO2 and the external CO2 concentration over different pH levels, regardless of total Ci or HCO3 concentration in the medium. In these cases, the low CO2 signal appears to be perceived at plasma membrane by a mechanism that has not yet been identified.

Although a change in Ci availability is the primary signal initiating changes in gene expression, some intermediate metabolites in carbon metabolism and some environmental signals also appear to be involved in the regulation of the CCM. Light or O2 or CO2/O2 ratio have been reported to affect the induction of the CCM and the expression of carbonic anhydrase (Spalding and Moroney 1982; Ramazanov and Semenenko 1984; Dionisio-Sese et al. 1990; Villarejo et al. 1996; Spalding 1998). It has been suggested that the regulation by light or O2 is associated with photosynthesis and photorespiration (Ramazanov and Cardenas 1992; Spalding 1998; Giordano et al. 2005). However, contradictory evidence has also been shown to question the light or O2 involvements in the regulation of the CCM. Induction of Cah1 has been reported to be light-independent (Rawat and Moroney 1995). Recent re-examination of O2 effects on acclimation failed to support any role of O2 or of photorespiration in regulating gene expression, at least that of CAH1 and GDH1, or the CCM function, as determined by photosynthetic characteristics (Vance and Spalding 2005).

Considering the complexity of interaction among different metabolic pathways and signaling systems in eukaryotes, it is not surprising that the regulation of the CCM is often entangled with other signal pathways. It has been demonstrated that blue light signaling, cell cycle and circadian rhythm are involved in the regulation of gene expression associated with the CCM (Dionisio et al. 1989a, 1989b; Rawat and Moroney 1995; Spalding 1998). Dionisio et al. (1989a) has proposed two light-requiring-steps, a photosynthetic-dependent step and a photosynthetic-independent (blue light) step, controlling Cah1 induction during the limiting CO2 acclimation. The induction of the CAH1 gene product, pCA1, also was inhibited by potassium iodide, a flavin quencher, indicating that a flavoprotein may be the blue light sensor (Dionisio et al. 1989b). Induction of the CCM and the regulation of CO2 responsive genes also are complicated by the cell cycle, which itself is regulated by circadian rhythm (Marcus et al. 1986; Rawat and Moroney 1995; Eriksson et al. 1999; Spalding 1998). The expression of CAH1 and CAH4/CAH5 has been reported to exhibit a circadian rhythm in synchronously grown cells (Rawat and Moroney 1995; Eriksson et al. 1998). This circadian pattern of gene expression may reflect the complex gene regulation by both Ci and the cell cycle in C. reinhardtii (Spalding 1998).

Gene expression in the CCM

Many de novo-induced genes in the CCM appear to be involved in the fundamental functions in limiting CO2 acclimation, including Ci transport systems, CA and photorespiration. Moreover, various C. reinhardtii genes involved in a wide spectrum of metabolic reactions and acclimation responses have been identified as quantitatively up- or down-regulated by changes in Ci supply.

The most abundant proteins induced by limiting CO2 include three CAs (CAH1, CAH4 and CAH5; Fujisawa et al. 1990; Fukuzawa et al. 1990; Eriksson et al. 1996) and several Ci transporter candidates (CCP1, CCP2, LCIB, LCIC, LCID, HLA3/MRP1 and LCIA/NAR1.2; Chen et al. 1997; Im and Grossman 2001; Miura et al. 2004; Wang and Spalding 2006). Some apparent constitutively expressed genes also exhibit CO2 responses, such as the thylakoid luminal CA (CAH3) which shows 2-fold increases in transcript abundance induced by low CO2 (Karlsson et al. 1998). Several genes involved in photosynthesis also have been identified as being either induced or up-regulated by limiting CO2, including AAT1 (alanine:C302-KG aminotransferase), PGPI (phosphoglycolate phosphatase), GDH1 (glycolate dehydrogenase), SHMT1 (serine hydroxymethyltransferase) and several other photorespiration-related genes (Chen et al. 1996; Mamedov et al. 2001; Im and Grossman 2001; Im et al. 2003; Miura et al. 2004; Nakamura et al. 2005).

Not only is the induction of gene expression associated with limiting CO2 the expression of many genes also is induced or up-regulated by high CO2. CAH2 and RHP1 are two genes which are induced by high CO2, and their functions appear to be associated with CO2 uptake in high CO2 conditions (Fujisawa et al. 1990; Fukuzawa et al. 1990; Rawat et al. 1990; Eriksson et al. 1999). Other high CO2 induced genes include H43, coding a periplasmic protein of unknown function (Shiraiwa and Kobayashi 1999; Hanawa et al. 2007) and many additional photosynthesis-related and nutrient-related genes (Miura et al. 2004).

Translational regulation and post-translational regulation have also been demonstrated to control the expression of limiting CO2 acclimation-related and CCM-related genes. Transient translational down-regulation of the large and small subunits of Rubisco is induced by limiting CO2 (Winder et al. 1992), and a posttranslational modification of CIA5 has been postulated to control the CCM (Fukuzawa et al. 2001; Xiang et al. 2001). Recently, the phosphorylation of some thylakoid proteins was reported in association with limiting CO2 acclimation (Turkina et al. 2006). Interestingly, in this latter research, the authors reported multiple phosphorylations of LC15, the gene product of a previously identified, low CO2 inducible gene, LC15 (Im and Grossman 2003; Miura et al. 2004). LC15 was found to be peripherally associated to the stroma side of chloroplast thylakoids. It is not clear whether this protein plays any specific role in the CCM or in other aspects of limiting CO2 acclimation.

The recent applications of genome-wide analyses have revealed a large number of CO2-regulated-genes. The functions of these gene products fall into several broad categories which include many metabolic pathways and stress responses (Im and Grossman 2001; Im et al. 2003; Miura et al. 2004). This is not surprising since, in essence, utilization of Ci affects many fundamental aspects of metabolism, growth and development of photosynthetic organisms. It has been noticed that many CO2-responsive-genes also are regulated by light (Im and Grossman 2001; Im et al. 2003), although the genes associated with the CCM can be distinct from non-CO2-responsive-genes by their sensitivity to CO2 and are controlled by the Cia5-associated signal transduction pathway. Several stress-related genes have been identified to be responsive to Ci change, but their relationship to limiting CO2 acclimation has not been proven (Im et al. 2003; Miura et al. 2004).

Signal transduction pathways associated with the CCM

The mechanism underlying the gene regulation associated with the CCM is largely unknown. The major impediment to understanding the signal transduction pathway(s) is inadequate information about the components in the pathway(s). So far only a very few elements associated with the CCM signaling have been identified.
The CIA5 mutant has played an unparalleled role so far in furthering our understanding the signal transduction in the microalgal CCM. This mutant lacks all known limiting CO2 inducible characteristics, including Ci transport activity, gene regulation, and biochemical and structural changes associated with the limiting CO2 acclimation (Moroney et al. 1989; Marek and Spalding 1991; Spalding et al. 1991). Since CIA5 appears to be a master regulator in the CCM, identification of CIA5 must be the first important advance to date in studying the signal transduction associated with limiting Ci acclimation and the CCM.

Two research groups simultaneously identified the CIA5 (or CCMI) gene, which encodes a putative transduction factor (Fukuzawa et al. 2001; Xiang et al. 2001). The deduced gene product of CIA5 is a 699-aa hydrophilic polypeptide with a putative zinc-finger motif in its N-terminal region and a glycine repeat region characteristic of transcriptional activators. Near the C-terminus of CIA5 several putative phosphorylation sites are found, and it was speculated that the C-terminus of CIA5 might be posttranslationally modified in response to a limiting CO2 signal. CIA5 is constitutively expressed irrespective of Ci conditions, and it is still not clear how CIA5 regulates gene expression or how this protein interacts with other members in the pathway(s). It seems likely that a limiting CO2 signal causes modification of the CIA5 protein, and that the modified CIA5 then either directly regulates CO2 responsive genes or regulates downstream signal transduction components.

CO2 responsive promoters have been identified in CAH1 and CAH/CAH5 by using the arylsulfatase (ARS1) gene as a reporter (Villard et al. 1997; Kuchu et al. 1999, 2003). Two regulatory regions were found to be present in the CAH1 promoter: a silent region responsible for repressing transcription in high CO2 and an enhancer region for activating transcription in low CO2 (Kuchu et al. 1999). Present in the enhancer region of CAH1 are two cis-acting elements, EE-1 and EE2, which were found to interact with some nuclear proteins (Kuchu et al. 2003). Since many CO2 responsive genes appear to be coordinately regulated, they must share some regulatory elements or be controlled by the same pathway. In deed, the core sequence motif, EE/C in CAH/EE-1 and EE-2 elements is also present in the CAH4 promoter. Recently, Yoshioka et al. (2004) identified and characterized LCR1, a Myb transcription factor which binds to the CAH1 promoter. LCR1 is involved in the regulation of several low-CO2-inducible genes, including CAH1, and its expression is induced by low CO2 under the control of CIA5. LCR1 appears to be associated with the CIA5 signalling cascades, but it is not clear whether its expression is regulated directly by CIA5, or through other signal mediator(s).

Several lines of evidence have indicated that many low CO2 inducible genes are differentially regulated. Even though expression of CAH1, CCP1/CP2 and CAH/CAH5 appear to be correlated, they do not always follow the same expression pattern (Villard et al. 1996, 1997). While high CO2 inducible by CAH/CAH5 and CCP1/CP2 appear to be regulated in the light. Inhibition of the glycolytic pathway appeared to repress the expression of CAH1 and CAH/CAH5, but had no effect on the expression of CCP1/CP2. Even being highly homologous, the CCP1 and CCP2 genes exhibited different timing in their expression upon limiting CO2 induction (Chen et al. 1997). It seems likely that, even sharing some regulatory elements, these CO2 responsive genes are regulated by divergent pathways. The differential regulation may reflect an association of these genes with different CO2 acclimation states, or with functions in distinct aspects of limiting CO2 acclimation.

**PROSPECTS**

As lack of detailed molecular composition is becoming a bottleneck for our understanding of the CCM in eukaryotic microalgae and its regulation, it is expected that more future work will continue to focus on identification of the functional components in the CCM. This task can be achieved by identification and characterization of non-acclimating mutants, global analysis of gene expression profiles, genome mining, and identification of proteins interacting with known CCM components. The recent availability of the genome sequences from C. reinhardtii and other microalgae, as well as the molecular tools increasingly available for C. reinhardtii genetics, provide an immense step forward.

Once candidate genes are available, their functions can be characterized by a reverse genetic approach, because RNA interference has been proven successful in silencing C. reinhardtii genes. Advances in the understanding of the CCM and its regulatory pathways in C. reinhardtii will give us better insight into the integrated network associated with carbon acquisition and utilization in photosynthetic organisms.

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