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Review

The evolution of inorganic carbon concentrating mechanisms in photosynthesis

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Inorganic carbon concentrating mechanisms (CCMs) catalyse the accumulation of CO_2 around rubisco in all cyanobacteria, most algae and aquatic plants and in C_4 and crassulacean acid metabolism (CAM) vascular plants. CCMs are polyphyletic (more than one evolutionary origin) and involve active transport of HCO_3^-, CO_2 and/or H^+, or an energized biochemical mechanism as in C_4 and CAM plants. While the CCM in almost all C_4 plants and many CAM plants is constitutive, many CCMs show acclimatory responses to variations in the supply of not only CO_2 but also photosynthetically active radiation, nitrogen, phosphorus and iron. The evolution of CCMs is generally considered in the context of decreased CO_2 availability, with only a secondary role for increasing O_2. However, the earliest CCMs may have evolved in oxygenic cyanobacteria before the atmosphere became oxygenated in stromatolites with diffusion barriers around the cells related to UV screening. This would decrease CO_2 availability to cells and increase the O_2 concentration within them, inhibiting rubisco and generating reactive oxygen species, including O_3.

Keywords: alga; cyanobacteria; crassulacean acid metabolism; C_4 photosynthesis; embryophytes; stromatolites

1. INTRODUCTION

Photosynthesis by O_2-producing organisms invariably involves the carboxylase rubisco (ribulose bisphosphate carboxylase–oxygenase) as the core enzyme involved in producing reduced carbon (Tabita *et al.* 2008). The different molecular forms of this enzyme, with different kinetics with respect to substrate affinities and maximum specific reaction rates, appear to function optimally within the constraints of the overall mechanism of the enzyme (Tcherkez *et al.* 2006). However, no known rubisco has a half-saturation value for CO_2 that saturates the carboxylase activity at the present atmospheric CO_2 concentration and at the temperature that the enzyme functions *in vivo*. Furthermore, all known rubiscos have an oxygenase activity competitive with the carboxylase activity that adds resource costs to net C assimilation in the present atmosphere with diffusive CO_2 entry, and they all have high molecular masses and relatively low CO_2-saturated specific reaction rates so that offsetting the relatively low CO_2 affinity by increasing the enzyme quota on a biomass basis is very costly in terms of energy and nitrogen inputs.

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One contribution of 15 to a Discussion Meeting Issue 'Photosynthetic and atmospheric evolution'.

These considerations can help to rationalize, in evolutionary terms, why about half of the total inorganic C fixation on Earth, almost all by oxygenic photosynthesis, involves inorganic carbon concentrating mechanisms (CCMs). These CCMs increase the CO_2 concentration, and the CO_2/O_2 ratio, at the site of rubisco activity. This increases the rate of net C assimilation per unit rubisco and decreases the resource costs associated with rubisco oxygenase activity, although involving additional resource costs for the synthesis, maintenance and operation of the mechanisms that bring about the active accumulation of CO_2. An additional resource cost comes from the need for additional pumping of inorganic C to make up for any inorganic carbon leakage that occurs from the internal pool (Raven 1991*a*; Sültemeyer & Rinast 1996). Since any barriers to leakage of CO_2 (the most likely species of inorganic carbon to leak) are also likely to act as barriers to the efflux of photosynthetically produced O_2, there is also likely to be the additional problem of higher steady-state O_2 concentrations around the photosynthetic apparatus with implications for rubisco catalysis and the generation of reactive oxygen species (Raven & Larkum 2007).

Here we outline the mechanism and environmental and phylogenetic distribution of CCMs in extant organisms, the times at which they evolved, and their probable future in relation to atmospheric composition. We first consider the CCMs in cyanobacteria

and algae, which account for well over half of the 50 Pg or so of carbon assimilated each year in the oceans, and then with the C_4 and crassulacean acid metabolism (CAM) found in terrestrial vascular plants that accounts for 18–30% of the 60 Pg or so of C assimilated each year on land (Berry 1994; Ehleringer *et al.* 1997). The cyanobacterial and algal CCMs, like those of many submerged vascular plants, involve energized transport of CO_2, HCO_3^- and/or H^+ across one or more membranes. C_4 and CAM involve a C_3/C_4 carboxylation–decarboxylation cycle preceding rubisco. In C_4, this cycle involves decarboxylation at different sites, but within seconds or tens of seconds, of the carboxylation, while in CAM the carboxylation and decarboxylation parts of the cycle are very close together spatially but are approximately 12 hours out of phase over the diel light–dark cycle.

2. THE RANGE OF ORGANISMS WITH CCMs

(a) *Background*

CCMs occur today in all the cyanobacteria examined, in all algae apart from a few members of the Chlorophyta and Rhodophyta as well as most or all members of the Chrysophyceae and Synurophyceae, as well as in some hornworts, lycopsids and ferns, and in a significant number of flowering plants (Winter & Smith 1995; Badger *et al.* 1998, 2002; Sage & Monson 1998; Colman *et al.* 2002; Keeley & Rundel 2003; Price & Badger 2003; Edwards *et al.* 2004; Giordano *et al.* 2005; Raven *et al.* 2005*a–c*; Price *et al.* 2007; Roberts *et al.* 2007*a*,*b*; table 1).

(b) *Cyanobacteria*

In cyanobacteria, the CCM is based on accumulation of HCO_3^- in the cytosol (table 1*a*,*b*), movement of this HCO_3^- through their proteinaceous shells into the carboxysomes containing rubisco, and conversion to CO_2 using one or more carbonic anhydrase (CA) enzymes (Badger *et al.* 2002; Price *et al.* 2007). The open ocean cyanobacteria with form IA rubiscos have a restricted suite of HCO_3^- accumulation processes and, probably, little capacity to acclimatize to decreased inorganic C availability (an unlikely event in the pelagic ocean). Coastal and freshwater cyanobacteria with form IB rubisco have a greater range of HCO_3^- accumulation mechanisms and an ability to acclimatize to decreased external inorganic carbon supply by expressing high-affinity HCO_3^- accumulation processes, yielding a greater whole-cell affinity for HCO_3^- and CO_2 (Price & Badger 2003; Price *et al.* 2007).

A lot is known about the components of the cyanobacterial CCMs at the molecular genetic level. It is known that regulation involves sensing the size of the intracellular HCO_3^- pool, with an auxiliary role for the concentration of O_2 (Price *et al.* 2007).

(c) *Algae*

There is apparently greater diversity of CCMs in algae than in cyanobacteria (table 1*c–g*,*i*) and less is known about the mechanisms and molecular genetics of the CCMs (Badger *et al.* 1998; Colman *et al.* 2002; Giordano *et al.* 2005). Pyrenoids, which are within the chloroplast stroma and contain rubisco, occur in many algae expressing CCMs. Pyrenoids are conjectured to be the analogues of cyanobacterial carboxysomes, although they are not present in all algae with CCMs (Badger *et al.* 1998; Giordano *et al.* 2005; Kevekordes *et al.* 2006). The most detailed knowledge is for the green freshwater alga *Chlamydomonas reinhardtii* (Moroney & Ynalvez 2007; Spalding 2007; cf. Im *et al.* 2003). Rather less is known for the biogeochemically very important diatoms (Roberts *et al.* 2007*a*,*b*), although there are now two completed diatom genome sequences. While very small (picoplanktonic) cells could, in principle, rely on diffusive CO_2 entry when similar, larger cells could not, the evidence for eukaryotic algae (and cyanobacteria) shows that even the smallest cells have CCMs (Raven 1991*b*; Iglesias-Rodriguez *et al.* 1998; Giordano *et al.* 2005).

Much research is based on the assumption that the algal CCM is based on the active transport of one or more of CO_2, HCO_3^- and/or H^+ and that the first acid-stable product of inorganic carbon assimilation is 3-phosphoglycerate, the immediate product of the carboxylase reaction of rubisco, that is, the photosynthetic biochemistry is C_3. However, there are tracer carbon and other data suggesting C_4 photosynthetic biochemistry in a green alga and possibly in a diatom (table 1*g*). However, more recent tracer carbon data (table 1*i*) suggest that the diatom *Thalassiosira weissflogii* has C_3–C_4 intermediate biochemistry while *T. pseudonana* has C_3 biochemistry; both have CCMs, presumably based on mechanisms in table 1*b–d* (Roberts *et al.* 2007*a*,*b*; cf. McGinn & Morel 2008).

Keeley (in Winter & Smith 1995) and Keeley (1998) concludes that there is little evidence of a significant contribution of CAM to the carbon budget of algae.

(d) *Symbioses with cyanobacteria and/or algae as the partner performing photosynthesis*

Raven (2003) reviews the available evidence; this shows that all of the cyanobacteria and dinoflagellates investigated, and some of the green algae examined, express CCMs when they are contributing photosynthesis to a symbiosis.

(e) *Embryophyta: anthocerotes, lycopsids, pteropsids and coniferopsids*

The hornworts are terrestrial; some of them have pyrenoids in their chloroplasts and have CCMs (Smith & Griffiths 1996; table 1*c*,*d*). The lycopsid *Isoetes* (an embryophyte clade at the fern level of organization) often lives submerged in freshwater and obtains most of its inorganic carbon through the roots and assimilates CO_2 by CAM in the leaves (Keeley 1998; table 1*j*). This is also true of amphibious species (e.g. *Isoetes andicola*, formerly *Stylites andicola*) that, even when their leaves are exposed, do not form functional stomata and behave ecophysiologically as if they are still submerged (Keeley 1998). A number of epiphytic ferns (pteropsids) have CAM (Keeley & Rundel 2005; table 1*j*). The coniferopsid cycadophyte *Dioon edule* (Vovides *et al.* 2002) and gnetophyte *Welwitschia mirabilis* have some features of CAM, which could constitute a CCM (Keeley & Rundel 2003).

Table 1. The distribution of CCMs among extant organisms.

mechanism	location within organism	phylogenetic distribution	references
a. Passive CO_2 entry, energized conversion to HCO_3^-	plasmalemma, thylakoid; $HCO_3^- \rightarrow CO_2$ and assimilation by rubisco in carboxysomes	cyanobacteria	Badger *et al.* (1998, 2002), Price & Badger (2003), Giordano *et al.* (2005) and Price *et al.* (2007)
b. Energized entry of HCO_3^-	plasmalemma, carboxysomes	cyanobacteria	Badger *et al.* (1998, 2002), Price & Badger (2003), Giordano *et al.* (2005) and Price *et al.* (2007)
c. Energized entry of CO_2	plasmalemma? plastid envelope? rubisco in stroma/pyrenoid	many algae, and either CO_2 or HCO_3^- in hornworts with CCMs and some of the aquatic vascular plants with CCMs (see *e* and *f*)	Smith & Griffiths (1996), Colman *et al.* (2002), Maberly & Madsen (2002), Giordano *et al.* (2005), Raven *et al.* (2005*b*), Kevekordes *et al.* (2006), Burey *et al.* (2007), Moroney & Ynalvez (2007), Roberts *et al.* (2007*a*) and Spalding (2007)
d. Energized entry of HCO_3^-	plasmalemma? plastid envelope? rubisco in stroma/pyrenoid	many algae, and either CO_2 or HCO_3^- in hornworts with CCMs and some of the aquatic vascular plants with CCMs (see *d* and *f*)	Smith & Griffiths (1996), Colman *et al.* (2002), Maberly & Madsen (2002), Giordano *et al.* (2005), Raven *et al.* (2005*b*), Kevekordes *et al.* (2006), Burey *et al.* (2007), Moroney & Ynalvez (2007), Roberts *et al.* (2007*a*) and Spalding (2007)
e. Energized flux of H^+ to cell wall, conversion of HCO_3^- to CO_2	plasmalemma, CO_2 flux to rubisco in stroma	some algae, including characean green algae, and either CO_2 or HCO_3^- in hornworts with CCMs and some of the aquatic vascular plants with CCMs (see *d* and *e*)	Walker *et al.* (1980), Beer *et al.* (2002), Maberly & Madsen (2002), Helblom & Axelsson (2003), Uku *et al.* (2005)
f. Energized flux of H^+ to thylakoid lumen, conversion of HCO_3^- to CO_2	thylakoids, CO_2 flux to rubisco in pyrenoid	freshwater green microalga *Chlamydomonas*	Pronina & Semenenko (1992), Raven (1997*a*), Giordano *et al.* (2005) and Moroney & Ynalvez (2007)
g. C_4 metabolism in single-cell type	inorganic C + C_3 acid $\rightarrow$ C_4 acid in cytosol, C_4 acid $\rightarrow$ C_3 acid + CO_2 in chloroplast stroma (or nearby), rubisco in stroma	marine green acellular macroalga *Udotea*, possibly a marine diatom, a few terrestrial and submerged flowering plants	Beardall *et al.* (1976), Reiskind *et al.* (1988), Raven (1997*a*), Sage & Monson (1998), Reinfelder *et al.* (2000), Keeley & Rundel (2003), Edwards *et al.* (2004), Giordano *et al.* (2005), Osborne & Beerling (2006), Roberts *et al.* (2007*a*,*b*)
h. C_4 metabolism in two-cell types	inorganic C + C_3 acid from bundle sheath (bs) cell $\rightarrow$ C_4 acid in cytosol of mesophyll (mes) cell, C_4 acid $\rightarrow$ bs cell, decarboxylated to C_3 acid ($\rightarrow$ mes) and CO_2 fixed by rubisco in stroma of bs chloroplasts.	most C_4 terrestrial flowering plants, a few amphibious/aquatic flowering plants	Sage & Monson (1998), Keeley & Rundel (2003) and Osborne & Beerling (2006)
i. C_3–C_4 intermediate	combinations of improved internal recycling of photorespiratory CO_2 and partial two-cell C_4 photosynthesis in flowering plants; in one cell in a diatom?	a few terrestrial flowering plants, and a diatom	Sage & Monson (1998) and Roberts *et al.* (2007*a*,*b*)
j. Crassulacean acid metabolism	inorganic C + C_3 organic acid in dark in cytosol of mes $\rightarrow$ C_4 acid stored for approximately 12 hours in vacuole; in light C_4 acid $\rightarrow$ cytosol $\rightarrow$ C_3 acid ($\rightarrow$ carbohydrate) and CO_2 fixed by rubisco in stroma	some aquatic/amphibious lycophytes, a few terrestrial ferns and gymnosperms, some terrestrial and aquatic vascular plants	Winter & Smith (1995), Keeley (1998) and Keeley & Rundel (2003)

(f) ***Embryophyta: magnoliophytes with CAM***

CAM was first known from terrestrial dicotyledonous plants where it has been associated with restricted inorganic carbon supply as a result of restricted stomatal opening in the light when evaporative demand is highest in habitats of limited or fluctuating water availability, for example, epiphytes and inhabitants of some arid zones (Winter & Smith 1995; Keeley & Rundel 2003).

CAM in freshwater aquatic plants was recognized much later, and has also been related to restricted inorganic carbon availability although not, in aquatics, as a result of water deficiency (Keeley 1998; Maberly & Madsen 2002). Inorganic carbon deficiency in freshwaters is not strongly coupled to atmospheric variations, being more dependent on autogenous factors in the water body (Keeley 1998; Keeley & Rundel 2003).

(g) *Embryophyta: magnoliophytes with C_4 photosynthesis*

A range of terrestrial flowering plants in a number of families has polyphyletically evolved the C_4 pathway of photosynthesis, which usually involves the division of biochemical functions between two cell types in 'Kranz anatomy', which could be a palaeontological indicator of this form of C_4 photosynthesis (table 1*h*), but sometimes the whole reaction sequence occurs in single cells (Sage & Monson 1998; Edwards *et al.* 2004; table 1*g*). A few submerged freshwater aquatic flowering plants also have C_4 photosynthesis (Maberly & Madsen 2002; Keeley & Rundel 2003). Sage *et al.* (2002) discuss why the evolution of C_4 and CAM in a single genus cannot occur, with the possible exception of *Portulaca*.

(h) *Embryophyta: magnoliophytes with C_3–C_4 intermediate photosynthesis*

This syndrome, initially defined on the basis of physiology, involves a range of metabolic and structural features in different species, is apparently limited to terrestrial flowering plants (and a diatom!) and is polyphyletic.

(i) *Embryophyta: aquatic magnoliophytes*

Some submerged freshwater aquatic magnoliophytes have constitutive or facultative CAM, while others have constitutive or facultative C_4 photosynthesis. However, other freshwater flowering plants, and seagrasses, have C_3 photosynthetic biochemistry, and many of these have CCMs based on active transport of HCO_3^-, CO_2 and/or H^+ (table 1*c–e,g,h,j*).

3. REGULATION OF CCMs

C_4 (and C_3–C_4 intermediate) photosynthesis in terrestrial plants (and, as far as it has been tested, in a diatom) is constitutive, as is CAM in many terrestrial CAM plants (Keeley & Rundel 2003; Roberts *et al.* 2007*a*,*b*). The expression of CAM in some terrestrial plants is facultative, with the expression when the organism has a limited supply of water relative to evaporative demand (Keeley & Rundel 2003). CAM in submerged vascular plants of the isoetid life form is facultative in some species, which revert to C_3 physiology when emersed, but other species of isoetids, with thick cuticles on their shoots and no openable stomata, retain CAM using CO_2 taken up through the roots even when the shoots are emersed. Other submerged CAM plants and those with C_4 that evolved after they returned to living in water show variability in expression of these pathways as a function of temperature and inorganic carbon availability. Cyanobacteria and algae with CCMs typically show constitutive expression of the CCMs within the range of inorganic carbon concentrations and speciation that they normally encounter in nature, although in many cases there is increased affinity for inorganic carbon of the overall CCM when the organism is grown at lower inorganic carbon concentrations (Giordano *et al.* 2005). The adaptation, and the acclimation mechanisms, of inorganic C acquisition mechanisms will have been influenced by the glacial–interglacial cycles of atmospheric CO_2 (180–280 ppm); this variation is not within the anthropogenic range of CO_2 increase, currently at 380 ppm.

The expression of CCMs in algae and cyanobacteria is not just regulated by the supply of inorganic carbon. The levels of PAR (photosynthetically active radiation), which are lower than those yielding the maximum rate of growth lead to a decreased affinity for inorganic carbon (Giordano *et al.* 2005), with the probable consequence that the reduced photosynthetic energy supply reduces the selective significance of CCMs relative to diffusive CO_2 entry. This is because, in the case of CCMs, a constant leakage rate of CO_2 needs to be offset, so that the energy cost per unit net carbon assimilation increases, whereas in the case of diffusive CO_2 entry, the energy loss due to photorespiration declines in proportion to carboxylation (Giordano *et al.* 2005). There are also effects of inorganic carbon supply for growth on acclimation to variations in PAR (MacKenzie *et al.* 2002; Burns *et al.* 2006).

The other aspect of electromagnetic radiation that has been investigated for its effect on CCMs is the effect of UV-B. The available data are for three species of eukaryotic algae, and range from showing that the CCM is less sensitive to UV-B than are other reactions of photosynthesis to finding that the CCM is more sensitive than the rest of photosynthesis (Beardall *et al.* 2002; Sobrino *et al.* 2004). The data from terrestrial flowering plants (Johnson & Day 2002; Correia *et al.* 2005) do not permit a decision as to whether the C_4 CCM shows specific sensitivity to UV. No work seems to have been performed on the effect of UV-A when energizing photosynthesis (Gao *et al.* 2007) on the functioning of CCMs.

At a constant inorganic C supply, the affinity for inorganic carbon increases when nitrogen (as NO_3^-) is limiting growth; this could be related to higher carbon assimilation per unit catalytic nitrogen when a CCM is operating (Raven 1991*a*,*b*; Raven *et al.* 2005*c*). However, when nitrogen is supplied as NH_4^+ there is a decreased affinity for inorganic carbon when nitrogen is limiting (Raven *et al.* 2005*c*). The effects of phosphorus deficiency on CCM expression are not consistent between the two datasets available (Raven *et al.* 2005*c*). Iron deficiency increases inorganic carbon affinity, perhaps, by a similar argument to that used for nitrate deficiency, because greater CCM expression increases net carbon assimilation per unit catalytic iron (Raven 1990, 1991*a*,*b*; Raven *et al.* 2005*c*). The inorganic C assimilation rate per unit catalytic Zn could be increased if the need for CA (which usually requires Zn) for photosynthesis is decreased or abolished by switching from C_3 to C_4 biochemistry if HCO_3^- enters the cells (Reinfelder *et al.* 2000; Raven *et al.* 2005*c*; cf. Roberts *et al.* 2007*a*,*b*).

The findings for increased inorganic C affinity related to NO_3^- and Fe limitations of growth, and the connection to predictions from biochemical modelling as to reductions in the N and Fe cell quotas needed for growth at a given rate as a function of increased engagement of CCMs, clearly need further examination by whole-cell elemental, and quantitative proteomic, analyses (cf. Finkel *et al.* 2006).

4. IMPLICATIONS OF CCMs FOR NATURAL ABUNDANCE OF C ISOTOPES

In terrestrial plants that lack CCMs, that is, embryophytes and lichens with diffusive CO_2 entry associated with C_3 physiology and biochemistry, the $\delta^{13}C$ values are in the range $-25‰$ to $-30‰$. By contrast, those with CCMs, i.e. C_4 biochemistry, CAM, and those based on active transport across membranes in some anthocerotes and lichens, have higher, i.e. less negative, $\delta^{13}C$ values, up to $-10‰$. The values for C_3–C_4 plants are more variable and frequently near the $\delta^{13}C$ values of those with C_3 physiology. For aquatic organisms, the picture is less clear (Raven *et al.* 2002*a*,*b*; Kevekordes *et al.* 2006), even in oceans where the $\delta^{13}C$ values of the source inorganic carbon are somewhat more variable than that in the atmosphere on yearly to decadal time scales, but much less variable than among freshwater bodies. The most negative values of organism $\delta^{13}C$ are generally correlated with diffusive CO_2 entry, while more positive values relate to either presence of CCMs, or diffusive limitation of organic supply to the organism, or a combination of the two (Raven *et al.* 2002*a*,*b*; Kevekordes *et al.* 2006). However, there are possibilities of CCMs which can also bring about 1:1 exchange fluxes of inorganic carbon across membranes, which would yield very negative $\delta^{13}C$ values (Raven *et al.* 2002*a*). The use of $\delta^{13}C$ values in detecting the occurrence of CCMs in the past requires a knowledge of the source inorganic C $\delta^{13}C$ value, and is also influenced by temperature and by inorganic carbon concentrations as well as the rate of photosynthesis and, especially in aquatic organisms, cell or organ size and boundary-layer thickness (Korb *et al.* 1996; Finkel *et al.* 2005; Katz *et al.* 2005).

5. EVOLUTIONARY ORIGINS OF CCMs: WHENCE?

The genes for many biochemical components of C_4 and CAM occur as parts of anaplerotic pathways and storage mechanisms in their C_3 ancestors (Winter & Smith 1995; Sage & Monson 1998; Keeley & Rundel 2003). This is not to say that the gene duplications, alterations in regulation and/or localization of the products of the duplicate (C_4 or CAM) genes, and other developmental changes involved in the evolution of C_4 and CAM are trivial, but it is clear that these evolutionary changes have occurred independently in C_4 approximately 30 times, and 20 times for CAM.

Raven (1980) pointed out that CCMs based on active transport processes were analogous to those accumulating other nutrient solutes (e.g. NH_4^+, NO_3^-, urea, amino acids, $H_2PO_4^-$, SO_4^{2-} and $Si(OH)_4$) prior to their assimilation. Some of these accumulation mechanisms could have evolved in relation to episodes of nutrient shortage over geological time (Raven *et al.* 2005*a*; cf. Konhauser *et al.* 2007). Raven *et al.* (2008) suggest that the CCMs could generally be interpreted in terms of the recruitment of many components from pre-existing transporters and, in the case of CO_2 acquisition by cyanobacteria, NAD(P)H dehydrogenase. The argument of Raven *et al.* (2008) could be further tested with the phylogenies of gene families containing CCM components when more is known of the genetic background of CCMs, especially for eukaryotes. The case for such bricolage is most obvious for secondarily aquatic flowering plants, which evolved less than 120 Myr ago (Raven *et al.* 2008). As Raven *et al.* (2008) point out, each stage in CCM evolution must have had selective significance, though identification of these stages and their significance is difficult.

6. EVOLUTIONARY ORIGIN OF CCMs: WHY?

The replacement of rubisco and the photosynthetic carbon reduction cycle in O_2 evolvers by one of the other inorganic carbon assimilation pathways found in autotrophs is apparently prevented by, among other things, oxygen damage to one or more of the enzymes involved in these alternative pathways (Berg *et al.* 2007; Raven *et al.* 2008).

The fact that O_2 evolvers cannot replace rubisco means that the 'obvious' role of CCMs is in improving the working conditions for rubisco in an environment in which diffusive gas exchange would yield significantly subsaturating concentrations of CO_2 at the active site of rubisco and/or a significant restriction on net carbon assimilation by the occurrence of rubisco oxygenase activity (Giordano *et al.* 2005; Raven & Larkum 2007). However, it must be remembered that not all photosynthetic O_2 evolvers have CCMs (Raven *et al.* 2005*a*,*b*); optimality criteria that compare the costs and benefits of the trait should be employed to investigate the distribution of CCMs (Rosen 1967; Raven *et al.* 2008). As will be seen under consideration of when CCMs evolved, a low CO_2 concentration, either globally or locally, is frequently considered as a selective factor in the evolution of CCMs (Keeley & Rundel 2003; Giordano *et al.* 2005; Osborne & Beerling 2006), if not always in their spread (Keeley & Rundel 2005). The role of O_2 in the evolution of CCMs is not clear (Giordano *et al.* 2005; Riding 2006). While not claiming that the environmental signal for inducible CCMs reflects the selective factors involved in their evolution, in cyanobacteria increased expression of CCMs involves decreased intracellular inorganic carbon concentration with a secondary role for increased oxygen (Price *et al.* 2007), while the eukaryotic algae tested respond to extracellular CO_2 with no observable effect of O_2 (Giordano *et al.* 2005; Vance & Spalding 2005). There are other factors that influence the expression of CCMs in vascular plants, for example, facultative CAM plants on land and water availability, and temperature and submerged C_4 (Keeley & Rundel 2003).

7. EVOLUTIONARY ORIGIN OF CCMs: WHEN?

The CCM with the most recent origin that can be relatively accurately dated is C_4 in land plants, with fossil anatomical and stable isotope evidence from a

grass at *ca* 12.5 Myr ago and from soil carbonate and herbivore teeth and skeleton carbon isotope ratios indicating sufficient local C_4 photosynthesis to substantially alter this proxy for plant organic carbon (Keeley & Rundel 2003). Molecular clock data suggest an origin 20–30 Myr ago (Keeley & Rundel 2003). For CAM, the pathway in terrestrial and aquatic flowering plants and *Welwitschia* evolved less than 120 Myr ago subsequent to the origin of the gnetophytes and flowering plants, the ancestral condition in these clades being C_3 physiology (Keeley & Rundel 2003). Carbon isotope evidence for CAM 200 Myr ago could be related to the occurrence of CAM cycling in an extant cycad (Decker & de Wit 2006; see Griffiths *et al.* (2007) for the interpretation of carbon isotope data in CAM). Extant ferns with CAM are in a clade that originated in the Cretaceous (120–65 Myr ago). The lycopsid *Isoetes*, expressing CAM when submerged (or effectively submerged), has a fossil record from palustrine (marsh or wetland) habitats from 230 Myr ago (Keeley & Rundel 2003). Inorganic carbon availability in submerged palustrine habitats is not, as Keeley & Rundel (2003) point out, closely coupled to the atmospheric CO_2 level. There are very few carbon isotope data consistent with terrestrial CCMs during the low CO_2 episode (Royer *et al.* 2007) in the Carboniferous some 300 Myr ago (Keeley & Rundel 2003; Osborne & Beerling 2006).

Badger *et al.* (2002), following Raven (1997*b*), suggest that CCMs in cyanobacteria and algae evolved in the low CO_2 (and high O_2) environment of the Carboniferous. There is also the possibility of CCMs evolving in ice house, and presumably low CO_2, episodes in the Proterozoic at approximately 2.4, 0.75 and 0.6 Gyr ago (Giordano *et al.* 2005; Harland 2007). Although there seem to be no carbon isotope data in support, we have seen that high $\delta^{13}C$ values are not inevitable consequences of the operation of CCMs. A major problem is the maintenance of CCMs in the high CO_2 intervals, up to hundreds of millions of years, between low CO_2 episodes (Giordano *et al.* 2005; Sheldon 2006; Came *et al.* 2007; Royer *et al.* 2007). There were clearly cyanobacteria at the earliest ice house time mentioned above, eukaryotic oxygen evolvers for the two later ice houses (Brocks & Pearson 2005; Giordano *et al.* 2005), although the biomarker and structural fossil evidence do not permit conclusions as to the occurrence of CCMs.

Although not necessarily indicating the time of origin of algal CCMs, there is carbon isotope evidence (not a result of changed atmospheric $\delta^{13}CO_2$) from marine sediments in the latter part of the Cenozoic (the last 15 Myr) that CCMs became more significant in the ocean as well as on land as the CO_2 content of the atmosphere (and the ocean) decreased (Katz *et al.* 2005). The increasing $\delta^{13}C$ through the latter part of the Cenozoic could also be explained in terms of diffusive CO_2 entry if there was an increasing cell size, based on the allometry of photosynthetic rate with the surface area per unit volume and the increasing restriction on photosynthesis by diffusion boundary layers around the cell (Korb *et al.* 1996; Finkel *et al.* 2005; Katz *et al.* 2005). However, the available data show that for diatoms, the dominant contributors to marine primary productivity at that time, the mean cell size decreased rather than increased with time in the Cenozoic (Finkel *et al.* 2005; Katz *et al.* 2005).

8. EVOLUTION OF CCMs: HYPOTHESES ABOUT STROMATOLITES

Giordano *et al.* (2005) and Raven & Larkum (2007) show that cyanobacteria in stromatolites could have low intracellular CO_2 concentrations and high O_2 concentrations, despite high external CO_2 and negligible O_2 (see Anbar *et al.* 2007; Kaufman *et al.* 2007) in the medium some 2.3–2.5 Gyr ago. The reasoning here is that the cyanobacteria in a stromatolite are separated from the medium by a millimetre or centimetre of unstirred interstitial water in a mineral and organic polymer, which adds tortuosity to the aqueous diffusion path (although the possibility of photomotion by the cyanobacteria, altering their distribution on the illuminated relative to the shaded side, must be acknowledged; Batchellor *et al.* 2004). Here we explore the implications of UV screening as a factor favouring the occurrence of cyanobacteria in stromatolites and other dense benthic populations. We discuss the indirect and direct effects of high UV flux on the intracellular environment and thus the influence on the potential environment that early CCMs would be exposed to.

Our suggestion is based on the observations that stromatolites occur, and occurred, intertidally or in shallow water, where fluxes of UV relative to PAR incident on the Earth's atmosphere were higher more than 2.4 Gyr ago, and that there was no stratospheric O_3 layer that would have attenuated UVC > UVB > UVA > PAR. The high UV flux at the surface of the ocean, and the absence of a great depth of seawater over the stromatolites even with plausible levels of organic compounds dissolved in the seawater, would not significantly attenuate UV relative to PAR at the stromatolite surface relative to the water surface. The minerals and UV-screening organic compounds produced by the cyanobacteria between the surface of the stromatolites and the cyanobacteria act as not only a UV screen (not a new suggestion!) but also, and incidentally, a barrier to diffusion of inorganic carbon and of oxygen (Giordano *et al.* 2005; Raven & Larkum 2007).

The restriction on inorganic C supply to the cyanobacteria could, as suggested by Giordano *et al.* (2005) and Raven *et al.* (2008), have implications for the evolution of CCMs as well as of form IA and form IB rubiscos. The corresponding build-up of O_2 has received rather less attention, except in relation to rubisco oxygenase activity (Giordano *et al.* 2005; Raven & Larkum 2007; Raven *et al.* 2008). Such O_2 build-up might be the first encounter that organisms had with high O_2 concentrations, and hence with high rates of production of reactive oxygen species such as $^1O_2$, $^{\cdot}OH$, $^{\cdot}O_2^-$ and H_2O_2. To these can be added O_3, since any UV not attenuated by the extracellular inorganic and organic screening compounds can generate O_3 from photosynthetically produced O_2 and from cyanobacterial mats in an initially anoxic atmosphere.

The production of ozone from photosynthetically produced oxygen was demonstrated in experiments subjecting cyanobacterial mats to a simulated early

Earth atmosphere (10% CO_2/90% N_2) and a short-wavelength UV source (see electronic supplementary material; C. S. Cockell 2002, unpublished data). The mats were incubated in quartz tubes purged with the simulated atmosphere and exposed to a 185 nm source modulated to deliver the same biologically effective irradiance as that expected on the surface of the Archaean Earth in the absence of any atmospheric UV screens apart from carbon dioxide.

After 10 min of exposure, tubes containing no mats or mats that had been heat killed did not exhibit significant production of ozone (approx. 0.2 ppm O_3). The present-day (21% O_2) atmosphere supported the formation of 1.4 ppm O_3. Tubes with live cyanobacterial mats and the simulated early Earth atmosphere showed a net production of 0.7 ppm O_3, formed from the interaction of low-wavelength UV radiation with the photosynthetically produced oxygen. This value was significantly higher than all the controls assessed by Student's *t*-test ($p < 0.01$).

Unlike the other reactive oxygen species, which are generated within the cells, O_3 can be generated outside the cells as well. There would have been opposing gradients of O_2 and of UV, with the concentration of O_2 highest within the cells and the UV flux lowest within the cells. Knowing the relationship between UV flux and O_2 concentration in producing O_3 would allow a quantitative model to be produced of the O_3 production rate along the gradient from the cells to the surface assuming an exponentially increasing UV flux and linearly decreasing O_2 concentration, further assuming no O_2 consumption by non-photosynthetic biota or inorganic reductants between the cyanobacteria and the surface of the structure. A problem with assessing the biological impact of the O_3 generated in and around the cyanobacterial cells is uncertainty as to the solubility of O_3 and the rate at which it would be consumed by chemical reactions with the organic and inorganic compounds outside the cells and, indeed, simply by being in aqueous solution. A further complication in assessing the role of reactive oxygen species and ozone in the evolutionary context is the possible role of the cyanobacterial sheath. A thick sheath will retard the diffusion of oxygen out of the cell, making it more likely that biologically detrimental concentrations of free radical species and ozone would be achieved intracellularly. However, a thick sheath will concomitantly protect the cell from free radicals and ozone in the intercellular environment that results from other cells' oxygen production. Conversely, thin sheaths provide less protection from the intercellular toxic species, but allow for faster diffusion of these species from the intracellular environment.

The effects of O_3 on aquatic photosynthetic organisms have not been investigated to the same extent as for their terrestrial counterparts, presumably owing to the uncertainties that have just been mentioned and the assumption that the effects of dissolved O_3 would be mediated largely by the other reactive oxygen species resulting from its reactions in solution. However, ozone readily mixes with water and its oxidizing abilities, more powerful than even those of chlorine, are used today to decontaminate drinking water and disinfect surfaces (Moore *et al.* 2000). At a concentration of 2 mg l^{-1} (equivalent to 2 ppm by weight), 90% inactivation of some bacterial species is achieved in less than 20 s (Lezcano *et al.* 1999). Concentrations as low as 0.33 mg l^{-1} can cause complete inactivation of *Staphylococcus* species in 7 min (Lezcano *et al.* 2000).

The scheme we have just outlined for cyanobacterial microbialites involves the addition of damage by reactive oxygen species (and effects of O_2 *per se*; Raven & Larkum 2007) to that from UV, with some of the load of reactive oxygen species resulting from absorbed UV radiation. This scheme presents the earliest occurrence in microcosm of the later global occurrence of damage from reactive oxygen species as well as of that from UV for organisms living in the surface ocean or on land. The UV screens in the microbialites (and then in other organisms) were supplemented, with atmospheric oxygenation, by the UV screening involving O_3.

Can we learn anything from the response of extant organisms to UV and reactive oxygen species that bear on the plausibility of the scheme that we have proposed on UV screening and the origin of CCMs? For the effects of UV on CCMs, we have no information on cyanobacteria and only a few data from algae, yielding responses varying from CCMs being less sensitive to being more sensitive to UV than is the rest of photosynthetic metabolism (Beardall *et al.* 2002; Sobrino *et al.* 2004). Data for terrestrial flowering plants do not permit definitive conclusions as to the UV sensitivity of the C_4 CCM (Johnson & Day 2002; Correia *et al.* 2005). For evidence as to any increased production of reactive oxygen species as a result of operation of CCMs and the related higher intracellular O_2 concentration (Raven & Larkum 2007) we can turn, in the absence of direct evidence, to changes in gene expression as the CCM expression is increased when cultures of the green alga *C. reinhardtii* and the glaucocystophyte alga *Cyanophora paradoxa* grown in high CO_2 are transferred to low CO_2. Here there is increased transcription of genes associated with increased production of oxidants (Im *et al.* 2003; Burey *et al.* 2007). Further investigation is needed.

9. CCMs IN THE FUTURE

Given the current and marked anthropogenically induced rise in atmospheric CO_2 concentrations, a widespread view is that CCMs will become less competitive relative to organisms relying on diffusive CO_2 influx to C_3 biochemistry (see Keeley & Rundel 2003; Giordano *et al.* 2005). However, as shown in §3, other interrelated environmental changes, such as temperature increase, changes in the depth of the ocean upper mixed layer and variations in the supply of resources other than C, will interact with the increase in CO_2.

In the much more distant future, the temporal trajectory of the Sun is such that the trend of increasing output of electromagnetic radiation seen over the Sun's existence will continue so that the Earth will be too hot to be habitable well before the Sun expands to a radius similar to that of the Earth's orbit as a supernova before it becomes a red dwarf (Franck *et al.* 2006). Thermal

death of life on Earth can be delayed if greenhouse gas levels in the atmosphere are minimized, although such a reduction would, for CO_2, have implications for photosynthesis (Rampino & Caldeira 1994). This point was first considered by Lovelock & Whitfield (1982) in the context of C_3 physiology of photosynthesis; they suggested that CO_2 would be down to the lower limit for photosynthesis in 100 Myr, if this gas was acting as a major component of Earth's temperature regulation (cf. Berner & Kothavala 2001). Caldeira & Kasting (1992) revisited this question, and pointed out that C_4 photosynthesis would, through a higher affinity for CO_2 and a lower CO_2 compensation concentration, permit photosynthetically powered ecosystems to persist for an additional several hundreds of millions of years to 0.9–1.5 Gyr. Such 'salvation through CCMs' is independent of the means by which the decreased biosphere CO_2 concentration is achieved.

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