The evolution of inorganic carbon concentrating mechanisms in photosynthesis

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Abstract

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, 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 acclamatory responses to variations in the supply of not only CO_2 but also of 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 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 which 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 N inputs.

These considerations can help to rationalise, 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 C leakage that occurs from the internal pool (Raven, 1991; Sültemeyer and Rinast 1996). Since any barriers to leakage of CO_2 (the most likely species of inorganic C 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 and 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 likely future in relation to atmospheric composition. We consider first the CCMs in cyanobacteria and algae which account for well over half of the 50 Pg or so of C assimilated each year in the oceans, and then with the C_4 and CAM (crassulacean acid metabolism) found in terrestrial vascular plants that accounts for less than half of the 60 Pg or so of

C assimilated each year on land. 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 site to, 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 about 12 h out of phase over the diel light-dark cycle.

2. The range of organisms with CCMs

(a) *Background*

Carbon concentrating mechanisms occur today in all the Cyanobacteria examined, in the majority of algae, in some hornworts, lycopods and ferns, and in a significant number of flowering plants (Winter & Smith 1995; Badger *et al.* 1998; Sage and Monson 1998; Badger *et al.* 2002; Colman *et al.* 2002; Keeley and Rundel 2003; Edwards *et al.* 2004; Price and Badger 2003; Giordano *et al.* 2005; Raven *et al.* 2005; Price *et al.* 2007; Roberts *et al.* 2007a.b): Table 1.

(b) *Cyanobacteria*

In Cyanobacteria the CCM is based on accumulation of HCO_3^- in the cytosol (Table 1a, 1b) 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 acclimate 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 acclimate to decreased external inorganic C supply by expressing high-affinity HCO_3^- accumulation processes, yielding a greater whole-cell affinity for HCO_3^- and CO_2 (Price and 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 more diversity of CCMs in algae than in cyanobacteria Table 1c, 1d, 1e, 1e, 1f, 1g, 1i) and less is known of the mechanisms and of the molecular genetics of the CMMs (Badger *et al.* 1998; Colman *et al.* 2002; Giordano *et al.* 2005). Pyrenoids, which are volumes within the chloroplast stroma containing RubisCO and which are conjectured to be the analogues of cyanobacterial carboxysomes; however, 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 and Ynalvez 2007; Spalding 2007; cf. Im *et al.* 2003). Rather less is known for the biogeochemically very important diatoms (Roberts *et al.* 2007a.b), although there are now two completed diatom genome sequences.

Much research is based on the assumption is 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 C assimilation is 3-phosphoglycerate, the immediate product of the carboxylase reaction of RubisCO, i.e. the photosynthetic biochemistry is C_3. However, there are data suggesting C_4 photosynthetic biochemistry in a green alga and possibly in a diatom (Table 1g). However, recent data (Table 1i) suggests that *Thalassiosira weissflogii* has C_3-C_4 intermediate biochemistry while *T. pseudonana* has C_3 biochemistry; both have CCMs, presumably based on mechanisms in Tables 1b-1d (Roberts *et al.* 2007a,b).

Keeley (in Winter and 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, coniferopsids***

The hornworts are terrestrial; some of them have pyrenoids in their chloroplasts and have CCMs (Smith and Griffiths 1996); Table 1c, 1d The lycopsid *Isoetes* , which

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 1j. This is also true of amphibious species (e.g. *I. 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 and Rundel 2005): Table 1j. The coniferopsid gnetophyte *Welwitschia mirabilis* has some features of CAM (Keeley and 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, e.g. epiphytes and inhabitants of some arid zones (Winter and Smith 1995; Keeley and Rundel 2003). CAM in freshwater aquatic plants was recognised 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 and 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 and Rundel 2003).

(g) ***Embryophyta: magnoliophytes with C_4 photosynthesis***

A range of terrestrial flowering plants in a number of families have polyphyletically evolved the C_4 pathway of photosynthesis which usually involves two cell types

(Kranz anatomy, which could be a paleontological indicator of this form of C_4 photosynthesis) (Table 1h), but sometimes the whole reaction sequence occurs in single cells (Sage and Monson 1998; Edwards *et al.* 2004) (Table 1g). A few submerged freshwater aquatic flowering plants also have C_4 photosynthesis (Madsen and Maberly 2002; Keeley and Rundel 2003).

(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 1c, 1d, 1e, 1g, 1h, 1j).

3. Regulation of CCMs

C_4 (and C_3-C_4 intermediate) photosynthesis in terrestrial plants (and, as far as has been tested, in a diatom) is constitutive, as is CAM in many terrestrial CAM plants (Keeley and Rundel 2003; Roberts *et al.* 2007a,b). Expression of CAM in some terrestrial

plants is facultative, with expression when the organism has a limited supply of water relative to evaporative demand (Keeley and 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 others 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 if 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 expression of CCMs in algae and cyanobacteria is not just regulated by the supply of inorganic carbon. Levels of PAR lower than those yielding the maximum rate of growth leads to a decreased affinity for inorganic carbon (Giordano *et al.* 2005). It is likely that the reduced energy supply reduces the selective significance of CCMs relative to diffusive CO_2 entry, since the energy costs of photorespiration per unit net carbon assimilation are constant despite reduced rates of carboxylation and oxygenation at low PAR, while constant leakage rates despite decreased rates of inorganic C pumping at low PAR increases the energy cost per unit net carbon assimilation (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 and 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 energising photosynthesis (Gao *et al.* 2007) on the functioning of CCMs

At a constant inorganic C supply the affinity for inorganic C increases when N (as NO_3^-) is limiting growth; this could be related to higher C assimilation per unit catalytic N when a CCM is operating (Raven, 1991; Raven *et al.* 2005c), However, when N is supplied as NH_4^+ there is a decreased affinity for inorganic C when N is limiting (Raven *et al.* 2005c). Effects of P deficiency on CCM expression are not consistent between the two data sets available (Raven *et al.* 2005c). Fe deficiency increases inorganic C affinity, perhaps, as for N, because in some cases greater CCM expression increases net C assimilation per unit catalytic Fe (Raven, 1990, 1991; Raven *et al.* 2005c). 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.* 2005c; cf Roberts *et al.* 2007a,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, i.e. embryophytes and lichens with diffusive CO_2 entry and C_3 physiology and biochemistry, the $\delta^{13}C$ value is in the range -25‰ - -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.* 2002a,b; Kevekordes *et al.* 2006), even in oceans where the $\delta^{13}C$ values of the source inorganic C are somewhat more variable than that in the atmosphere on a yearly to decadal timescales, 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.* 2002a,b; Kevekordes *et al.* 2006). However, there are possibilities of CCMs with 1:1 exchange fluxes of inorganic C across membranes which would yield very negative $\delta^{13}C$ values (Raven *et al.* 2002a). 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 C concentrations as well as the rate of photosynthesis and, especially in aquatic organisms, on 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 and Smith 1995; Sage and Monson 1998; Keeley and Rundall 2003). This is not to deny 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 about 30 times and about 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-}, $Si(OH)_{4)}$) 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.* 2005a; 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 Ma ago (Raven *et al.* 2008).

6. Evolutionary origin of CCMs: Why?

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 occurrence of Rubisco oxygenase activity (Giordano *et al.* 2005; Raven and Larkum 2007). However, it must be remembered that not all photosynthetic O_2-evolvers have CCMs (Raven *et al.* 2005a,b); optimality criteria should be employed to investigate the distribution of CCMs (Rosen 1997; Raven *et al.* 2008) As will be seen under consideration of when CCMs evolved, a low CO_2 concentration, either globally or locally, are frequently considered as a selective factor in the evolution of CCMs (Keeley and Rundel 2003; Giordano *et al.* 2005; Osborne and Beerling 2006) if not always in their spread (Keeley and 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 evolutionary selective factors involved in their evolution, in cyanobacteria acclimation of CCMs involves intracellular inorganic C concentration with a secondary role for 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 and Spalding 2005). There are other factors that influence the expression of CCMs in vascular plants, e.g. facultative CAM plants on land and water availability, and temperature and submerged C_4 (Keeley and 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 about 12.5 Ma 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 C (Keeley and Rundel 2003). Molecular clock data suggest an origin 20-30 Ma ago (Keeley and Rundel 2003). For CAM the pathway in terrestrial and aquatic flowering plants and *Welwitschia* evolved less than 120 Ma ago subsequent to the origin of the gnetophytes and flowering plants, the ancestral condition in these clades being C_3 physiology (Keeley and Rundel 2003). Extant ferns with CAM are in a clade which originated in the Cretaceous. The lycopsid *Isoetes*, expressing CAM when submerged (or effectively submerged), has a fossil record from palustrine habitats from 230 Ma ago (Keeley and Rundel 2003). Inorganic C availability in submerged palustrine habitats is not, as Keeley and 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 Ma ago (Keeley and Rundel 2003; Osborne and Beerling 2006).

Badger *et al.* (2002), following Raven (1997b) 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 similar to 2.4 Ga, 0.75 Ga and 0.6Ga (Giordano *et al.* 2005; Harland 2007). Although there seem to be no C 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 Ma, between low CO_2 episodes (Giordano.*et al.* 2005; Sheldon 2006; Came *et al.* 2007; Royer *et al.* 2007).

Although not necessarily indicating the time of origin of algal CCMs, there is carbon isotope evidence from marine sediments in the latter part of the Cenozoic, suggesting CCMs became more significant in the ocean as well as on land as the CO_2 content of the atmosphere (and the ocean): 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 and 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 Ga ago. The reasoning here is that the cyanobacteria in a stromatolite are separated from the medium by mm or cm of

unstirred interstitial water in a mineral and organic polymer which adds tortuosity to the diffusion path. 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, that fluxes of UV relative to PAR incident on the Earth's atmosphere were greater 2.4 Ga ago, and that there was no stratospheric O_3 layer which attenuates 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 that would, with almost all of the plausible organic compounds dissolved in the seawater, 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 acts as 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 and Larkum 2007).

The restriction on inorganic C supply to the cyanobacteria could, as suggested by Giordano *et al.* (2005), have implications for the evolution of CCMs. The corresponding build-up of O_2 has received rather less attention, except in relation to Rubisco oxygenase activity (Giordano *et al.* 2005; Raven and Larkum 2007). 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 $^1O_2$, $^{\bullet}OH$, $^{\bullet}O_2^-$ 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 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 shortwavelength UV source (see supplementary material, Cockell, unpublished). 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 Archean Earth in the absence of any atmospheric UV screens apart from carbon dioxide.

After 10 minutes of exposure, tubes containing no mats or mats that had been heat-killed did not exhibit significant production of ozone (~ 0.2 pm 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 as inside them. 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 is would be consumed by chemical reactions with 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 has not been investigated to the same extent as for their terrestrial counterparts, presumably because of 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 (equivalent to 2 ppm by weight), 90% inactivation of some bacterial species is achieved in less than twenty seconds (Lezcano *et al*. 1999). Concentrations as low as 0.33 mg/L can cause complete inactivation of *Staphylococcus* species in 7 min (Lazcano *et al.* 2001).

The addition of damage by reactive oxygen species (and effects of O_2 *per se*: Raven and Larkum 2007) to that from UV, with some of the load of reactive oxygen species resulting from absorbed UV radiation, in the scheme we have just outlined in cyanobacterial microbialites 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 only 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; Sobrano *et al.* 2004). Data for terrestrial flowering plants does not permit definitive conclusions as to the UV sensitivity of the C_4 CCM (Johnson and 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 and 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 *Chlamydomonas*

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 Keeling and Rundal 2003; Giordano *et al.* 2005). However, as shown in Section 3, other, inter-related 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 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 minimised, although such a reduction would for CO_2, have implications for photosynthesis (Rampino and Calderia 1994).. This point was first considered by Lovelock and 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 Ma if this gas was acting as a major component of

Earth's temperature regulation (cf. Berner and Kothvala 2001). Caldeira and 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 Ga. Such 'salvation through CCMs' is independent of the means by which the decreased biosphere CO_2 concentration is achieved.

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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	1,2,8,16.17
b.Energized entry of HCO_3^-	Plasmalemma, carboxysome	Cyanobacteria	1,2,8,16,17
c.Energized entry of CO_2	Plasmalemma? Plastid Envelope? Rubisco in stroma/pyrenoid	Algae, hornworts(?),aquatic vascular plants(?)	5,6,8,12,13, 14,20,23,26,27
d.Energized entry of HCO_3^-	Plasmalemma? Plastid Envelope? Rubisco in stroma/pyrenoid	Algae, Hornworts(?), aquatic vascular plants(?)	5,6,8,12,13, 14,20,23,26,27
e.Energized flux of H^+ to cell wall, conversion of HCO_3^- to CO_2	Plasmalemma. CO_2 flux to Rubisco in stroma	Characean algae, aquatic vascular plants	4,9,13,28,29
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 alga *Chlamydomonas*	8,14,18,19
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*, a marine diatom(?), a few terrestrial and submerged flowering plants	3,7,8,11,15,19, 21,22,23,24,25,
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 terrrestrial flowering plants, a few amphibious/aquatic flowering plants	11,15,25
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.	23,24,25
j.Crassulacean acid metabolism	Inorganic C + C_3 organic acid in dark in cytosol of mes $\rightarrow$ C_4 acid stored for about 12 h 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, a terrestrial gnetophyte gymnosperm, some terrestrial and aquatic vascular plants.	10,11,30

Key to references in Table 1

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Supplementary Methods

Microbial mats were collected from a pond on Devon Island in the Canadian High Arctic. These mats are composed of oscillatorian cyanobacteria similar to those described previously in other ponds of the high arctic (Vezina and Vincent 1987). The ~0.5 cm thick mats take advantage of the briefly available water during the summer months and remain in a dormant, desiccated state during the rest of the year. They can be considered analogous to early cyanobacteria in their mode of energy acquisition (photoautotrophy). The mats were collected in July 2003 and stored in a dry state in the laboratory. Sections of mat of size 2 x 2 cm were rehydrated and placed into quartz tubes of size 2.5 (diameter) x 15 cm (length) that allowed greater than 95% transmission of UV radiation used in these experiments. The tubes were purged with a 90:10 nitrogen:carbon dioxide, oxygen free mixture (Air Products Ltd.) as a simulated Archean anoxic atmosphere, and they were sealed.

The estimates of Kasting (1987) were used to determine the composition of the atmosphere (10% CO_2/90% N_2) used in the experiments as an analog for that of the Archean. The suggestion that at approximately 2.5 Ga, just before the oxygenation of the atmosphere, pCO_2 may have been about 10 kPa has been supported by more recent estimates, based on the occurrence and distribution of redox-sensitive minerals in paleosols, of ~ 4 kPa (Rye et al. 1995). The remainder of the atmosphere is assumed to be nitrogen, although trace amounts of other gases, which are not considered here,

were likely present. Because this experiment is focused on the production of O_3, the most critical aspect of this experiment lies not with the exact CO_2 or N_2 concentration, but in the possibility of generating significant quantities of O_3 from localized accumulations of O_2 and a high UV flux.

The mats were allowed to photosynthesize for six hours under ambient light levels during January 2004. After this period they were exposed to light from a low pressure mercury source (UV Products Ltd.) that emits radiation at the ozone-forming wavelength of 185 nm. We used the absorption profile of oxygen provided by Yung and DeMore (1999) to match the effective oxygen splitting (ozone-forming) energy from the 185 nm source to the expected effective ozone-forming energy on the surface of Archean Earth in the UV region of 195 to 242 nm. We assumed that there are no other UV absorbers in the atmosphere other than CO_2.

Ozone concentrations in the tubes were measured using an A-21ZX ozone monitor (EcoSensors Corporation) with the sensor sealed into the tubes. Control experiments were run using: no microbial mats, but with nitrogen:carbon dioxide gas, microbial mats that had been boiled for 30 minutes to kill the micro-organisms and purged with nitrogen:carbon dioxide gas, live mats in the simulated Archean atmosphere which were not exposed to photosynthetically active radiation and ambient atmosphere (21% oxygen). All experiments were repeated five times.

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