Carbon concentration mechanisms in photosynthetic microorganisms

Durba Ghoshal and Arup Goyal

Department of Biology, University of Minnesota-Duluth, Duluth, MN 55812, USA

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Unicellular green algae and cyanobacteria have mechanisms to actively concentrate dissolved inorganic carbon into the cells, only if they are grown with air levels of CO$_2$. The carbon concentration mechanisms are commonly known as “CCM” or “DIC-pumps”. The DIC-pumps are environmental adaptations that function to actively transport and accumulate inorganic carbon (HCO$_3^-$ and CO$_2$; C) within the cell and then use this C pool to actively increase the concentration of CO$_2$ at the site of ribulose bisphosphate carboxylase-oxygenase (Rubisco). The current working model for dissolved inorganic carbon concentration mechanism in unicellular green algae includes several isoforms of carbonic anhydrase (CA), and ATPase driven active transporters at the plasmalemma and at the inner chloroplast envelopes. In the past fifteen years, significant progress has been made in isolating and characterizing the various isoforms of carbonic anhydrase at the biochemical and molecular level. However, we have an inadequate understanding of active transporters that are located on the plasmalemma and at the chloroplast envelopes. In this mini-review we focus on certain aspects of the induction, function and significance of the dissolved inorganic carbon concentration mechanisms in aquatic photosynthetic microorganisms.

Introduction

Atmospheric CO$_2$ level is controlled, in part, by the amount of photosynthesis and photosynthesis by plants and algae. Two limiting reactions are catalyzed by a chloroplastic enzyme, ribulose bisphosphate carboxylase/oxygenase (Rubisco). Rubisco, a bifunctional enzyme at the branch point of carbon flow, catalyzes both the carboxylase reaction of the C-3 reductive photosynthetic carbon cycle and the oxygenase reaction of the C-2 oxidative photosynthetic carbon cycle. These are competing reactions based upon the availability of the two gaseous substrates, CO$_2$ and O$_2$. The carboxylase reaction initiates photosynthesis, whereas the oxygenase activity initiates glycolate synthesis and photosynthesis.

Both reactions of Rubisco and subsequent C-2 and C-3 carbon cycles coexist in the overall system of photosynthetic carbon metabolism (Fig. 1). The net fixation of CO$_2$, followed by the C-3 cycle, produces carbohydrates, regenerates ribulose bisphosphate (RuBP) and is accompanied by O$_2$ evolution. The fixation of O$_2$ initiates the C-2 cycle, which also regenerates RuBP at the expense of one CO$_2$ per two glycylate molecules (Fig. 2). The complete system, including both C-2 and C-3 cycles of carbon metabolism, is a complex self regulating process that coexists and limits atmospheric CO$_2$ removal and O$_2$.

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*Author for correspondence
Tel: 218-726 7566; Fax: 218-726 8142
E-mail: agoyal@d.umn.edu

Abbreviations: CA, carbonic anhydrase; C, inorganic carbon (HCO$_3^-$ and CO$_2$); CCM, carbon concentration mechanisms; DIC, dissolved inorganic carbon; Rubisco, ribulose bisphosphate carboxylase-oxygenase

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Fig. 1—A schematic bicycle model of photosynthetic carbon metabolism from the dual activities of Rubisco. [The C-3 reductive photosynthetic carbon cycle is on the left side of the bicycle (solid line), and the C-2 oxidative photosynthetic carbon cycle initiates on the right side of the bicycle and continues around the left circle (dashed line)].
accumulation. Photosynthetic carbon metabolism has been reviewed in Regulation of Atmospheric CO₂ and O₂ by Photosynthetic Carbon Metabolism edited by Tolbert and Preiss; Carbon Dioxide Fixation and Reduction in Biological and Model Systems edited by Branden and Schneider[3].

The rate of photosynthesis and the effective ratio of Rubisco carboxylase to oxygenase activity in plants and algae are increased in nature by mechanisms for concentrating CO₂ at the site of Rubisco. These mechanisms are commonly known as carbon concentration mechanisms (CCM). There are two known processes for increasing CO₂ in the microenvironment of Rubisco. In macro algae, bundle sheath cells of C-4 plants (like corn) and CAM plants (orchids and cactus), an oxygen-insensitive PEP-carboxylase is the primary carboxylating enzyme, the products of which are transported and decarboxylated at the site of Rubisco by C-4 organic acid cycles. In cyanobacteria, green algae and aquatic plants, the dissolved inorganic carbon (DIC) is concentrated at the site of Rubisco by active transport of inorganic carbon (C) and dehydration of HCO₃⁻ to CO₂. This process is commonly known as DIC-pump. Therefore CCM in C-4 and CAM plants is different from the DIC-pump that is present in aquatic photosynthetic microorganisms. The C-4 photosynthesis and the CAM-photosynthesis are relatively well characterized; however we have an inadequate understanding of components involved in the uptake, transport and concentration of inorganic carbon into C-3 plant cells, cyanobacteria, and unicellular green algae. This mini-review summarizes the progress that has been made in our understanding of the components of the carbon concentration mechanism in aquatic photosynthetic organisms and its possible application for the improvement of photosynthetic CO₂ fixation and plant productivity.

Carbon concentration mechanism (CCM or DIC-Pump)

The CO₂ concentrating mechanism (CCM or DIC-pump) in cyanobacteria, green algae, and aquatic plants is an environmental adaptation that has a special function to maximize the efficiency with which Rubisco fixes CO₂ into organic molecules. The ambient level of free CO₂ in water is insufficient for rapid growth of algae and aquatic plants. In addition, the passive diffusion of CO₂ into the cell is an extremely slow process. Therefore, the dissolved inorganic carbon (DIC) pump is necessary in most, if not all, aquatic photosynthetic organisms including cyanobacteria and unicellular green algae. The DIC-pump increases the rate of photosynthesis and suppresses the oxygenase activity of Rubisco, thus
reducing the energetically wasteful process of photorespiration. In the last fifteen years, three conferences on carbon concentration mechanisms and its published proceedings provided a forum for the discussion on possible mechanisms of inorganic carbon uptake and its role in the physiology and ecology of aquatic photosynthetic organisms.

**CCM model in cyanobacteria**

Cyanobacteria have an active uptake mechanism for dissolved inorganic carbon that results in accumulation of inorganic carbon species \((\text{C}_1)\) in the cell. The CCM is absolutely essential for cyanobacteria to perform efficient photosynthesis. Different species and strains have been used to study CCM in cyanobacteria: *Synechococcus* PCC 7942 \(^{7,9}\), *Synechococcus* UTEX 625 \(^{10,11}\), *Synechocystis* PCC6803 \(^{12,13}\), *Anacystis nidulans* R2 \(^{14}\). The current working model of cyanobacterial CCM consists of at least two basic components: a \(\text{C}_1\) transport system and a Rubisco-rich polyhedral microcompartment carboxysome (Fig. 3A and B) \(^{15}\). Three types of inorganic carbon transporters have been reported: (a) a primary \(\text{CO}_2\) transporter that delivers bicarbonate inside the cell using a CA-like moiety \(^{8,16}\). The energization of this transport event may require a NDH-I complex or some other components of the respiratory chain or the assistance of \(\text{CoT}A\); (b) a \(\text{Na}^+\) - independent, ATPase-dependent high affinity \(\text{HCO}_3^-\) transporter that is probably coded by a \(\text{cppABCD}\) cluster (Fig. 3B) \(^{17}\); (c) a constitutive \(\text{Na}^+\) dependent \(\text{HCO}_3^-\) transporter which functions as a direct \(\text{HCO}_3^-/\text{Na}^+\) symport or as an indirect \(\text{Na}^+/\text{H}^+\) antiport which is probably involved in \(\text{pH}\) regulation \(^{15}\). Inorganic carbon is accumulated in the cell as \(\text{HCO}_3^-\) that diffuses into the carboxysomes. In the carboxysomes, a specific isoform of carbonic anhydrase aids in the generation of an elevated concentration of \(\text{CO}_2\). Leakage of \(\text{CO}_2\) from the carboxysome is possible; however, an unidentified property of the carboxysome reduces the \(\text{CO}_2\) leakage from this microcompartment.

**Components of CCM in cyanobacteria**

The two major components of cyanobacterial CCM are CA, and multiple forms of \(\text{C}_1\) transporters which actively transport \(\text{CO}_2\) and \(\text{HCO}_3^-\). These transporters have specificity towards distinct substrates (\(\text{CO}_2\) or \(\text{HCO}_3^-\)). The \(\text{C}_1\) transport results in \(\text{HCO}_3^-\) accumulation in the cell and in Rubisco-containing carboxysomes. In addition, an unknown component that minimizes the leakage of \(\text{CO}_2\) from carboxysomes has also been suggested \(^{15}\). The \(\text{HCO}_3^-\) transport involves a \(\text{Na}^+\) dependent pump (\(\text{Na}^+/\text{HCO}_3^-\) symport), but its induction characteristics are not known. It is believed that the \(\text{Na}^+\) dependent uptake system is responsible for the majority of \(\text{HCO}_3^-\) transport in both the high and the low \(\text{DIC}\) grown cells. Recently a high affinity \(\text{Na}^+\) independent \(\text{HCO}_3^-\) transporter has been shown to be present under limiting \(\text{C}_1\) levels. This newly reported transporter seems to be responsible for a minor proportion of \(\text{HCO}_3^-\) transport \(^{18}\).

It is now very well established that carboxysomes are an integral part of CCM in cyanobacteria \(^{15,19-22}\). The strong evidence for the involvement of carboxysomes in CCM was developed using different approaches: (i) replacement of Rubisco (L888 from *Synechocystis*) with the dimeric form of Rubisco from *Rhodospirillum rubrum* led to the loss of carboxysomes \(^{22}\); (ii) mutational approach that resulted in a change in the shape or presence of carboxysomes produced high \(\text{CO}_2\) requiring phenotypes \(^{23}\); (iii) immunogold labeling revealed that a major proportion of Rubisco resides in the carboxysomes \(^{24}\); (iv) a low affinity carbonic anhydrase was also identified to be associated with the Rubisco in the carboxysome \(^{25}\).

**Carboxysomal CA**

Rubisco can assimilate \(\text{C}_1\) only in the form of \(\text{CO}_2\), therefore the internal pool of \(\text{HCO}_3^-\) in the carboxysomes has to be dehydrated to \(\text{CO}_2\) by a carboxysomal isoform of CA. The \(\text{HCO}_3^-\) is envisaged to be diffused into carboxysomes through its proteinaceous shell. The activity of this isoform is sufficient to provide enough free \(\text{CO}_2\) to saturate the carboxylating site of Rubisco. It is important to recognize that although carboxysomal CA generates a high concentration of \(\text{CO}_2\), its leakage to the cytosol is minimal. The gene and the protein for this isoform are similar to the enzyme from *E. coli* and the chloroplastic CA from higher plants \(^{26}\). The carboxysomal CA contains an extension of 60-70 amino acids at the carboxyl end, giving a monomer size of 31 kD. This extension sequence may be required for proper targeting into carboxysomes or may be involved in possible protein-protein interaction with Rubisco within the microenvironment.

It seems that the CCM in cyanobacteria involves only two major components; however, the number of genes that may be involved in the CCM, directly or indirectly, have been reported to be in excess of 50.
Fig. 3 — Basic components of the CCM in *Synechococcus* species (A and B). [Three types of inorganic carbon transporters are shown: (a) a primary CO₂ transporter that delivers bicarbonate inside the cell owing to a CA-like event inside the transporter; energization may require an NDH-1 complex or some other components of the respiratory chain or the assistance of CotA; (b) a Na⁺-independent, high-affinity HCO₃⁻ transporter (ABC transporter-traffic ATPase) that is powered by ATP, coded by a cmpABCD cluster; (c) a constitutive Na⁺ dependent HCO₃⁻ transporter; Na⁺ involvement may be direct (HCO₃⁻-Na⁺ symport) or indirect (Na⁺-H⁺ antiport and pH regulation). CO₂ is accumulated in the cell as HCO₃⁻ in chemical disequilibria, followed by diffusion into the Rubisco-containing carboxysomes. Here a low activity of CA aids in the generation of an elevated CO₂ concentration and some unresolved property of the carboxysome reduces the CO₂ leakage from the microcompartment.

The genetic analysis of different mutants has revealed that more than 10 genes are directly related to CCM, most of them responsible for the assembly and functioning of carboxysomes. Nineteen other genes have the potential to code for distinct type-1 NADH dehydrogenase complexes. Recently, a novel NDH-1 complex on the cytoplasmic membrane has been reported to be involved in the energization of high affinity CO₂ transport activity.

The expression of many genes has been found to be altered when cells are grown under low-CO₂ conditions. While certain genes are specifically transcribed, the rate of transcription of some of the constitutively expressed genes increases. For example, cmpA gene is expressed under low-CO₂ conditions which has led to the identification of positive and negative regulatory elements. The sequence information for various CO₂ responsive genes and their promoter region is now available, but the data needs to be analyzed to understand its significance. The regulation also occurs at the post-translational level. For example a change in phosphorylation of PII protein, the gene product of glnB, has been observed in a number of cyanobacteria under CO₂ stress. It was suggested that the PII protein participates in coordinating C/N metabolism. Similar phosphorylation activity was also observed under nitrogen and saline stress conditions; therefore, this property might be attributed to the general stress signal. It is, however, not clear if the post-translational regulation would account for the increase in apparent photosynthetic affinity observed in Synechococcus PCC7942 and PCC7002 within minutes of change to low C₃ conditions. Investigators working with cyanobacteria are currently dissecting regulation of gene expression using mutational and molecular approaches.

CCM model in unicellular green algae

Unicellular green algae concentrate dissolved inorganic carbon inside the cell and chloroplasts by mechanism(s) that are conceptually similar to cyanobacteria. However the structure and specific function of various components are different. One of the reasons for this difference is the presence of organelles like chloroplasts. Various investigators have used different species to study CCM in green algae. These species include but are not limited to, Chlamydomonas, Chlorophyta, Chlorococcum, Dunaliella, and Scenedesmus.

Fig. 4 — A proposed working model for the DIC-concentrating mechanism in unicellular green algae like Chlamydomonas, Chlorophyta, Dunaliella and Scenedesmus

The current working model of DIC uptake in green algae includes two pathways that may or may not be present in the same algae (Fig. 4). In the first pathway, the "CO₂ pump", external carbonic anhydrase (CA) enhances the replenishment of CO₂ from external HCO₃⁻ as CO₂ enters the cell. At intracellular pH, CO₂ is predominantly reconverted to HCO₃⁻ and actively transported by an ATPase-dependent bicarbonate transporter into the chloroplast. The ATPase-dependent bicarbonate transporter is located at the inner chloroplast envelope. In the chloroplast, HCO₃⁻ is dehydrated by an isofrom of CA to CO₂, the substrate for carboxylase activity of Rubisco. In the second pathway, the "HCO₃⁻ pump", an ATPase-dependent transporter is proposed for HCO₃⁻ uptake at the plasmalemma as well as at the chloroplast envelope. By using inhibitors like vanadate and salicylhydroxamic acid (SHAM), several components of active transport of C₃ have been suggested. For example, the ATPase-dependent bicarbonate transport activity is inhibited by Vanadate, a known inhibitor of P-type ATPase, whereas the CO₂ transport activity is inhibited by SHAM, an inhibitor of alternative oxidation/respiration. The presence of an active CO₂ transport has been established but the nature of the transporter is not described. (Ghoshal and Goyal, unpublished results). Isoforms of CA in different compartments have been characterized, but the membrane associated active DIC transporters are not.

Components of CCM in unicellular green algae

In the natural environment, dissolved C₃ exists predominantly as HCO₃⁻. Several components of the
CCM in unicellular green algae have been identified in species of *Chlorella*[^56-58], *Chlamydomonas*[^59], *Scenedesmus*[^60], and *Dunaliella*[^61]. These components are compartmentalized in three subcellular locations: (i) periplasmic carbonic anhydrase that dehydrates the \( \text{HCO}_3^- \) to \( \text{CO}_2 \) and plasmalemma bound \( \text{C}_i \) transporter(s); (ii) isoforms of cytosolic carbonic anhydrase; (iii) an ATP-dependent transporter at the inner chloroplastic envelope that transports \( \text{HCO}_3^- \) from the cytosol into the stroma; and (iv) a chloroplastic isoform of the CA that converts \( \text{HCO}_3^- \) to \( \text{CO}_2 \), to be fixed by Rubisco[^62]. Recent studies are focused on the isolation and characterization of membrane transporters[^63-64], fast-induced CCM[^65-67], structure-function-relationships of various isoforms of CA[^68], developing a transformation system for *Dunaliella*, (Ghoshal and Goyal, work in progress), and regulation of gene expression using mutational and other molecular approaches[^69].

### Carbonic anhydrase(s)

Carbonic anhydrase (CA; carbonate-lyase, carbonate dehydratase; EC 4.2.1.1) is a zinc-containing metalloenzyme that catalyzes the reversible conversion of the two forms of inorganic carbon, carbon dioxide and bicarbonate ion that predominate in aqueous media at physiological pH values. This enzyme is universally distributed in all photosynthetic organisms.

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\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3 \rightarrow \text{H}^+ + \text{HCO}_3^- 
\]

The CA enables algae to efficiently utilize low levels of carbon dioxide that could otherwise limit their growth. There is considerable evidence that CA is an important component of the CCM in unicellular green alga, *Chlamydomonas reinhardtii*. The catalytic activity of CA appears concomitantly with the induction of the CCM when cells are transferred for growth on a \( \text{CO}_2 \)-limited medium from a \( \text{CO}_2 \)-rich medium. The role of CA in unicellular green algae is to convert \( \text{HCO}_3^- \) to \( \text{CO}_2 \). The converted \( \text{CO}_2 \) is then transported into the cytoplasm and/or utilized by Rubisco in the chloroplasts. Many isoforms of CA have been identified in various species. These isoforms are localized at the plasmalemma[^62], in the periplasmic space[^60-63], cytoplasm[^64-65], mitochondria[^66], and chloroplasts[^67]. The use of *Chlamydomonas* and *Chlorella* species has contributed a great deal to our current understanding of the role of CA in CCM; however, other species like *Dunaliella* has also been used to identify unique isoforms of CA. Atleast five genes encoding isoforms of CA have been identified in *Chlamydomonas reinhardtii*. The gene products of *cahl* and *cahl2*, are directed to the periplasmic space[^68], the *cahl3* encodes for chloroplastic CA[^69] and the other two genes encode mitochondrial CA[^70].The role and function of extracellular/periplasmic CA in microalgae have been reviewed in detail[^71-73].

### Periplasmic CA (pCA)

The first step of CCM is the transfer/transport of \( \text{C}_i \) species from the aquatic environment to inside the cell. When high-\( \text{CO}_2 \) grown cells of microalgae (e.g., *Chlorella*, *Chlamydomonas* and *Dunaliella*) are adapted to grow under ambient air levels of \( \text{CO}_2 \) in light, a dramatic increase in the activity of the DIC-pump and extracellular/periplasmic CA has been observed[^74]. The extracellular CA in *Chlamydomonas reinhardtii* accelerates the conversion of \( \text{HCO}_3^- \) to \( \text{CO}_2 \); thus, the CA enzyme is of primary physiological importance at high pH where \( \text{HCO}_3^- \) is the predominant form of \( \text{C}_i \) in solution at equilibrium. Based on its location, the external CA was named as periplasmic CA (pCA) and its activity can be assayed in intact cells. The pCA is responsible for dehydrating \( \text{HCO}_3^- \) to \( \text{CO}_2 \), which is actively transported into the cell. The level and time required for the induction of pCA varies from species to species and with environmental conditions such as light, pH and temperature. For example, pCA accounts for about 99% of total CA in *Chlamydomonas* whereas it only accounts for about 50% of the total activity in the case of cell wall-less algae *Dunaliella*, a close relative of *Chlamydomonas* (Goyal, unpublished results). The pCA from *Chlamydomonas* is a soluble enzyme that is located predominantly in the periplasmic space, whereas in case of *Dunaliella* sp., the enzyme is associated with the membrane via ionic interactions. The pCA from *Chlamydomonas* is inhibited by a high concentration of salts in comparison with *Dunaliella* in which expression is induced by salt and the enzyme requires high saltions for its optimum activity[^75].

The characterization of pCA has been made possible by its abundance and easy purification protocols[^76]. The mature pCA is synthesized as a 41.6 kD precursor that is post-translationally cleaved and glycosylated to 35-38 kD and a 4 kD small subunit. The holoenzyme is believed to be a heterotetramer composed of two large and two small subunits, each joined to a large subunit by a disulfide bond[^77]. The mature subunit of the enzyme is a glycoprotein and
Chemical deglycosylation results in a polypeptide with an apparent molecular mass of 32 kD as determined by SDS-PAGE. Classical sulfonamide inhibitors of CA like ethoxzolamide and acetazolamide inhibit the activity of pCA (ref. 32). Two genes (cahl and cah2) encoding pCA from *Chlamydomonas reinhardtii* have been characterized. These two genes are encoded by the nuclear genome and are 92% identical at the level of amino acids. However, gene products from cahl and cah2 show only 20% identity to the animal CA. The *cahl* is expressed only under low-CO₂ conditions whereas cah2 is expressed under high-CO₂ conditions. In addition to pCA from *Chlamydomonas*, novel isoforms of salt inducible pCA have been described from *Dunaliella* species. The pCA from *Dunaliella* species was immunoreactive with the antibody against the pCA from *Chlamydomonas reinhardtii*; however, its apparent molecular weight on SDS-PAGE appeared to be 30kD. Recently, a low-CO₂ inducible salt resistant pCA from *Dunaliella salina* has been cloned and characterized. The unique feature of *D. salina* pCA is that it is a single polypeptide that is equivalent to the four subunits of pCA from *Chlamydomonas*, which is a heterotetramer of two large and two small subunits. The *D. salina* pCA does not require disulfide bonds for its stabilization as is the case of *Chlamydomonas*. Therefore, unlike *Chlamydomonas*, the pCA from *D. salina* is a monomer that is formed essentially by internal gene duplication. This clearly indicates that *Dunaliella* has evolved enzymes that are adapted to function optimally under extreme external environmental conditions such as high salt stress.

**Cytosolic CA (cytCA)**

A significant level of intracellular CA activity has been described in various microalgae. These intracellular isoforms include cytosolic CA (cytCA), chloroplastic CA (chlCA) and mitochondrial CA (mitCA). The cytCA appears not to be directly involved in CCM, rather it maintains the HCO₃⁻ pools to reduce leakage of free CO₂ from the cytosol. Based on immunoreactive properties, it is now clear that the cytCA is quite different from both chlCA and pCA (ref. 70). The cytCA from *Chlamydomonas reinhardtii* is a 110 kD polypeptide that is less reactive to sulfonamide inhibitors as compared to pCA. The mutational analysis revealed that cytCA is under different regulatory control than pCA. Multiple isoforms of intracellular CA have been found in *Chlorella*, *Dunaliella* sp., and in *Coccomyxa*.

Interestingly, *Coccomyxa*, an organism lacking CCM, contains about 100 fold cytCA (that has characteristics similar to *E. coli* and higher plant enzyme) compared to *Chlamydomonas*, *Chlorella*, and *Dunaliella*, however its role is not clear.

**Chloroplastic CA (chlCA)**

The stromal pH in light is around 8. At this pH, the spontaneous conversion of HCO₃⁻ to CO₂ would be too slow to account for the observed rate of CO₂ fixation. Therefore, it is absolutely necessary that chloroplasts contain an isoform of CA to convert the large pool of HCO₃⁻ to CO₂. The ratio of pCA to cytCA and chlCA activity varies greatly with different species. In the past 5 years, several investigators have provided direct evidence to demonstrate the presence of CA in the stroma of the chloroplasts (chlCA). These studies include: (a) CA-directed photoaffinity labeling, (b) isolation and characterization of chlCA protein and its corresponding gene, and (c) direct activity assay with isolated chloroplasts. (Ghoshal and Goyal unpublished results). There are considerable differences in the data of various investigators on the reported induction of chlCA by direct assay, CA mutants, and mass spectrophotometric methods, when cells were adapted to the ambient air-level of CO₂. The chlCA activity in the chloroplasts isolated from the air-adapted cells of *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta* has been reported to increase by 3 to 8 fold compared to high-CO₂ grown cells. In our laboratory, using direct CA assay, no significant increase in activity in *Chlamydomonas reinhardtii* and *Dunaliella* could be detected (Ghoshal and Goyal, unpublished results). It appears that chlCA activity is similar in both the low-CO₂ and high-CO₂ adapted cells.

The chlCA is a 29.5 kD protein that is predominantly found in the insoluble fraction of the whole cell homogenate. The enzyme could be solubilized in the presence of 200 mM NaCl and KCl. The chlCA belongs to the α-type CA family that has been identified as *Cah3* that is highly sensitive to inhibition by sulfonamides. Recently a gene coding for chlCA has been isolated and characterized by genomic complementation of the ca-l mutant of *Chlamydomonas reinhardtii*. The gene encoding chlCA is constitutively expressed even under the high-CO₂ condition. The level of expression increases upon the transfer of the cells to a low-CO₂ condition.
environment. The cDNA encoding cah3 has also been isolated and characterized. This cDNA contains a putative signal peptide, which is similar to sequences that are believed to target the protein into the lumen of the thylakoids.

The physiological function of the proposed luminal CA is not clear. The luminal pH in light is around 5. It is proposed that the possible mechanism of luminal CA (Cah3) works through the conversion of HCO3- to CO2 at the inner side of the thylakoid membrane, using the light generated acidification of the illuminated thylakoids, where HCO3- may be acting as an uncoupler. The HCO3- is proposed to be transported along with light dependent movement of a proton (H+) across the thylakoid membrane into the lumen, where a membrane-bound CA converts it to CO2. The CO2 then leaks into the stroma to be fixed by Rubisco. This proposal suffers from the fact that no direct experimental evidence is presented to support the hypothesis, and that in light, stromal pH is around 8 where any leaked CO2 will instantaneously convert back to HCO3-. An additional complication arises from the fact that pyrenoids contain a significant amount of Rubisco along with a minor activity of an uncharacterized CA. The pyrenoids are considered to be one of the main sites for CO2 fixation but their role in CCM is not very clear and is circumstantial. Thylakoids traverses the pyrenoids; therefore, it is important to evaluate whether the luminal CA is found only in the thylakoids or it is a contaminating activity from CA that is localized in the pyrenoids. The identification of mutants with disrupted pyrenoids may help in establishing a clear role of luminal CA in CCM.

Mitochondrial CA (mtCA)

Until a few years ago, most investigators believed that chloroplasts were the major site for CCM in green algae; however, recently a novel isoform of CA associated with mitochondria was identified. In the past, several observations in relation to ultra-structural changes and the 36 kD peptid e is associated with the chloroplast envelope. All others were in the soluble fraction, with the exception of 22 kD mtCA, that could only be detected by 35S labeling. Therefore it is unlikely that these peptides represent an active transporter. Because the active DIC transporter is one of the major functional activities in the plasmalemma.
and in the chloroplast envelopes, it was expected that they should be present in relatively large quantity in their respective membranes. After purification of plasmalemma and chloroplast envelopes, we have identified three peptides of apparent molecular weight of 45, 47, and 90 kD in *Dunaliella tertiolecta*\(^{45,87}\). We could not detect any other peptide by Coomassie or by silver staining, however we will continue our search for additional protein(s) that may be directly or indirectly related to the DIC-pump.

### Identification of membrane proteins associated with the DIC-pump

As discussed above, based on physiological and inhibitor studies, it was proposed that an ATPase-dependent active bicarbonate transporter is present at the chloroplast envelope and at the plasmalemma\(^{45,87}\). There are a few details regarding ATPases and active transporters or ion channels that may be associated with the membranes of either the chloroplast envelope or the plasmalemma. Characterization of purified plasmalemma and chloroplast envelopes may lead to the identification of membrane proteins that are induced by low CO\(_2\). One approach to identify and characterize components of an active inorganic carbon transporter is to identify specific membrane bound proteins and their corresponding genes that are induced with the DIC-pump during adaptation to low CO\(_2\). A limitation of this approach has been to obtain large amounts of chloroplasts needed for envelope separation and the lack of procedures for isolating the plasmalemma from unicellular green algae. We have addressed this problem by developing methods for isolating pure plasmalemma, chloroplasts and their envelopes. After purification of membranes, we discovered that during the induction of the DIC-pump in *Dunaliella* three peptides were specifically induced: a 90 kD peptide at the plasmalemma, and 45 and 47 kD peptides at the inner chloroplast envelope\(^{45,87}\).

The three newly discovered peptides have been abbreviated as Low-CO\(_2\) Induced peptides (LCI): LCI-45, LCI-47, and LCI-90. These three peptides are induced at the same time and by the same treatments that induces the DIC-pump activity. Darkness or stress conditions do not induce these peptides. Therefore, it is unlikely that newly identified membrane peptides are stress proteins (Ghoshal and Goyal, Unpublished results). The three Low-CO\(_2\) Induced peptides are not expressed in *Dunaliella* cells grown: [1] in high-CO\(_2\) environment\(^{45,87}\), [2] in light if photosynthetic electron transport is inhibited by DCMU, and [3] in the presence of a low levels of UV-B that inhibits induction of the DIC-pump proteins (Ghoshal and Goyal, Unpublished results). These membrane peptides are not recognized by polyclonal antibodies raised against LIP-36\(^{79}\). All of the above results have established the similarity between known features of physiological regulation of the induction of the DIC-pump and these peptides.

### Why *Dunaliella* as a model system?

Any protein that would be involved in the active transport of DIC is most likely to be present in the plasma membrane and/or in the chloroplast envelopes. The major barrier to a biochemical and molecular understanding of the postulated ATPase-dependent bicarbonate transporters has been obtaining enough pure chloroplast envelopes and plasmalemma to identify and purify substantial quantities of inducible proteins that are associated with the DIC-pump. Species of *Chlorella*, *Chlamydomonas* and *Scenedesmus* have been used for studying the DIC-pump at the whole cell level. From these three organisms, it has not been possible to isolate large quantities of chloroplasts (at least a gram equivalent chlorophyll), a pre-requisite for the isolation of chloroplast envelopes. Another problem is that *Chlorella* and *Chlamydomonas* grow very slowly at alkaline pH and contain very little plasmalemma associated bicarbonate transport activity. *Scenedesmus* has been the organism of choice for studying bicarbonate transport by whole cells, but it is not possible to isolate chloroplasts and plasmalemma from it.

On the other hand, *Dunaliella* is a naturally occurring cell wall-less alga, a pre-requisite for the isolation of organelles in high yield from unicellular algae, and it has a DIC-pump system that is similar to *Chlorella*, *Chlamydomonas* and *Scenedesmus*. *Dunaliella* contains appreciable amount of bicarbonate transport activity both at the plasmalemma and at the chloroplast envelope and can be adapted to grow on high salt and alkaline pH to contain a HCO\(_3^-\) pump. Because of these advantages, *Dunaliella* was adapted as a model system for isolating chloroplasts in high yield (about 40%), inner and outer chloroplast envelopes, and pure plasmalemma.

### What triggers the induction?

The nature of the signal that induces the response
to the CO₂ level is still not clear. The total C₃ level, the ratio of [CO₂]/[O₂], and the concentration of dissolved CO₂ are assumed to play a major role. It seems that cells respond to specific C₃ species that they are capable of utilizing under that particular physical condition. It is possible that the signal is coming through the level of an intermediary metabolite in the carbon metabolic pathway. Supply of acetate or glucose to cyanobacteria and green algae suppresses the DIC-pump. The effect of [CO₂]/[O₂] ratio on assimilation suggests that photorespiration may play a role. Inhibition of the glycolate pathway by salicylhydroxamic acid (SHAM) also inhibits DIC accumulation in *Chlamydomonas, Scenedesmus* and *Dunaliella*. Aminoxyacetate, an inhibitor of glycolate metabolism, suppressed the synthesis of two low CO₂ induced peptides of apparent molecular weight of 21 and 37 kD in *Chlamydomonas* but the LIP-36 was not affected. It is evident that there is more than one mechanism that triggers the signal for the carbon concentration mechanism to act. Moreover, there are other factors like blue light, which may also be acting at the secondary level to trigger the reaction.

**Perspectives for the future**

In the last two decades we have made significant progress on our understanding of CCM. Various components of CCM have been identified and many have been characterized at the level of protein and gene. However, we believe that there are many unanswered questions about the CCM that need urgent attention. A list of some of the challenges includes but are not limited to: [1] Identification and characterization of several transport components of cyanobacteria and unicellular green algae; [2] Several isoforms of CA have been identified and described; however, what they really do, at least the function of mitochondrial, luminal and chloroplast envelope associated CA needs to be described; [3] Selection of new mutants defective in various components of CCM. Generation of specific mutants is a challenge, better screening strategies for insertional mutants has to be developed; [4] The role of carboxysomes and pyrenoids and their interaction with other biochemical components of CCM needs urgent attention; [5] Nature of signal that triggers induction or suppression of DIC-pumps; [6] Requirement of quantity and quality of light, including effects of UV on the induction of DIC-pump and its repair mechanism by blue light; [7] The energization of DIC pumps, and how increasing atmospheric CO₂ levels may affect DIC-pumps; [8] Explore other aquatic photosynthetic organisms for the presence and functioning of the DIC-pumps; [9] Use of Microarray technology and other modern techniques to dissect function of different genes involved.

**Significance of DIC-pump(s) and it’s potential application**

By some estimates aquatic photosynthesis contributes more than 70% of the global CO₂ utilization including fixation and deposition of carbon as salts for long term storage. The algal DIC concentrating mechanisms and the C-4 cycles in plants or macro algae accomplish the same goal, namely to increase photosynthetic CO₂-fixation by altering the ratio of CO₂ and O₂ in the chloroplast at the site of Rubisco. This results in more photosynthetic biomass production and maintaining of the atmospheric CO₂ equilibrium.

Understanding the mechanism and function of the algal DIC-concentrating process is important for at least two reasons. First, it adds to our understanding of a basic cellular process, i.e. structure, function relationship and regulation of the mechanism by which algae concentrate inorganic carbon to suppress inevitable oxygenase activity of the Rubisco. Second, it may have practical significance to reduce photorespiration, an energetically wasteful process in plants, using genetic engineering techniques.

Nature has developed and used carbon concentrating mechanisms such as the C4-cycle in plants and DIC-pump in algae to increase the carboxylase activity relative to the oxygenase activity of Rubisco and to increase the efficiency of CO₂ fixation by photosynthesis. In the past much research has been directed, unsuccessfully, at the elimination of photorespiration in C-3 plants by Rubisco modification. Aquatic photosynthetic machinery is very similar to C-3 plants. It is important to recognize that the pH gradient across the chloroplast envelope alone can not support high rates of CO₂ fixation in C-3 plants. Therefore, it is likely that an active inorganic carbon transport system may be present in C-3 plants. The C-4 pathway is highly complex to be considered for transfer to C-3 plants by genetic engineering. The DIC-pump in algae consists of only two main components, CA (already present in plants) and an active DIC-transporter at the chloroplast envelope and/or at the plasmalemma. Therefore, it may be
feasible to transfer the system to higher plants and an engineered bicarbonate transporter system might be able to use part of the energy wasted on photorespiration to concentrate inorganic carbon into the chloroplast.

We are now convinced that the photorespiratory pathway cannot be eliminated. However, reduction of photorespiration may be possible by genetically transferring either algal active transporters or by modifying genes (if a putative transporter gene is found in the C-3 plants) encoding for an active inorganic carbon transporter at the chloroplast envelopes of C-3 plants of economic importance. The DIC-concentrating mechanism is an essential part of the photosynthetic carbon metabolism, which in itself has a significant importance in the use of algae for biomass, chemical production, and phytoremediation using solar energy. Similarly, engineering a DIC-carbon concentrating system into higher plant cells grown in cultures would greatly improve the potential for using green plant cells in photoautotrophic cultures for the production of pharmaceutical products.

In conclusion, although much progress has been made in the field of carbon concentration mechanism in the past fifteen years, we are still a long way from a complete understanding of various components of the DIC-pumps in cyanobacteria and unicellular green algae. The development of new molecular tools, identification and characterization of novel genes, mutants defective in CCM, and innovative new strategies will contribute to further progress in dissecting the mechanism of active dissolved inorganic carbon concentration in microalgae.

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