Chapter 7

Structural and Biochemical Features of Carbon Acquisition in Algae

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I. Introduction

Cyanobacteria, eukaryotic algae and vascular plants ultimately depend on the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) for assimilation of CO_2 into organic matter, initially, via a 6C carboxyketone intermediate, in the form of 3-phosphoglycerate (Eq. 7.1).

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$$CO_2 + H_2O + D - \text{ribulose} - 1,5 - \text{bisphosphate} \rightarrow 2 \times 3 - \text{phosphoglycerate} \quad (7.1)$$

The 3-phosphoglycerate so formed undergoes a series of reactions, leading to the net production of one molecule of triose phosphate for every 3 CO_2 assimilated, in the Calvin-Benson-Bassham Cycle (Photosynthetic Carbon Reduction Cycle; PCRC) and the regeneration of one ribulose-1,5-bisphosphate. Each turn of the PCRC uses 2 NADPH plus 3 ATP per CO_2 assimilated, involving at least 9 mol photons absorbed per mol CO_2 assimilated (Raven et al. 2014) although, as will be described below, the precise energetic costs involved in net incorporation of CO_2 into carbohydrate usually exceed this.

There are a number of alternative pathways leading to net CO_2 assimilation in autotrophs (Raven et al. 2011, 2012) but, as discussed later in this chapter, cyanobacteria and algae appear to use only the PCRC (Beardall and Raven 2016). Indeed, 99% of primary productivity on the planet is carried out by processes that involve Rubisco and the PCRC (Raven 2009; Beardall and Raven 2016).

In this chapter, we examine the biochemical and structural constraints on carbon assimilation in cyanobacteria and eukaryotic algae and discuss the need for CO_2 concentrating mechanisms (CCMs) if these photoautotrophs are to achieve significant rates of net photosynthesis. In order to determine whether cells possess a capacity for CCMs, a number of criteria need to be satisfied, but there are currently misconceptions in the literature about what comprises proof of CCM activity. Accordingly, we discuss the mechanisms of biochemical and biophysical CCMs and the strengths and weaknesses of the various criteria that have been employed as yardsticks for CCM determination.

II. Carbon Assimilation

A. *The Characteristics of Most Rubiscos Necessitate Operation of a CCM*

In addition to the, relatively low catalytic rate, carboxylase activity shown in Eq. 7.1, Rubisco also possesses an oxygenase activity, leading, via a 5C peroxyketone intermediate, to the formation of one molecule of phosphoglycerate and one molecule of phosphoglycolate (Eq. 7.2).

$$O_2 + D - \text{ribulose} - 1,5 - \text{bisphosphate} \rightarrow 3 - \text{phosphoglycerate} + 2 - \text{phosphoglycolate} \quad (7.2)$$

The phosphoglycolate so formed can be acted upon by 2-phosphoglycolate phosphatase, leading to formation of glycolate. The latter can be excreted from cyanobacteria or plastids, leading to a net loss of 2 organic C. Alternatively 2 molecules of glycolate can enter the sequence of reactions known as the photorespiratory carbon oxidation cycle (PCOC, photorespiration) leading to formation of one molecule of 3-phosphoglycerate and the net loss of 1 C (Beardall and Raven 2016; Raven et al. 2011; Beardall et al. 2003). Eisenhut et al. (2008) showed that a cyanobacterium has two alternative pathways of glycolate metabolism in addition to the PCOC; deletion of all three of the pathways is lethal, so in this organism glycolate excretion alone is not an adequate sink for glycolate. The role of photorespiration as well as alternative pathways of electron flow and oxygen consumption in algae and cyanobacteria are discussed in a separate chapter in this volume (Raven et al. 2019).

CO_2 and O_2 compete for the same active site on Rubisco, and the achieved rates of the two carboxylase and oxygenase activities depends on the O_2:CO_2 ratio at the active site of the enzyme, according to the Selectivity Factor S_{rel} shown in Eq. 7.3.

$$S_{rel} = \left(K_{0.5}O_2 \times k_{cat}CO_2\right) / \left(K_{0.5}CO_2 \times k_{cat}O_2\right) \quad (7.3)$$

where $k_{cat}(CO_2)$ is the CO_2-saturated specific rate of carboxylase activity of Rubisco (mol CO_2 mol^{-1} active sites s^{-1}), $K_{1/2}$ (CO_2) is the concentration of CO_2 at which the CO_2 fixation rate by Rubisco is half of $k_{cat}(CO_2)$, $k_{cat}(O_2)$ is the O_2-saturated specific rate of oxygenase activity of Rubisco (mol O_2 mol^{-1} active sites s^{-1}) and $K_{1/2}$ (O_2) is the concentration of O_2 at which the O_2 fixation rate by Rubisco is half of $k_{cat}(O_2)$.

Autotrophs contain a broad range of different forms of Rubisco. These have been discussed extensively in the literature (Badger et al. 1998; Raven and Beardall 2003; Beardall and Raven 2016; Tcherkez et al. 2006; Whitney et al. 2011; Raven et al. 2011, 2012; Studer et al. 2014; Griffiths et al. 2017; Bathellier et al. 2018), so their characteristics are only summarised briefly here. There are 3 known main forms of Rubisco, referred to as Forms I, II and III. A fourth group Form IV) consists of Rubisco-like proteins which lack carboxylase activity and which may instead function in S metabolism (Hanson and Tabita 2001). Cyanobacteria and many algae have Form I Rubisco with 8 large and 8 small subunits (L_8S_8). Form I Rubiscos can be further categorised into Form IA and Form IB. *Prochlorococcus* and many marine *Synechococcus* species produce Form IA Rubisco, obtained by lateral gene transfer from an autotrophic proteobacterium (Raven et al. 2012). The vast majority of marine and freshwater cyanobacteria however possess the ancestral Form IB Rubisco, which was transferred in primary endosymbiosis to glaucocystophyte and thence chlorophyte algae and then by secondary endosymbiosis to chlorarachniophyte and euglenophyte algae (Raven et al. 2012). All red algae on the other hand possess Form ID RUBISCO, obtained by lateral gene transfer from an autotrophic proteobacterium (displacing the Form IB Rubisco transferred in primary endosymbiosis). Through secondary endosymbiosis this form of Rubisco appears, at least in those species that have been examined, to have been passed on to cryptophytes, haptophytes (e.g. cocolithophores) and heterokonts (= stramenopiles or ochristans, e.g. diatoms). Some Form IB Rubiscos have the very high selectivity for CO_2 over O_2 (Raven et al. 2012), though Tcherkez et al. (2006) suggest that Form 1D has the highest affinity for carbon dioxide and the highest carbon dioxide:oxygen selectivity.

On the other hand, the ancestral dinoflagellates and *Chromera veria*, both alveolates, possess Form II Rubisco, believed to have been obtained by lateral gene transfer from an autotrophic proteobacterium; Form II Rubisco comprises only 2 large subunits (L_2). Form III Rubiscos also lack small subunits (but can have more complex structures based on the L_2 basal structure ($(L_2)_4$, $(L_2)_5$), but are found only in Archaea so will not be considered further here.

These various forms of Rubisco have differing kinetic properties and in particular differing selectivity factors. Form IBc Rubiscos, found in most marine and freshwater β-cyanobacteria, have high $K_{1/2}(CO_2)$ values of 105–290 (with most values falling in the range of 200–260 μM). Selectivity factors vary from 38 to 56 mol mol^{-1} and CO_2-saturated specific reaction rates (k_{cat}) values range from 2.6 to 11.4 mol CO_2 mol^{-1} active sites s^{-1}. In contrast, the Form IAc Rubisco of the marine α-cyanobacterium *Prochlorococcus* MIT9313 has the highest known $K_{1/2}(CO_2)$ of a Form I Rubisco of 750 μM, combined with a moderate CO_2-saturated specific reaction rate of 4.7 mol CO_2 mol^{-1} active sites s^{-1} (Scott et al. 2007). On the other hand, green algae have Form 1B Rubiscos with higher affinity with $K_{0.5}(CO_2)$ values of 29–38 μM and higher S_{rel} of 61–83 mol mol^{-1} being reported, but with lower k_{cat} values (Raven and Beardall 2003).

The Form 1D Rubiscos found in heterokont and haptophyte algae (see Chap. 2) show values that are very variable. This can even be the case for the same organism; values of $K_{0.5}(CO_2)$ for partially purified Rubisco of the coccolithophore *Emiliana huxleyi* have been

reported as 72 μM (Boller et al. 2011), or 200 μM (Shiraiwa et al. 2004). Diatom Form ID Rubiscos are also variable (Boller et al. 2015; Young et al. 2016) with $K_{0.5}(CO_2)$ values varying from 23 to 68 μM, CO_2 selectivity from 57 to 116 mol mol^{-1} and specific reaction rates of 2.1 to 3.7 $site^{-1}$ s^{-1}. Form 1D Rubiscos in the Synurophyceae have reported $K_{0.5}(CO_2)$ values in vitro of 18.2 μM (*Mallomonas papulosa*), 28.4 μM (*Synura petersenii*) and 41.8 μM (*Synura uvella*) (Bhatti and Colman 2008; Raven and Giordano 2017). These $K_{0.5}(CO_2)$ should be taken in context of typical air-equilibrium CO_2 concentrations of 10–25 μM, depending on temperature, salinity etc.

Dinoflagellates (see Chap. 2) are unusual in being the only eukaryotic organisms possessing Form II Rubiscos. These enzymes are unstable *in vitro* and are thus poorly characterized, but appear to have very low selectivity factors (~37). Some idea of their kinetic properties can be obtained from work on Form II Rubiscos from photosynthetic proteobacteria, from which it is believed the dinoflagellate Form II Rubiscos originated by lateral gene transfer (Badger et al. 1998; Whitney et al. 1995). These have very low S_{rel} values (Whitney and Andrews 1998; Leggat et al. 1999; Raven and Beardall 2003) and dinoflagellates would thus struggle to perform net CO_2 assimilation at air-equilibrium CO_2 levels (Tortell 2000).

The general trend across all autotrophs is that a low $K_{½}(CO_2)$, and a high S_{rel} are correlated with a low $k_{cat}(CO_2)$, and *vice versa* (Tcherkez et al. 2006; Raven et al. 2012). Given the relatively low affinities and selectivity factors of most of the algae and cyanobacteria as discussed above, achievement of significant rates of net photosynthesis necessitates the operation of a CO_2 concentrating mechanism (CCM) to elevate CO_2 concentrations, and increase CO_2:O_2 ratios at the active site of Rubisco. The various forms which these mechanisms can take are discussed in more detail below.

It is worth noting in the context of enhancement of photosynthetic rates that, in contrast to terrestrial C_3 vascular plants, Rubisco in algae and cyanobacteria represents a relatively small proportion (~2–6%) of the total protein pool and hence investment in N (Losh et al. 2013; Raven 2013a; Flynn and Raven 2017), a resource which is frequently in limiting supply in aquatic, especially marine, systems. This may be related to CCM activity as terrestrial plants possessing a C4 biochemical CCM have lower Rubisco N:Total leaf N than C3 plants lacking CCMs (see Raven 2013a and references within). Furthermore N-limitation has been shown to cause upregulation of CCMs in some algae (Beardall et al. 1982, 1991; Young and Beardall 2005; Hu and Zhou 2010), improving N-use efficiency, though this is apparently not so in *Chlamydomonas reinhardtii* (Giordano et al. 2003; Chap. 4) or the diatom *Phaeodactylum tricornutum* (Li et al. 2012; Chap. 16).

B. The PCRC and Other Pathways for C Assimilation

Six pathways for the assimilation of CO_2 into organic matter have been identified in autotrophs, including those (C_4 photosynthesis among them) relying on activity of Rubisco in the PCRC. However, of those pathways found in nature, the only example that is not inhibited by oxygen, while exhibiting carboxylase activity with an ecologically relevant (at least in terms of the photic zone in marine and freshwater systems) affinity for CO_2 and has a lower energy (absorbed photon) cost than the PCRC, is the 3-hydroxypropionate bi-cycle (Bar-Even et al. 2010, 2011, 2012; Raven et al. 2012; Raven and Beardall 2016). However, fixation of CO_2 via the PCRC acting alone is the dominant pathway for carbon assimilation in cyanobacteria and algae. There is some evidence for C_4 photosynthesis in the marine ulvophycean alga *Udotea flabellum* (Reiskind et al. 1988; Reiskind and Bowes

1991) and, controversially, there are also reports of C_3-C_4 single cell intermediate photosynthesis in one species of a marine diatom (*Thalassiosira weisflogii*) (Reinfelder et al. 2000; Morel et al. 2002; Roberts et al. 2007a, b; Reinfelder 2011; Haimovich-Dayan et al. 2013), and although there is better evidence for C_4 photosynthesis in freshwater macrophytes and in seagrasses (Holaday and Bowes 1980; Salvucci and Bowes 1983; Magnin et al. 1997; Reiskind et al. 1997; Bowes et al. 1978; Bowes et al. 2002; Maberly and Madsen 2002; Bowes 2011; Koch et al. 2013; Larkum et al. 2017; Raven and Giordano 2017), the five alternative pathways for C assimilation described in Raven et al. (2012) are not represented in algal photosynthetic C assimilation.

Although the basic reactions of the PCRC are similar across the range of cyanobacteria and algae examined, recent work is indicating that there is considerable phylogenetic variation in the way the cycle is regulated. In terrestrial vascular plants, enzymes of the PCRC such as phosphoribulokinase (PRK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-1,6-bisphosphatase, and sedoheptulose-1,7-bisphosphatase are inactivated in the dark and activated in the light, while the key enzyme of the oxidative pentose phosphate (OPP) pathway, glucose-6-phosphate dehydrogenase shows the reverse. Of these, the two key enzymes in PCRC regulation are PRK and GAPDH. In green algae regulation of these 2 enzymes is under redox control, but PRK is not redox-regulated in the marine centric diatom *Odontella sinensis* (Michels et al. 2005) or the freshwater pennate diatom *Asterionella formosa* (Boggetto et al. 2007). In contrast *A. formosa* does show redox-activation of NADPH-dependent GAPDH (Boggetto et al. 2007), but this is lacking in *O. sinensis* GAPDH (Michels et al. 2005). Maberly et al. (2010) investigated the redox regulation of PRK and GAPDH in more detail, including the role of the protein CP12, and have been able to show considerable variation across different algal groups with the cryptophytes and haptophytes studied showing differing regulatory properties to another clade containing chromalveolates and a third with a mix of vascular plants, a diatom, a xanthophycean and an eustigmatophycean. Though the phylogenetic trends in regulation of the PCRC across photoautotrophs as discussed by Maberly et al. (2010) and more recently by Jensen et al. (2017) are not clear cut, the significance to the evolutionary history of algae is worthy of further investigation.

III. Occurrence of CCMs

It is apparent from the discussion above that the kinetics of Rubisco in most cyanobacteria and algae operating at, or below, air-equilibrium levels of CO_2 require operation of a CO_2 concentrating mechanism (CCM) to improve the supply of CO_2 to the active site, minimise photorespiration and improve net rates of carbon assimilation. Notable exceptions appear to be species that occur where CO_2 levels are high, such as in the Chrysophyceae and Synurophyceae (Maberly et al. 2009; Raven and Giordano 2017), freshwater red algae belonging to the Batrachospermales (Raven et al. 1982; Raven et al. 2005), as well as some marine algae where low light levels constrain photosynthesis so that CO_2 diffusion is sufficient to satisfy demand (Kübler and Raven 1994, 1995). In this regard it is interesting to note that the florideophycean red alga *Heminura frondosa*, thought to lack CCM activity on the basis of work on fresh material isolated from low light environments, expressed a CCM capacity when exposed to high light (Catriona Hurd, personal communication). Other exceptions are the coccoid symbiotic trebouxiophycean green alga *Coccomyxa*, using CO_2 from soil or host cell respiration (Raven and Colmer 2016), though this is apparently not the case for the Antarctic species *Coccomyxa subellipsoiea* (Blanc et al. 2012), and the aerophytic, terrestrial trebouxiophycean spe-

cies *Stichococcus minor* (Munoz and Merrett 1989). All other species examined, admittedly a small fraction of the conservative estimate of >70, 000 extant algal species (Guiry 2012), appear to have CCMs. Reports of a lack of CCM activity in the coccolithophore *Emiliania huxleyi* are now believed to be unfounded (Rost et al. 2007; Reinfelder 2011; Stojkovic et al. 2013). It could be expected that size might play a role in whether a CCM is expressed or not, as decreasing size would decrease diffusion resistance of CO_2 potentially diminishing the need for CCMs in smaller species (Raven 1986; Raven 1999). However, *Micromonas pusilla* (cell volume 2.1×10^{-18} m^3) has an active CCM (Iglesias-Rodriguez et al. 1998) and contains pyrenoids (Meyer and Griffiths 2013), and although *Ostreococcus* (cell volume ~ 0.48×10^{-18} m^3) lacks pyrenoids (Meyer and Griffiths 2013) and has an unclear CCM status (Schaum and Collins 2014); as discussed below an absence of pyrenoids does not equate with absence of CCM activity (see Chap. 9).

However, the expression of CCM activity varies greatly. Cyanobacteria with the low CO_2 affinity Form IA or Form IB Rubisco show highly expressed CCMs, while diatoms with Form ID Rubiscos with higher CO_2 affinity (Young et al. 2016) show lower CCM activity and green algae with relatively high affinity (see above) show relatively low capacity for CCM expression. Tortell (2000) and Griffiths et al. (2017) showed a broad inverse relationship between carbon concentration factor and Rubisco specificity factor, and a positive relationship between specificity factor and paleo CO_2 levels, though the data need to be interpreted carefully and expression of CCM activity is modulated by a range of environmental factors including CO_2 concentration, light level, temperature and nutrient availability (Beardall and Giordano 2002). The role of CCM activity in controlling competition between species is complex and involves interactions between Rubisco characteristics, dissolved inorganic carbon concentrations, CO_2 concentrating capacity and other environmental factors such as light (Ji et al. 2017; Beardall and Raven 2017). As stated above, members of the Chrysophyceae and Synurophyceae, lacking CCMs, became dominant when aqueous CO_2 concentrations were significantly above air equilibrium (Maberly 1996; Maberly et al. 2009). Van de Waal et al. (2011) showed that two strains of *Microcystis aeruginosa,* with differing affinities for DIC, were shown to sequentially dominate a culture based on the available CO_2 and Lines and Beardall (2018) attributed the success of the cyanobacterium *Cylindrospermopsis raciborskii* (= *Raphidiopsis raciborskii*: Aguilera et al. 2018) in a reservoir in Queensland Australia to its high CCM activity and affinity for CO_2 at low environmental concentrations. Shapiro (1990, 1997) and Low-Décarie et al. (2011, 2015) have shown from ecological observations and competition experiments that cyanobacteria have the capacity to outcompete other groups of photoautotrophs in freshwater phytoplankton communities. However, Ji et al. (2017) have shown that this is not always the case (see also Beardall and Raven 2017).

IV. Mechanisms of CCMs Versus Diffusive CO_2 Fluxes

A. Definition of CCMs and What Do We Need in Order to Demonstrate Operation of CCMs?

Unfortunately, despite many years of CCM-related research, there is still a good deal of misunderstanding about what comprises reliable and robust evidence for CCM activity. In general, irrespective of the mechanisms discussed below, CCM activity is characterised by several cellular physiological properties. (i) By definition, there needs to be a net positive gradient of $CO_{2\ in} > CO_{2\ out}$ Simple measurements showing intercellular [DIC] is higher than extracellular [DIC] are

insufficient as gradients in DIC could simply be a consequence of CO_2 equilibration between inside and outside the cell with higher pH internally than externally. Thus proof of CCM activity requires it to be demonstrated that dissolved CO_2 in the cells is higher than outside. Such measurements were originally done using radioisotopes measuring $[DIC]_{in}$, $[DIC]_{out}$ and internal (and external) pH (see e.g. Badger et al. 1980; Kaplan et al. 1980), but more recent approaches involve measurements using mass spectroscopy (see e.g. Sültemeyer et al. 1991) and are the preferred approach for laboratories with access to such instrumentation. (ii) Characteristically cells with active CCMs have $K_{0.5}CO_2$ values for DIC-dependent photosynthesis less than that for their Rubiscos and measurements of $K_{0.5}CO_2$ are useful and relatively simple approaches to measuring CCM capacity of cells. This approach requires that the CO_2-saturated *in vivo* Rubisco activity is not so high as to account for the ratio of *in vivo* to *in vitro* $K_{0.5}CO_2$. (iii) Diffusive supply of CO_2 followed by assimilation by Rubisco leads to a discrimination against heavier isotopes of C by about 30‰. Consequently, measuring carbon isotope discrimination $\delta^{13}C$ (or more accurately $\Delta^{13}C$ if measurements of source discrimination are made) in the organic component of algae can give an indication of possible CCM activity. Thus cells using CO_2 diffusion alone show $\Delta^{13}C$ values ~ −30‰ and discrimination values become less negative as CCM activity increases (Raven et al. 2005; Stepien 2015). However, this method depends on a known and constant discrimination between $^{13}CO_2$ and $^{12}CO_2$ in fixation by Rubisco, and it is known that there is significant variation in this discrimination in cyanobacteria and algae (Tcherkez et al. 2006; Scott et al. 2007; Boller et al. 2011; Boller et al. 2015). Furthermore, the ability of cells to express the full RubisCO fractionation factor also depends on the extent to which diffusive CO_2 supply exceeds cellular demand. As demand approaches the supply rates the effective fractionation factor is reduced (Laws et al. 1995).

Other parameters, more specific to particular mechanisms involved in CCMs, have often been mis-interpreted. These include using the presence of sequences for enzymes such as PEP carboxylase in genomes of algae as *prima facie* evidence for C_4 metabolism and the misconception that possession of external carbonic anhydrase can alone result in CO_2 accumulation. 'C_4 genes' are widespread in organisms lacking CCMs, or even photosynthesis (Aubry et al. 2011; Chi et al. 2014), so more than genomics is needed to show that C_4 photosynthesis occurs. The use of transcriptomics in combination with physiological measurements and pulse-chase labelling can, however, be informative (Roberts et al. 2007a).

Thus, in order of increasing importance, genomic information, transcriptomics and proteomics, enzyme activity measurements and short-term labelling experiments are required in order to distinguish between biochemical C_4 CCMs and biophysical CCMs based on active transport of inorganic carbon species (Fig. 7.1).

B. CCMs Based on Active Transport of Inorganic C Species

There are five variants of inorganic carbon transport in cyanobacteria, each with different physiological characteristics (Table 7.1). CO_2 can diffuse across the plasma membrane of cyanobacteria, probably with a least some CO_2 uptake effected by aquaporins (Kaldenhoff et al. 2014; Raven and Beardall 2016). However, on either the cytosolic face of the plasma membrane or on the thylakoid membrane there is an energized conversion of CO_2 to HCO_3^- via a NAD(P)H dehydrogenase, which effectively acts like a Ci-pump, even though direct active transport of CO_2 has not occurred. There are two NAD(P)H dehydrogenase systems: an inducible, high-CO_2 affinity, system (NDH-I_3) at the thyla-

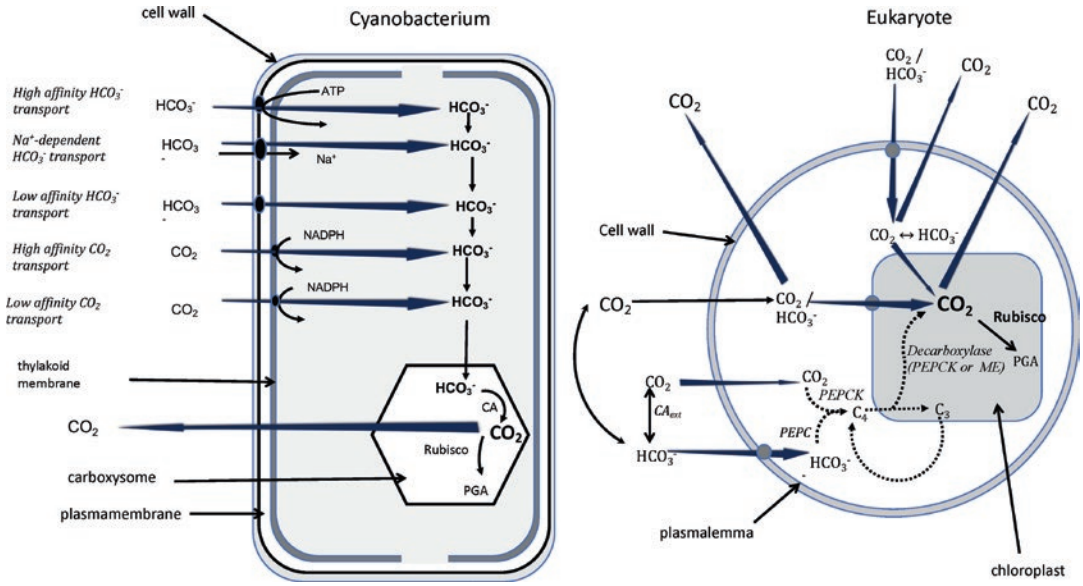

Fig. 7.1. Basic mechanisms of CCMs in a cyanobacterium (left) and a eukaryotic alga (right). No attempt has been made to represent the role of the pyrenoid in those algae that possess them (see Chap. 9). (See the text for details. Redrawn after Giordano et al. 2005)

Table 7.1. Inorganic carbon transporters in cyanobacteria

Transporter	Substrate	Affinity	Flux	Comments
BCT1	HCO_3^-	High	Low	ABC-type transporter found exclusively in freshwater β-cyanobacteria; low-CO_2 inducible
SbtA	HCO_3^-	High	Low	Sodium-dependent transporter
BicA	HCO_3^-	Low	High	Sodium-dependent transporter
NDH-13	CO_2	High	Low	Energized conversion of CO_2 to HCO_3^-
NDH-14	CO_2	Low	High	Energized conversion of CO_2 to HCO_3^-

koid membrane and a constitutive, low affinity, one (NDH-I_4, located probably at the plasma membrane) (Price et al. 2008). Cyanobacteria can also actively take up HCO_3^- from the medium using a number of different HCO_3^- pumps. In freshwater β-cyanobacteria only, the genes for a low-CO_2 inducible, high affinity HCO_3^- transporter, BCT1, are encoded by the *cmpABCD* operon which belongs to the traffic ATPase family (Omata et al. 1999). These genes are absent from the genomes of all marine α- and β-cyanobacteria so far sequenced. In *Synechocystis* 6803 and various other β-cyanobacteria, a high affinity, inducible, Na^+-dependent, HCO_3^- transporter (SbtA) is present (Shibata et al. 2002). BicA is another Na^+-dependent HCO_3^- transporter, and this is aligned with the SulP family of transporters (Price et al. 2004). Unlike SbtA though, this is a low affinity system. BicA and SbtA may both be forms of Na^+/HCO_3^- symporters, although to date this there is no conclusive evidence for this. Whatever the form of inorganic carbon transported, the outcome is for HCO_3^- delivered, directly or indirectly, to the cytosol. The HCO_3^- then diffuses into the polyhedral protein-walled bodies termed carboxysomes, which contain all the cell quota of Rubisco and show the only carbonic

anhydrase (CA) activity in the cell. CO_2 generated within the carboxysomes by this CA leads to the build-up to a higher steady state concentration than in the bulk medium, thus strongly favouring the carboxylase over the oxygenase activity of Rubisco (Smith and Ferry 2000; Price et al. 2002).

The mechanisms of CCMs in eukaryotic algae are not as well defined as they are in cyanobacteria and are more complicated because of the additional membranes the DIC needs to traverse. Inorganic carbon needs to be transported across the plasmalemma, then across the membranes of the chloroplast envelope and CO_2 then needs to be provided at a higher than ambient concentration to the active site of Rubisco, which is within the pyrenoids in those cells that possess them and in the stroma in cells without pyrenoids. Although all pyrenoid-containing algae have CCMs (Badger et al. 1998; Raven 1997a, b; Raven and Beardall 2003), not all algae with CCMs have pyrenoids (Badger et al. 1998; Morita et al. 1998, 1999; Raven 1997b, c; Raven and Beardall 2003; Kevekordes et al. 2006; Raven and Giordano 2017). Active transport mechanisms for DIC could thus be based on the plasma membrane, or the inner plastid envelope membrane, or both.

More recent molecular evidence has begun to characterise the various bicarbonate transporters in algal cells and a large number of candidate proteins have been identified. In diatom genomes there are genes for a number of solute carrier (SLC)-type transporters, also found in mammalian systems (Bonar and Casey 2008). Several of these have been implicated in bicarbonate transport in diatoms with, in *Phaeodactylum tricornutum*, the plasmalemma associated PtSLC4-2 being low-CO_2 inducible and having a high requirement for Na^+ (Nakajima et al. 2013). Similar transporters (PtSLC4-1, and PtSLC4-4) are also plasmalemma-located in *P. tricornutum* and likewise appear to be involved with HCO_3^- influx from low-CO_2 environments. Nakajima et al. (2013) also showed the existence of orthologous SLC4 genes in another diatom species, *Thalassiosira pseudonana*. Recent evidence (Tsuji et al. 2017a) suggests that in these diatoms plasmalemma HCO_3^- transport is driven, directly or indirectly, by energy generated by linear electron flow from photosystem II to photosystem I, contrasting with previous work suggesting a role for ATP from cyclic photophosphorylation (Ogawa and Ogren 1985; Ogawa et al. 1985; Palmqvist et al. 1990; Spalding et al. 1984), with some eustigmatophycean algae appearing to be unusual in having a CCM driven by respiratory ATP (Huertas et al. 2002). Plasmalemma-based bicarbonate transporters have also been demonstrated in the green alga *Chlamydomonas reinhardtii* (Ohnishi et al. 2010; Yamano et al. 2015) though the genes for these transporters (HLA3 and LCI1) do not appear to share homology with the SLC systems in diatoms (Tsuji et al. 2017a, b).

Physiological investigations have shown that algae can also take up CO_2 and this, in the absence of hard evidence for an active CO_2 transporter, is assumed to take place by passive diffusion (Patel and Merrett 1986; Colman and Rotatore 1995; Mitchell and Beardall 1996; Johnston and Raven 1996; Korb et al. 1997; Burkhardt et al. 2001; Rost et al. 2003; Trimborn et al. 2008; Kaldenhoff et al. 2014; Raven and Beardall 2016), possibly assisted by aquaporin channels, though a high permeability of cell membranes to CO_2, can have consequences for leakage (Tchernov et al. 2003; Raven and Beardall 2016).

Though there are some species in which CCM activity appears to be based solely at the plasmalemma (Rotatore and Colman 1990, 1991), there is also evidence for a role of the plastid envelope in CCMs in a range of other species, based on a demonstrable capacity for active transport of DIC. Thus, photosynthetically active chloroplasts from high- and low-CO_2 grown cells of two species of the Chlorophyceae have been shown

to possess low- and high-affinity DIC uptake systems respectively, as do the corresponding intact cells (Amoroso et al. 1998). Active uptake of both CO_2 and HCO_3^-, and CO_2 accumulation, have been demonstrated in isolated chloroplasts of *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta* (Amoroso et al. 1998) and *Tetraedon minimum* and *Chlamydomonas noctigama* (van Hunnik et al. 2002). Molecular studies have identified genes for SLC4- type transporters associated with the chloroplast envelope membranes in diatoms (Nakajima et al. 2013; Tsuji et al. 2017b) and a number of putative transporters (LCIA, CCP1 and CCP2) have been suggested for the chloroplast envelope of *Chlamydomonas* (Wang et al. 2015; Yamano et al. 2015; Machingura et al. 2017), though the work of Mackinder et al. (2017) suggests CCP1 and CCP2 are less important. Matsuda et al. (2017) have also postulated a range of transporters for inorganic carbon transport across all 4 of the diatom chloroplast envelope membranes and the pyrenoid-penetrating thylakoids, though such transport systems remain uncharacterised and speculative at present.

CCMs also involve a range of CAs that maintain equilibrium between CO_2 and bicarbonate in the various cellular compartments (see De Mario et al. 2017 for a recent review). Importantly, in many algae an extracellular carbonic anhydrase (CA_{ext}) associated with the cell wall converts bicarbonate to CO_2, assisting the diffusion of the latter across the cell wall. Within the cell, internal CAs facilitate the interconversion of bicarbonate and CO_2 with active inorganic transport across the chloroplast envelope then occurring as described above. In green algae such as *Chlamydomonas*, the external CA is an α-CA, while in some diatoms this role is carried out by a β-CA. In other diatoms a ζ-CA is involved (Hopkinson et al. 2013) and δ-CA_{ext} has been reported in a dinoflagellate (Lapointe et al. 2008). The full range of carbonic anhydrases found in algae and cyanobacteria is discussed in DiMario et al. (2017). CA_{ext} activity is inducible by low CO_2 levels and in some cases is only found when rates of CO_2 consumption exceed the rate of uncatalysed supply from bicarbonate (Smith-Harding et al. 2018), which may explain in part at least the contradictory reports of external CA presence/absence in some species (John-McKay and Colman 1997).

As well as being involved in the supply of inorganic carbon across the plasmalemma, CAs may also be involved in CCMs based on acidification in the thylakoid. An α-CA (Cah3) is based on the inner side of the thylakoid membrane and is involved in the conversion of bicarbonate to CO_2 following transport of bicarbonate to the thylakoid lumen, the CO_2 thus produced could then leak out of the lumen to the site of Rubisco in the stroma or pyrenoid. However, direct evidence for such a mechanism is not yet available, though in *Chlamydomonas* there is CA compartmentalization evidence that is at least consistent with such a process (Pronina and Semenenko 1992; Pronina and Borodin 1993; Raven 1997c; Sinetova et al. 2012).

Acidification can also play a role in enhancing inorganic carbon supply in macroalgae and aquatic vascular plants where localised low pH and external carbonic anhydrase(s) at the cell surface shifts the bicarbonate:CO_2 ratio in favour of CO_2, increasing its concentration and enhancing its diffusion (or in some cases active transport) into the cell (Raven and Hurd 2012; Raven 2013a, b; Raven et al. 2014; Raven and Beardall 2016). Similar mechanisms are unlikely in microalgae due to the thinner diffusive boundary layer and enhanced CO_2 and proton leakage in small cells (Flynn et al. 2012; Raven and Beardall 2016).

C. C4 Photosynthesis as a CCM in Algae?

In some vascular plants photosynthetic carbon assimilation is based on an initial assimilation of bicarbonate, catalysed by the enzyme PEP carboxylase, which has a high affinity for its inorganic carbon substrate. The initial stable resulting products are the C_4 dicarboxylic acids malate or aspartate (depending on species) and these compounds are then transported from mesophyll cells to another type of cell, the bundle sheath cells, where their decarboxylation leads to enhanced supply of CO_2 at the active site of Rubisco, located therein (Sage 2004; Sage et al. 2011). This process thus acts as a biochemical CO_2 pump improving CO_2 supply. Studies in the late 1970's by Beardall and coworkers proposed the existence of single-cell C_4-like photosynthetic metabolism in diatoms (Beardall et al. 1976), though later work (Morris et al. 1978) ascribed their labelling patterns and other data to high rates of anaplerotic β-carboxylation through PEPCase. Such reactions are necessary to top up the intermediates of the TCA cycle as these are used to support biosynthetic processes such as protein synthesis (Aubry et al. 2011; Chi et al. 2014). Subsequently, Reinfelder et al. (2000) and Morel et al. (2002) revisited the topic and proposed that the marine diatom *Thalassiosira weisflogii* was also capable of C_4 photosynthesis. Subsequent work (Morel et al. 2002; Reinfelder et al. 2004; Roberts et al. 2007a, b) has provided better evidence for some form of C_4 (or more likely C_3-C_4 intermediate) mechanism in *Thalassiosira weisflogii*, though operation of a similar mechanism in other diatoms (*T. pseudonana* and *Phaeodactylum tricornutum*) has not been substantiated (Haimovich-Dayan et al. 2013; Clement et al. 2017; Ewe et al. 2018). Similarly, high levels of enzymes putatively involved in C_4 photosynthesis in the haptophyte *Emiliania huxleyi* were shown by pulse chase labeling experiments to instead be associated with anaplerotic β-carboxylation (Tsuji et al. 2009). Thus *T. weisflogii* remains the only microalga to date for which C_4 photosynthesis is a possibility, though it is more likely better aligned with C_3-C_4 intermediate metabolism (Roberts et al. 2007a). Nonetheless, C_4 metabolism in the macroalga *Udotea flabellum,* using PEP carboxykinase as the carboxylase, is well established (Reiskind et al. 1988; Reiskind and Bowes 1991).

V. Structural Aspects of CO_2 Acquisition

The role of carboxysomes in cyanobacterial inorganic carbon acquisition has been dealt with in detail recently by Kerfeld and Melnicki (2016) so will only be mentioned briefly here.

The polyhedral bodies known as carboxysomes are present in all cyanobacteria and contain most of the cyanobacterial cell's Rubisco and carbonic anhydrase, enclosed within a semi-permeable proteinaceous shell (Kinney et al. 2011; Espie and Kimber 2011). Details of carboxysomal structure and function can be found in Kerfeld and Melnicki (2016), but in brief HCO_3^- is transported into the carboxysome lumen and converted to CO_2 via carbonic anhydrase, thereby elevating CO_2 concentrations at the active site of Rubisco. The protein shell is thought to act in minimising CO_2 leakage, though as discussed by Raven and Beardall (2016) leakage is still likely to be significant given the large accumulation factor for CO_2 found in cyanobacteria. Indeed, Hopkinson et al. (2014) showed that approximately 50% of the inorganic carbon transported into cells of a species of *Prochloroccus* that lacks CO_2 recovery capacity was lost as CO_2 efflux.

In eukaryotic algae, the analogous structure to the carboxysome is the pyrenoid, a microcompartment within the chloroplast (see Chap. 9). Clearly, in algae that possess pyrenoids, the majority of the Rubisco is found there, with only a small portion of cel-

lular Rubisco in the stroma (McKay and Gibbs 1991). Green algal pyrenoids also contain Rubisco activase, supporting the notion that Rubisco in pyrenoids is catalytically active (McKay and Gibbs 1991). Non-green algal species do not express Rubisco activase, but utilise another protein, CbbX, which has activase-like properties (see Kroth 2015).

In contrast, in species without pyrenoids Rubisco is found throughout the stroma (McKay and Gibbs 1991 and references therein). Interestingly, if *Chlamydomonas reinhardtii* is grown at elevated CO_2 in order to down-regulate CCM expression, increased levels of Rubisco (and a three fold higher proportion of cellular Rubisco) appear in the stroma, though pyrenoid Rubisco levels remain high and similar to those in low-CO_2 grown cells (Borkhsenious et al. 1998). It is clear however, that possession of a pyrenoid is not a prerequisite of a CCM. While all algae with pyrenoids have CCMs, not all algal species with CCMs have pyrenoids (Giordano et al. 2005; Kevekordes et al. 2006), though pyrenoid loss from *Chlamydomonas* results in loss of CCM function (Meyer et al. 2012; Mitchell et al. 2017).

The fine structure, development and composition of pyrenoids in *Chlamydomonas reinhardtii* have been dealt with recently in the excellent reviews by Meyer et al. (2017), Meyer and Griffiths (2013), Mackinder (2018) and Mackinder et al. (2017) and so are not dealt with here in detail. The pyrenoid is surrounded by a starch sheath (although mutant studies suggest that this is not essential for the CCM: Villarejo et al. 1996) and, important for CCM activity, is traversed by membrane tubules, sometimes termed pyrenoid lamellae, or transpyrenoid thylakoids, that are contiguous with the photosynthetic thylakoid membranes (Engel et al. 2015). However, these tubules are distinct from stromal thylakoids in lacking O_2-evolving PSII centres and light harvesting antenna, and this is the case in red algae and diatoms as well as in the green algae (McKay and Gibbs 1991; Mustardy et al. 1990; Pyszniak and Gibbs 1992; Tsekos et al. 1996). The pyrenoid tubules do however, contain the α-CA (Cah3) described above, and this could be responsible for conversion of bicarbonate to CO_2 within the lumen of the pyrenoid tubules, which would then diffuse out of the lumen to the Rubisco in the bulk of the pyrenoid. A similar protein, Pt43233, is found in the diatom *Phaeodactylum tricornutum* (Kikutani et al. 2016), suggesting this could be a widespread mechanism in pyrenoid-containing algae.

Such a mechanism would also require transport of bicarbonate into the transpyrenoid thylakoid lumen; this could be downhill (passive) transport driven by the proton-motive force across the thylakoid (Raven 1997c), but no such transporter has been identified to date. It is important to note that many pyrenoids lack thylakoid tubules or lamellae (Dodge 1973; Badger et al. 1998).

In addition to the pyrenoid tubules as discussed above, for green and red algae with CCMs, the plasma membrane, or the inner plastid envelope membrane, or both, could be the location of the active transport mechanism(s) (Amoroso et al. 1998; Moroney and Chen 1998; Kaplan and Reinhold 1999; Villarejo et al. 2001; Young et al. 2001; Giordano et al. 2005). Other algae have one (dinophytes, euglenoids) or two (chlorarachniophytes, cryptophytes, haptophytes and heterokonts) additional chloroplast envelope membranes which are frequently, but incorrectly, termed the chloroplast endoplasmic reticulum (Cavalier-Smith 2000). The involvement of these additional envelope membranes in a CCM based on active transport processes as discussed above has not yet been examined. Hopkinson et al. (2011) and Matsuda et al. (2017) have suggested that, with 4 bounding membranes, diatoms might require a HCO_3^- transporter at each membrane, which would be a large energetic constraint

both in terms of running costs and capital investment. Gee and Niyogi (2017) showed that the carbonic anhydrase CAH1, expressed in the space between the outer and next innermost of the membranes round the plastids of *Nannochloropsis oceanica*, is essential for operation of the CCM in this alga with active HCO_3^- influx at the plasmalemma.

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