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Algal Evolution in Relation to Atmospheric CO_2: Carboxylases, Carbon Concentrating Mechanisms and Carbon Oxidation Cycles

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Abstract.

Oxygenic photosynthesis evolved at least 2.4 Ga ago; all oxygenic organisms use the ribulose bisphosphate carboxylase-oxygenase (Rubisco) - photosynthetic carbon reduction cycle rather than one of the five other known pathways of autotrophic CO_2 assimilation. The high CO_2 and (initially) O_2-free conditions permitted used of a Rubisco with a high maximum specific reaction rate. As CO_2 decreased and O_2 increased Rubisco oxygenase activity increased and 2-phosphoglycolate was produced, with evolution of pathways recycling this inhibitory product to sugar phosphates. Changed atmospheric composition also selected for Rubiscos with higher CO_2 affinity and CO_2/O_2 selectivity correlated with decreased CO_2-saturated catalytic capacity and/or for CO_2 concentrating mechanisms (CCMs). These changes increase the energy, nitrogen, phosphorus, iron, zinc and manganese cost of producing and operating Rubisco - PCRC while biosphere oxygenation decreased the availability of nitrogen, phosphorus and iron. The majority of algae today have CCMs; the timing of their origins is unclear. If CCMs evolved in a low CO_2 episode followed by one or more lengthy high CO_2 episodes, CCM retention could involve a combination of environmental factors known to favour CCM retention in extant organisms which also oocur in a warmer high-CO_2 ocean. More investigations, including studies of genetic adaptation, are needed.

Keywords CO_2 concentrating mechanism – combined nitrogen – inorganic carbon – iron – mixing depth – phosphorus – photorespiration - photosynthetically active radiation - Rubisco – temperature – UVA – UVB

Introduction

Algae are oxygenic photosynthetic organisms other than embryophytic plants, and by this definition include the cyanobacteria as well as a wide range of eukaryotic lineages. Cyanobacteria, as indicated by the occurrence of oxygenic photosynthesis, evolved at least 2.4 Ga ago, although fossil (including chemical biomarker) evidence for cyanobacteria does not go back beyond 2.1 Ga ago. [1]. Eukaryotic algae have occurred for not less than 1.2 Ga ago [2-4] and from freshwaters, and possibly lake margins, since 1.1 Ga ago [5] (Table 1). Since 2.4 Ga ago the biosphere has become increasingly oxygenated [1] reflecting the colonization of the oceans by cyanobacteria after their origin in freshwater habitats, with a corresponding increase in the capacity of these organisms to have global biogeochemical influence [6, 7] (Table 1). A significant increase in oxygen, with oxygenation of the deep oean, occurred in the Neoproterozoic 0.54 – 1 Ga ago [8] with variations in the Phanerozoic including the highest known level in the Permo-Carboniferous glaciation [9]. Carbon dioxide varied with a general downward trend with minima generally related to glaciations [10, 11]. CO_2 was relatively constant at about 23 (range of estimates 10 – 100) times the present level between 2.5 and 1.8 Ga ago, with a very significant decrease between 1.8 and 1.1 Ga ago [12], further variations in the Neoproterozoic [13-15] and relatively well established changes in Phanerozoic with minima in the Permo-Carboniferous and in the Pleistocene glaciations [9, 16]. In this article we consider how these environmental changes have influenced algal evolution, both through direct effects of the concentrations of CO_2 and O_2 on photosynthesis and related metabolism, and through indirect effects of changes in temperature (CO_2) and the availability of combined nitrogen, phosphorus and iron (CO_2 and O_2).

Autotrophic Carboxylases

Six pathways of autotrophic CO_2 fixation are known in extant organisms, including ribulose bisphosphate carboxylase-oxygenase carboxylase activity in the photosynthetic carbon reduction cycle (Rubisco – PCRC), using CO_2 as the inorganic carbon species assimilated, which is at the core of inorganic carbon assimilation in extant oxygenic photosynthetic organisms [17-22]. These pathways are summarised in Table 2 with respect to their stoichiometric requirement for reductant and ATP, their affinities for inorganic carbon expressed in terms of the half-saturation value for CO_2 and the influence of oxygen on their functioning.

Converting one CO_2 to the oxidation-reduction level of carbohydrates (CH_2O) requires four reducing equivalents at, or lower than, the midpoint redox potential of the $NADPH.NADP^+$ couple. The values given in Table 2 indicate this minimal stoichiometry, assuming no redox side reactions or futile cycles. An example of a side reaction for the Rubisco – PCRC pathway is the Rubisco oxygenase activity in the photosynthetic carbon oxidation cycles(s) (Rubisco-PCOC) which occurs at a relatively low $[CO_2]/[O_2]$ ratio. All of the autotrophic CO_2 fixation pathways also require ATP (Table 2), with a range of stoichiometries from one to three ATPs for CO_2 converted to carbohydrate. The Rubisco – PCRC pathway has the

equal highest energy cost of converting CO_2 to carbohydrate, with the further energy cost of the side-reaction of the Rubisco – PCOC at low $[CO_2]/[O_2]$ ratios.

The Rubisco – PCRC pathway seems more appropriate to CO_2 fixation at the present atmospheric CO_2 level when the half-saturation value for CO_2 is considered. The forms of Rubisco with the highest affinities for CO_2 (Form IB from some algae and vascular plants relying on CO_2 diffusion to Rubisco; Form ID from other algae) have half-saturation values for CO_2 almost as low as those of two other pathways, while the values for the other three pathways are considerably higher (Table 2). The final criterion in Table 1 is the effect of oxygen on the pathways. While the Rubisco – PCRC pathway is competitively (with CO_2) inhibited by O_2, the enzymes of this pathway are not damaged by O_2: some of the other pathways have one or more enzymes which are subject to irreversible inhibition by O_2 (Table 2). Not shown in Table 2, for lack of accurate information, is the resource cost of synthesising the enzymic machinery needed to fix one mole of CO_2 per second from the present atmospheric CO_2 concentration. This value is a function of the stoichiometry of the enzymic protein components in the pathway, with that of the carboxylase(s) set by their CO_2 affinity, and the M_r (relative molecular mass) values of the enzymes [17, 18]. Here the relatively low specific reaction rate and high M_r of Rubisco would tend to make the Rubisco – PCRC pathway expensive at present CO_2 concentrations, even without the O_2 effect, though some other pathways are probably at least as expensive as a result of the low CO_2 affinity of their carboxylases and the consequent large quantity of carboxylase needed to fix one mole of CO_2 per second from the present atmospheric CO_2 concentration.

The occurrence of Rubisco – PCRC as the core carboxylase in oxygenic photosynthetic organisms can be related to opportunity and functionality. By opportunity is meant the occurrence of the pathway at the time (about 2.4 Ga ago) at which the earliest evidence for oxygenic photosynthetic organisms is known. The Rubisco – PCRC pathway originated before oxygenic photosynthesis evolved (see below). By functionality is meant the carboxylase CO_2 affinity and carboxylase M_r values as determinants of the quantity of carboxylase (mass of protein) needed to fix one mole of CO_2 per second from the current atmosphere, the extent to which O_2 competitively inhibits or damages the enzymes of the pathway, and the ATP cost of the pathway per CO_2 assimilated (Table 2). The CO_2 affinity criterion apparently rules out three of the five pathways (two of which are also very oxygen-sensitive), leaving the 3-hydroxypropionate pathway and the Rubisco – PCRC pathway. While not oxygen inhibited in the manner found for Rubisco, the 3-hydroxypropionate pathway may be sufficiently O_2 sensitive to restrict its functionality in oxygenic organisms once O_2 had begun to accumulate in the part of the biosphere occupied by oxygenic photosynthetic organisms (Tables 1 and 2). In such ways can the role of the Rubisco – PCRC pathway in all known oxygenic photosynthetic organisms be rationalised.

Rubisco carboxylase activity and the PCRC

Rubisco evolved before the origin of oxygenic photosynthesis [23-25]. The Rubisco gene family not only contains the Forms I, II and III Rubiscos which catalyse the Rubisco

carboxylase and Rubisco oxygenase reactions, but also the Form IV Rubisco-Like Protein (RLP) which does not catalyse the typical Rubisco reactions and which is involved in methionine salvage in some Bacteria [23-25]. Molecular phylogenetic analysis [23-25] suggests that an ancestral Form III Rubisco arose in a methanogen (i.e. a member of the Archaea) and gave rise, by two vertical transmissions, to all other Form III Rubiscos and to Form IV. Horizontal gene transfer then moved Form III and Form IV Rubiscos to an ancestral bacterium where Form III gave rise to the ancestors of Forms I and II, and hence by vertical transmission to the Form IV RLP of Bacilli and to Forms I, II and IV of an ancestral proteobacterium.

Vertical descent and further diversification gave rise to the Forms IA, IC, ID and II, and the Form IV RLP in extant Proteobacteria. Horizontal gene transfer from the Proteobacteria transferred Form IA to cyanobacteria where Form IB evolved from Form IA [23-26]. However, there is also evidence that extant α-cyanobacteria with Form 1A Rubisco (α-cyanobacteria) acquired their Form IA Rubisco (and other α-carboxysomal proteins) from a proteobacterium, displacing the Form IB Rubisco and associated β-carboxysomal proteins [27-32]. Endosymbiosis of a heterocystous β-cyanobacterium [33] gave rise to the plastids of the Archaeoplastida (= Plantae) where the Form IB Rubisco of the endosymbiont was retained by glaucocystophyte and green algae and hence by vertical transfer to embryophytic ('higher') plants, and was moved by secondary endosymbiosis to the (rhizarian) chlorarachniophytes and the (excavate-discicristate) euglenoids. Horizontal gene transfer from a proteobacterium accounts for the presence of Form ID Rubisco in red algae and in the chromistan algae (ochrista or heterokontophytes, cryptophytes and haptophytes) whose plastids arose by secondary endosymbiosis of a red alga. The peridinin-containing dinoflagellate and basal apicomplexans (represented today by the photosynthentically competent *Chromera velia* and an as yet un-named close relative) also obtained their plastids by secondary endosymbiosis from a red alga, but horizontal transfer of Form II Rubisco from a proteobacterium replaced the Form ID Rubisco [34]. Other dinoflagellates have other forms of Rubisco as a result of tertiary endosymbioses. Finally, a second primary endosymbiosis involving an α-cyanobacterium with Form IA Rubisco accounts for the presence of plastids and Form IA Rubisco in the (rhizarian) euglyphid amoeba *Paulinella* [30, 31].

Some of the enzymes of the PCRC of vascular plants are derived from cyanobacteria in the primary endosymbiosis leading to the Archaeoplastida (= Plantae), while others are of host origin [35]. This work has now been extended to include the glaucocystophyte and red algal members of the Archaeoplastida [36] and the diatoms whose plastids arose from secondary endosymbiosis of a red alga [37] following an earlier secondary endosymbiosis of a green alga [38]. There are also differences among algae in the regulation of enzymes of the PCRC [39]. Further work is needed to establish the relevance, if any, of atmospheric changes to

these differences in the regulation of enzymes of the PCRC [39, 40], and also the absence of Rubisco activase from algae with the Form ID Rubisco in the few cases examined [37].

It is likely that the earliest Rubiscos in chemolithotrophic and anoxygenic photosynthetic Archaea and Bacteria were operating in a high CO_2 environment and, because there was no oxygenic photosynthesis, in the essential absence of O_2. Such an environment would have provided little or no selective pressure for a high CO_2 affinity or a high CO_2/O_2 selectivity relative to a high CO_2-saturated maximum catalytic rate, using the mechanistic arguments of [41] that a high maximum catalytic rate is incompatible with high CO_2 affinity and higher CO_2/O_2 selectivity.

The early organisms using Rubisco as a carboxylase would, then, be able to use diffusive entry of CO_2, with none of the resource costs associated with Rubisco operating at below the CO_2-saturated rate in the presence of O_2 when Rubisco oxygenase activity is expressed. The additional resource costs can be considered as capital (synthetic) costs and running costs. The capital costs are those of synthesising additional Rubisco enzyme per cell if the per cell rate of CO_2 assimilation is to be retained, as well as the costs of making the enzymes and transporters related to the operation of a PCOC and/or a CO_2 concentrating mechanism (CCM), and include energy, carbon and nitrogen as well as phosphorus for any additional riboisomes that are required [42]. The running costs are in terms of energy, both for synthesising 2-phosphoglycolate and metabolising it back to sugar through a PCOC and for operating a CCM.

The early Rubisco-containing organisms could still permit CO_2 saturation of a high specific reaction rate Rubisco, with corresponding savings of resources in constructing the enzymic machinery able to assimilate one mole of CO_2 per second. In other words, the conditions described would give the lowest possible energy, carbon and nitrogen costs of producing the amount of Rubisco capable of fixing one mole of CO_2 per second, and also give the lowest possible energy cost of operating Rubisco, i.e. 2 NADPH and 3 ATP per CO_2 converted to carbohydrate. A correlated saving associated with this minimal requirement for NADPH and ATP in autotrophic CO_2 assimilation is in the quantity of redox and ATP synthesis machinery needed for NADPH and ATP production. Minimizing the protein requirements for autotrophy also decreases the phosphorus requirement for mRNA in ribosomes and in tRNA and mRNA needed to produce the Rubisco and associated enzymes. These characteristics would minimize the nitrogen needed to produce the machinery associated with a given rate of CO_2 fixation, and thus the phosphorus in RNA needed to synthesise the proteins [42]. Costs in energy, nitrogen, phosphorus, iron and manganese will be considered below in the context of decreasing CO_2 and increasing O_2 [42-50].

The mechanistically constrained co-variation in Rubisco kinetics mentioned above, i.e. a high CO_2-saturated specific reaction rate correlated with low CO_2 affinity and CO_2/O_2 selectivity and vice versa [41] has parallels with the growth strategies of vascular plants [51] and of algae [52] according to the Competitive – Stress-Tolerant –Ruderal (CSR) paradigm, for which there are also mechanistic bases in terms of having high rates of metabolism and

195 growth in ruderals and lower rates but perhaps a more effective use of resources in stress-
196 tolerators [53-55].

197 **Oxygen accumulation, Rubisco oxygenase and the metabolism of phosphoglycolate**

198 The build-up of O_2 has had many effects on algal evolution, permitting respiration and, via
199 the occurrence of stratospheric ozone, a decreased UVB flux, and production of reactive
200 oxygen species from O_2 rather than UVB action on cell constituents [55, 56]. The presence of
201 O_2 does not, however, inhibit O_2 production by oxygenic photosynthetic organisms [57, 58].
202 The accumulation of O_2 in the habitats of oxygenic photosynthetic organisms using Rubisco
203 as their autotrophic carboxylase permits Rubisco oxygenase activity to occur, provided the
204 CO_2 concentration is below saturation for Rubisco. Such decreased CO_2 concentrations,
205 combined with at least local O_2 accumulation, probably occurred about 2 Ga ago, when there
206 is evidence of an ice age extending to low palaeolatitudes [12, 59].

207 The product of the Rubisco oxygenase activity is, as well as one 3-phosphoglycerate per O_2
208 consumed, one 2-phosphoglycolate. In addition to sequestering the often limiting resource
209 phosphorus if 2-phosphoglycolate continues to accumulate, 2-phosphoglycolate is also an
210 inhibitor of some reactions involving phosphate esters, including some in the PCRC [60].
211 Accordingly, all organisms using Rubisco in the presence of O_2, i.e. oxygenic
212 photolithotrophs and some chemolithotrophs, have 2-phosphoglycolate phosphatase [60, 61].
213 This enzyme would not have been needed to deal with 2-phosphoglycolate from Rubisco
214 oxygenase activity in anoxygenic photosynthetic proteobacteria, or in any anaerobic
215 chemolithtrophs, using the Rubisco-PRCR pathway before build-up of O_2 in the biosphere. 2-
216 phosphoglycolate phosphatase also occurs in some non-autotrophic bacteria which have no
217 Rubisco, where it is thought to be involved in some forms of DNA repair [62, 63]. Since
218 DNA damage and its repair must have occurred before oxygenic photosynthesis, the 2-
219 phosphoglycolate phosphatase in oxygenic photosynthetic organisms could have been
220 recruited from bacteria lacking autotrophic 2-phosphoglycolate synthesis. However, the 2-
221 phosphoglycolate phosphatase from eukaryotes does not seem to have been derived from the
222 2-phospho-glycolate phosphatise of cyanobacteria [64].

223 The organic carbon product of 2-phosphoglycolate phosphatase is glycolate. This can be
224 excreted, with loss from the organism of the energy and carbon used in its synthesis.
225 Alternatively, glycolate can be salvaged by metabolism to 3-phosphoglycerate, and hence
226 triose phosphate, which occur in the PCRC, albeit with the input of energy and the release of
227 CO_2 [61]. The cyanobacteria have two variants on pathways converting glycolate to 3-
228 phosphoglycerate.

229 One means of converting glycolate to 3-phosphoglycerate is the pathway via glycine and
230 serine, with recycling of ammonia, as in the classic PCOC of embryophytic plants and at least
231 some eukaryotic algae [61, 64-68]. The pathway through glycine and serine seems to have
232 been gained by eukaryotic photosynthetic organisms during the primary endosymbiosis
233 yielding the plastids of the Archaeplastida (= Plantae), although some of the genes in
234 eukaryotes came from α-proteobacteria rather than cyanobacteria [64]. The β-cyanobacterial

plastids ancestor gave rise to the glycolate oxidase, glycerate kinase and hydroxypyruvate reductase of algae and embryophytes, while serine hydroxymethyl transferase and the L, P and T subunits of glycine decarboxylase came from α-proteobacteria by horizontal gene transfer [64]. The origin of the other eukaryotic PCOC genes, i.e. those encoding the H subunit of glycine decarboxylase, glutamate-glyoxylate aminotransferase and serine-glyoxylate aminotransferase, has not yet been extablished [64].

The other pathway from glycolate to 3-phosphoglycerate involves tartronic semialdehyde, and is called the tartronic semialdehyde pathway by phycologists; bacteriologists call it the glycerate pathway, even though glycerate is also an intermediate of the PCOC [61, 64-66]. Parts of the PCOC (glycerate dehydrogenase, serine transminases, serine hydroxymethyltransferase) could have been recruited from core metabolism synthesising serine and glycine from glycolytic intermediates, while others (glycolate dehydrogenase/oxidase, glycine decarboxylase, glycerate kinase) have no known roles other than in the metabolism of glycolate to PCRC intermediates [69, 70].

It seems likely that at least one of the metabolic pathways from glycolate to 3-phosphoglycerate evolved in oxygenic photosynthetic organisms relying on diffusive CO_2 entry before CCMs evolved. Not only is there at least a minimal flux through Rubisco oxygenase and thence to intermediates of a glycolate metabolism pathway despite high levels of expression of CCMs [61, 65, 66, 68, 71], but elimination of the pathways of glycolate metabolism is fatal to the organism [61, 66]. Previous misgivings [67, 68] about the occurrence of the complete PCOC in diatoms have now been largely overcome, although there are still doubts as to the glycerate kinase step [37, 64].

The changes to CO_2 fixation in oxygenic photolithotrophs in relation to decreasing CO_2 and, especially, increasing O_2 is part of a wider range of resource cost increases as the biosphere becomes oxygenated. Falkowski and Godfrey [48] point out that not only is Rubisco impacted by increasing O_2 with decreasing CO_2, but that the potential for oxygen damage to nitrogenase becomes manifest, and the very source of the O_2, the reaction centre of photosystem II, is itself subject to photodamage both directly through excitation energy transfer to the reaction centre but also indirectly through the accumulated O_2 forming reactive O_2 species (see [42]). As Raven [42, 44-46] points out, the effects on Rubisco demand additional nitrogen in the enzyme itself and in related enzymes, more iron and manganese in additional thylakoid redox agents, and more phosphorus in the RNA needed to make the additional protein if the rate of photosynthesis is to be maintained. More energy input as NADPH and ATP is also needed to run CO_2 assimilation [42, 45, 46]. For oxygen damage to nitrogenase, there is generally synthesis of 'reserve' reserve nitrogenase in addition to what is needed in the absence of oxygen damage to satisfy the combined nitrogen requirements of cell growth. Synthesis of the 'reserve' nitrogenase requires the nitrogen and energy needed for the synthesis of any protein, but also the iron and (in almost all cases) molybdenum used in the nitrogenise cofactors. When oxygen damage does occur and reserve nitrogenase is used catalytically, more energy (but not nitrogen, iron and molybdenum) is needed to synthesise nitrogenase to replace what is damaged. In both cases, the production of more nitrogenase

than would be required in the absence of oxygen involves the use of more phosphorus for the RNA required for the additional protein synthesis. In the case of photoinhibition, more nitrogen and energy is needed to synthesise reserve photosystem II reaction centres, more energy is needed to synthesise replacement photosystem II reaction centres, and more phosphorus is needed for the RNAs needed for the extra protein synthesis. A further aspect of damage to proteins by O_2 concerns the absence of any core autotrophic CO_2 assimilation pathway other than Rubisco – PCRC from oxygenic photosynthetic organisms. In addition to the CO_2 affinity problems outlined for some of the alternative pathways, there would also be the requirement for additional resources (PAR, nitrogen and phosphorus) to make and use additional RNA needed for the additional resynthesis of O_2-damaged protein (see [42] and above, for other cases).

Compounding this need for additional nitrogen and phosphorus per unit CO_2 or N_2 assimilated and photons used in photochemistry is the effect of increased O_2 on the availability of nitrogen and phosphorus. Falkowski and Godfrey ([48]) point out that oxygenation of the biosphere not only decreases the potential for diazotrophy, but allows nitrifying microbes to convert NH_4^+ to NO_3^-, which in hypoxic or anoxic microhabitats can be denitrified to produce N_2O and N_2. This nitrification – denitrification sequence decreases the availability of combined nitrogen to non-diazotrophic primary producers. In the case of phosphorus, the availability of O_2 converts Fe(II) to oxidised iron (Fe(III), which binds phosphate and thus decreases global phosphorus availability [72, 73]. Phosphorus is one of the biogeochemical regulators of the O_2 content of the atmosphere [72, 73]. This topic will be returned to below in the context of ocean deoxygenation as a function of increases CO_2 and temperature.

Introduction to CCMs

Diffusive entry of CO_2 to Rubisco was presumably the ancestral mechanism of autotrophic CO_2 assimilation in oxygenic photosynthetic organisms. Entry of CO_2 to Rubisco by diffusion is found today in the majority, by species number and contribution to global primary productivity, of terrestrial oxygenic photosynthetic organisms, but in a minority of oxygenic photosynthetic organisms in aquatic environments where photolithotrophs with CCMs predominate [10, 43, 55, 74-80]: see Table 1. The references just cited show that CCMs are very widely distributed among algae, both phylogenetically and geographically, although they seem to be absent from chrysophycean and synurophycean algae [81]. The mechanistic, including molecular, details of the CCMs of cyanobacteria are now known [27-29, 32, 82]. The CCMs of eukaryotic algae are less clearly understood at both the molecular and the mechanistic levels, although they are clearly polyphyletic[10, 11, 43, 55, 80, 83, 84].

As to the evolutionary origin of CCMs, the selective factors were presumably decreasing CO_2 and increasing O_2. The variability of other gases over the last 2.4 Ga suggests there were several periods at which CCMs could have been resource-effective (energy, nitrogen, phosphorus, iron, zinc, manganese) alternatives to diffusive CO_2 entry to Rubisco with attendant high activity of Rubisco oxygenase and expression of high levels of enzymes

converting 2-phosphoglycolate to sugar phosphate [11, 43-47, 50, 85, 86]. We shall return to the question of the timing(s) of the origin(s) of CCMs and how, if they originated early, they were retained through intervening high-CO_2 episodes. First, we consider what the transition from diffusive CO_2 entry to CCM-based delivery of CO_2 to Rubisco involves.

The functioning of CCMs in comparison with the diffusive entry of CO_2

One factor in the evolution of a CCM in an organism previously relying on diffusive CO_2 movement from the medium to Rubisco as CO_2 availability decreases is the kinetics of the Rubisco used by the organism. A Rubisco with a high CO_2-saturated catalytic capacity, and a correspondingly low CO_2 affinity and CO_2/O_2 selectivity, such as occurs in the Form IA and Form IB Rubiscos of extant cyanobacteria, would be expected to present a stronger evolutionary case for a CCM at a given CO_2 concentration and CO_2/O_2 ratio than would eukaryotic Form IB and Form ID Rubisco with higher CO_2 affinity and CO_2/O_2 selectivity but a lower CO_2-saturated maximum catalytic rate [41]. Young et al. ([87]) have used molecular phylogenetic evidence to show that there was positive selection of the Form ID Rubiscos of in some eukaryotes which correspond to low CO_2 and low CO_2/O_2 episodes in the geological record, and that these episodes of positeive election could have corresponded to the time of evolution of CCMs. To be effective, the CCM must maintain a higher CO_2 concentration at the site of Rubisco than would be possible by CO_2 diffusion alone [11].

The essential component of a CCM is the accumulation of CO_2 in the compartment containing Rubisco to a higher steady-state concentration than occurs in the growth medium, and hence even higher than the steady-state concentration near Rubisco which could occur with diffusive CO_2 entry. For algae, one mechanism of accumulation could involve C_4-like photosynthetic metabolism with an ATP-dependent ($C_3 + C_1$) carboxylation in the cytosol, using HCO_3^- obtained directly or indirectly (via CO_2 entry from the medium followed by carbonic anhydrase catalysis) from the medium, followed by a (C_4-C_1) decarboxylation in the chloroplast [84]. The alternative mechanisms do not involve the inorganic carbon transferred from the medium to Rubisco forming organic intermediates. These alternative mechanisms involve transmembrane active transport mechanisms which move an inorganic carbon species (CO_2 or HCO_3^-), or H^+, against a free energy gradient. Such transporters could be (and have been) derived from transporter gene families by change of specificity of the transported substrate (to CO_2 or HCO_3^-), with changes in regulation and, perhaps, changes in intracellular targeting [27-29, 32]. An exception to the need for active transport across a membrane is CO_2 use in the cyanobacterial CCMs, where diffusive CO_2 entry across the plasmalemma is followed by energized conversion to HCO_3^- by the NAD(P)H – PQ oxidoreductase of the thylakoid membrane [27-29, 32]. Here a high CO_2 permeability of the plasmalemma is required. Such a high membrane permeability to CO_2 is needed for diffusive CO_2 entry all the way to Rubisco in organisms lacking a CCM, and (as noted above) in CO_2 'active transport' in cyanobacteria. A high CO_2 permeability is also necessary in organisms with a CCM mechanism involving CO_2 production from HCO_3^- in a compartment acidified by a H^+ pump followed by transmembrane movement of CO_2 into the compartment containing Rubisco [80]. The energized conversion of CO_2 to HCO_3^- in the cyanobacterial cytosol could increase

the chance of any CO_2 that leaks out of the carboxysomes being trapped as HCO_3^- in the cytosol. In all other cases a CCM is most energetically efficient with minimal CO_2 flux from the compartment in which it is accumulated back to the medium, i.e. with very low membrane permeabilities to CO_2.

The energetic savings that could result from a low CO_2 permeability of the plasmalemma, and of the inner plastid envelope (if that is the membrane involved in active transport of inorganic carbon), in eukaryotes with CCMs based on active transport of an inorganic carbon species would, if verified, suggest a phylogenetic, and in many cases an acclimatory (changes from growth in high to low CO_2) decrease in CO_2 permeability. This question has been addressed by a number of workers (e.g. [83, 88]), who found relatively high CO_2 permeability in the eukaryotic algal membranes examined, regardless of whether the algae had been cultured in high (CCM repressed) or low (CCM expressed) inorganic carbon concentrations. The permeability values for CO_2 of the plasmalemma of high-CO_2 and low-CO_2-grown cells of *Chlamydomonas reinhardtii* range from $0.76 – 1.8.10^{-3}$ cm s^{-1} [88], while for four species of diatom the range is $15 – 56.10^{-3}$ cm s^{-1} [83], with the range of values probably related to methodological as well as phylogenetic differences. While models for CCMs in diatoms consistent with the available data show relatively modest energy losses during CCM operation, they do involve constraints such as the membrane(s) at which active inorganic carbon transport occurs, and the chemical species involved in this active transport [83]. The same argument applies to the protein shell of the carboxysome, for which a restriction on CO_2 diffusion, but not on the diffusion of anionic substrates ($HCO_3^-$and ribulose-1,5-bisphosphate) and product (3-phosphoglycerate) has been demonstrated [89].

Most CCMs also involve one or more carbonic anhydrase enzymes: an exception is a C_4 mechanism which involves, as indicated above, HCO_3^- entry to the cytosol, ($C_3 + C_1$) carboxylation using HCO_3^-, and ($C_4 – C_1$) decarboxylation in the plastid stroma with CO_2 as the inorganic carbon product [68, 71, 80, 84, 90]. All other well-investigated CCMs seem to involve 'normal' carbonic anhydrases, i.e. those catalysing the equilibration of CO_2 and HCO_3^- [43, 80, 91]: this is the case for 'active CO_2 influx' in cyanobacteria which involves a carbonic anhydrase in the carboxysome as well as the energized conversion of CO_2 to HCO_3^- at the thylakoid membrane, which is effectively a unidirectional carbonic anhydrase [32].

The functioning of CCMs is influenced by a number of factors other than the availability of inorganic carbon (and, in some cases, O_2), e.g. photosynthetically active radiation, UV-B radiation, the form and concentration of combined nitrogen, the phosphate concentration and the iron concentration [42, 43, 50, 85]. The influence of these factors on the expression and functioning of CCMs is presented in Table 3 [42, 43, 50]. There are also predicted effects of expression of CCMs rather than reliance on diffusive entry of CO_2 on the resource costs of synthesis of the photosynthetic apparatus, and of its operation; these are discussed below. We next discuss the possible influence of these interactions on the evolution of CCMs, their retention through any high-CO_2 episodes between their origin in a low CO_2 habitat and today, and their fate in a future higher CO_2 and warmer world.

The origins of CCMs.

The 'why' of the origin of CCMs presumably concerns the occurrence of low CO_2, both in absolute terms and in relation to O_2, which was indicated above as requiring additional protein (hence RNA and phosphorus) in more Rubisco and in enzymes metabolising 2-phosphoglycolate to sugar phosphate, as well as additional energy input per net CO_2 assimilated by diffusive CO_2 entry of CO_2 to Rubisco and metabolism of 2-phosphoglyclate. Depending on the circumstances, e.g. the form of Rubisco present and the environmental conditions as well as the mechanism of the particular CCM [10, 11, 44-47, 50, 55, 67, 83, 85], a CCM could require less energy, nitrogen, phosphorus, zinc and iron for its synthesis, and less energy for its operation, than diffusive CO_2 entry with metabolism of 2-phosphoglycolate (Table 2). Other factors that could have influenced the evolution of CCMs include the decreasing UV-B flux with the increased stratospheric O_3 resulting from the build up of O_2, which is itself an influence on the evolution of CCMs [55]. UV-B radiation causes damage to Rubisco and to Photosystem II, but has less effect on Photosystem I. The limited data available suggest that UV-B has little effect on CCM activity in the green alga *Dunaliella tertiolecta* [92] but elevated CO_2 can increase the sensitivity of microalgae to UV-B [93, 94]. There is no information about the possibility of a differential impact of UV-B on the various forms of Rubisco, though it would be interesting to know if changes in UV-B in the past relate to the evolution of different Rubiscos.

The 'how' of the origin of CCMs concerns the ancestry of the various components of the pathway. For the active transport components H^+ pumps are ubiquitous and anion transporters/pumps (hence HCO_3^- transporters/pumps) are also widespread, as could be the ancestors of CO_2 pumps [10, 11, 27-29, 32, 55, 80, 82, 84]. Facilitators of downhill transmembrane CO_2 transport, yielding permeabilities in excess of those due to the lipid phase alone, are required for cyanobacterial active transporters and for the mechanism involving an acidified compartment generating CO_2 from HCO_3^- with subsequent transmembrane CO_2 diffusion to Rubisco. Such facilitators would presumably have originally been components of the diffusive pathway for CO_2 from the medium to Rubisco. Carbonic anhydrases could have had a number of roles prior to their co-option into CCMs, including that of facilitating diffusion of CO_2 (as HCO_3^-) in the diffusive entry of CO_2 from the medium to Rubisco. Cyanobacterial carboxysomes are part of a larger family of bacterial micro-compartments [95]. This brief view may be over-optimistic as to the ease of co-opting existing mechanisms into CCMs [10], and does not address the origin of the eukaryotic pyrenoid [96]; however, it does indicate some possibilities.

There are a number of options as to the 'when' of the evolution of CCMs. We initially consider times of relatively low CO_2, based on low palaeotemperatures (with the requirement that greenhouse gases corrected for the faint young sun) or on biogeochemical or biological proxies. Glacial/low CO_2 episodes occurred 2.4-2.1 Ga ago and at 750, 650 and 320-270 Ma ago, as well as the Pleistocene (last 2.4 Ma) years [11]. All but the earliest of these times would have been relevant to at least some of the eukaryotic as well as cyanobacterial oxygenic photosynthetic organisms (Table 1).There is no direct fossil evidence as to the

origin of CCMs, and little help from molecular clocks [11, 43, 55], although recent work by Young et al. ([87]) shows episodes of positive selection of Form ID Rubisco in diatoms and haptophyters which correspond to low CO_2 episodes and hence possibly relate to the origin of CCMs. Assuming, as seems very likely, that cyanobacterial and at least some algal CCMs evolved before the Pleistocene, they would have had to have survived intervening period(s) of higher CO_2 and higher temperatures. The mechanisms of retention of CCMs in these apparently unfavourable environments is now considered in the context of the response of present day CCMs to such environments.

Retention of CCMs in high CO_2 episodes

It is possible to argue for long-lasting low-CO_2 micro-habitats, e.g. in benthic microbial mats, including stromatolites, where inorganic carbon diffusion from the bulk medium into the cells is restricted by thick diffusion boundary layers [55]. Biogeochemically more important are planktonic habitats where such low CO_2 refuges are less plausible. CCM retention is considered in the context of present work on the response of phytoplankton to current and expected environmental change, and especially increasing CO_2 and the associated warming.

More widespread (planktonic) retention of CCMs in relatively high CO_2 concentrations has been argued by [11] in the context of what might happen with increasing CO_2 and temperature over the next several decades. The argument here is that the increased buoyancy of warmer surface waters will lead to a shoaling of the thermocline which will decrease fluxes of combined nitrogen and of phosphorus from the deeper ocean where mineralisation of sinking particulate organic matter occurs which have been modelled as decreasing global marine primary productivity [97-99]. This decreased nutrient supply, with the increased mean flux of photosynthetically active radiation (and UV-B) incident on the cells in the shallower upper mixed layer will, on the basis of observations on extant phytoplankton (Table 3), favour retention of CCMs despite higher CO_2 concentrations [11].

A further relevant consideration which was not mentioned by Raven et al. ([11]) is that a warmer upper mixed layer has decreased oxygen solubility. For the same rate of net oxygen production at two temperatures there will be greater degree of oxygen superaturation and hence a greater loss of oxygen to the atmosphere at the higher temperature. Together with the decreased solute transfer between the upper mixed layer and deeper ocean there is less transfer of oxygen below the thermocline [100-102]. The widespread, but not universal, decrease in calcification by calcified plankton [103-105], and the much smaller effect on silicification by diatoms [106] in a higher-CO_2, warmer ocean means less ballasting of sinking particulate organic matter, hence slower sinking and more microbial mineralisation just below the thermocline ([107] cf. [108]). While this higher nutrient concentration just below the thermocline might be expected to partly offset the lower rate constant for nutrient transfer to the upper mixed layer, another factor must be considered. The combination of more microbial respiration and increased oxygen supply can lead to hypoxia and even anoxia in certain sub-surface waters, with implications for loss of the nitrate and nitrite forms of combined nitrogen produced from organic matter by mineralisation and nitrification in less

deoxygenated zones followed by denitrification or the anammox reaction in more deoxygenated places [102], with a decrease in the nitrogen:phosphorus ratio in the nutrients reaching the upper mixed layer. Although, in the long term (thousands of years and more), a warmer world would heat the ocean interior as well as the upper mixed layer and potentially decrease the extent to which the thermocline shoals, there is a well-established correlation of wide-spread deep-ocean anoxia (and even euxinia) with warmer, high CO_2 episodes in Earth history [109], so at least a decreased upward flux of combined nitrogen across the thermocline would continue, favouring retention of CCMs.

These arguments are based on the response of extant algae to the changes occurring, and predicted, in their environment as a result of increased CO_2 and temperature, with downstream effects on the marine and inland water inorganic system, the mixed layer depth and water body oxygenation. This can be used to inform us of how CCMs were retained in past episodes of high CO_2. Dealing first with the organisms studied, almost all of the work has been carried out with organisms which have only been exposed to the increased CO_2 and associated changes in temperature and the availability of other resources (Table 3) for time periods of days to weeks. This time period allows 1 – 100 generation, meaning that only regulatory (altering the existing proteome by post-translational modification, and changes in the metabolome) and acclimatory (altering the expressed proteome based on the existing genome) [110] responses of extant algae can be expressed. In very few cases has relevant evolutionary evidence been sought in laboratory experiments for increased CO_2 [110-117] and, using different methods, higher temperatures [118]. An example of where evolution has been unable to cope with natural CO_2 enrichment present for several decades concerns calcified red algae growing on seagrass leaves near an underwater vent in the Mediterranean [119]. Even for studies of regulation and acclimation there can be problems with the length of time that an alga has been in culture and frequently exposed to CO_2, nitrogen, phosphorus, PAR, UV and temperature which has little relevance to their natural environment [120]. Furthermore, there is a relative lack of studies in which changes in CO_2 have been combined with other relevant environmental changes: Table 3 and [11] analyse, and give references to the important work in which such interactions have been studied. Finally, there are some relatively poorly constrained factors of ocean chemistry in the past [56, 121-125], and until recently little consensus as to the appropriate methodology [105, 126-129] for laboratory and mesocosm experimentation on increased CO_2.

Despite these reservations, which also apply to other models of past and future atmosphere – ocean – organism interactions, the suggestions of Raven et al. ([11]) provide a lead into further studies in how CCMs could be retained in lengthy episodes between shorter low-CO_2 episodes. Any retention of CCMs in high CO_2 episodes would be a further complication in the use of stable carbon isotope ratios of phytoplankton-derived organic carbon from marine sediments as a palaobarometer for CO_2, since most marine phytoplankton today have CCMs [43, 74, 130, 131]. Organisms lacking CCMs, e.g. terrestrial liverworts and mosses, do not suffer from this problem when used in palaeobarometry of CO_2 [132].

Conclusions

519 The changing CO_2 and O_2 concentration over the last 2.4 Ga have had significant effects on
520 the physiology and ecology of cyanobacteria and algae. From the presumed ancestral
521 diffusive CO_2 entry to Rubisco all extant cyanobacteria have CCMs, Rubiscos with high CO_2
522 saturated catalytic activity and low CO_2 affinity and CO_2/O_2 selectivity, and an essential role
523 for the capacity to convert the 2-phosphoglycolate formed as a very small fraction of the total
524 carbon flux into triose phosphates. Most eukaryotic algae have CCMs: a greater fraction have
525 CCMs in the sea than in freshwaters, and there is no strong relationship to water
526 temperatures. The evolution of CCMs can apparently be related to decreased CO_2 availability
527 and to the presence of oxygen, modulated by the kinetics of the form of Rubisco in the
528 organisms, with some components of the CCMs adapted in evolution from the roles in other
529 pathways. The retention of CCMs during the high CO_2 episodes predominant through Earth
530 history could have been related in part to the interaction of CCM expression with other
531 environmental factors which change in high CO_2 water bodies.

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1000 **Table 1**

1001 Inorganic carbon acquisition characteristics of cyanobacteria and algae related to earliest
1002 known occurrence of the taxon[1,2]

Taxon	Occurrence of CCM in Extant organisms	Oldest Known fossil	References 1003
Cyanobacteria	CCM ubiquitous	2.15 Ga (bio-markers). 2.45 Ga (O_2)	[1, 6, 7, 133]
Chlorophyta: Prasinophyceae	CCM ubiquitous	1.3Ga	[134, 135]
Chlorophyta: Chlorophyceae	CCM present in all?	(450 Ma)[32]	[135, 136]
Chlorophyta: Trebouxio-phyceae	CCM present or absent	450 Ma	[135-139]
Chlorophyta: Ulvophyceae	CCM usually present; absent in some; C_4 in one	540 Ma	[135, 136, 140]
Streptophyta: Charophyceae	CCM present in all?	450 Ma	[136, 140]
Streptophyta: Embryphyres	CCM usually absent; pyrenoid-based CCM is some anthocerophytes, C_4 or CAM in some freshwater tracheophytes, CCMs not involving C_4 and CAM in some freshwater and all marine tracheophytes	475 Ma	[140-142]
Rhodophyta: Bangiophyceae	CCM in all?	1.2 Ga	[2]
Rhodophyta: Florideophyceae	CCM in many, absent from some marine, many freshwater	600 Ma	[143, 144]
Ochrista: Bacillariophyceae	CCM in all?	120 Ma	[145, 146]
Ochrista: Fucophyceae	CCM in all?	(570 Ma?)	[144, 147]
Ochrista: Tribophyceae	CCM in all?	600 Ma	[3, 148]
Ochrista: Chrysophyceae and Synurophyceae	CCM absent in all	?	[81]

1004

1005 Footnotes

1006 [1]General referencess on earliest known occurrence of algae: [140, 149, 150]

1007 [2]Refences on presence or absence of CCMs: [11, 78-81, 96]

1008 [3]Based on the finding of Trebouxiophyceae in the Ordovician, and branching order of the Chlorophyceae, Trebouxiophyceae and
1009 Ulvophyceae from molecular phylogenetics [136].

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Table 2

Energy (NADPH and ATP) stoichiometry, affinity for inorganic carbon expressed as the half-saturation concentration for CO_2, competitive inhibition by O_2 and damage by O_2 for six autotrophic inorganic carbon assimilation pathways. Based on [17-22].

Pathway from inorganic carbon to carbohydrate	NAD(P)H per CO_2	ATP Per CO_2	$K_{1/2}$ CO_2 mmol m^{-3}	O_2 competitive inhibition	O_2 damage to one or more enzymes
Rubisco-PCRC, saturating CO_2 no O_2	2	3	≥10	Yes	No
Reverse TCAC	2	1.67	>1,500	No	Yes[1]
3-HO-Propionate	2	2	10	No	No[2]
3-HO-Propionate-4-HO-Butyrate	2	3	>2,000	No	O_2-insensitive pathway in Some organisms living in microaerobic habitats
Dicarboxylate-4-HO-Butyrate	2	2.67	>2,000	No	No; some organisms live in microaerophilic habitats
Wood-Ljungdahl pathway	2	1	40,000[2]	No	Yes

Footnotes

[1]The reverse TCAC can occur in thermophilic aerobic chemolithotrophs as a result of low O_2 solubility and high respiratory rates maintaining a low O_2 concentration inside the cells, and expression of an O_2-insensitive version of the 2-oxoglutarate-ferredoxin oxidoreductase which has t least a five-fold lower specific activity than the O_2-sensitive enzyme [20].

[2]The most oxygen-sensitive enzyme, methylmalonyol-CoA mutase, can be assayed and even purified at atomospheric equilibrium O_2 concentrations, it may not be sufficient O_2-tolerant to function in illuminated cells of oxygenic photosynthetic organisms [20].

[3]Although acetogens live in habitats with higher CO_2 concentrations than correspond to atmospheric equilibrium [151], the *in vitro* $K_{1/2}$ value cited does seem very high. .

Table 3

Influence of environmental conditions on the expression of CCMs and the resource cost of CCM operation versus diffusive entry of CO_2

Factor	Change to algal environment caused by variation in the factor	Effects on expression of CCMs and on their affinity for CO_2	Predicted effect of CCM expression on resources costs of synthesis and (for PAR) operation of the photosynthetic apparatus
CO_2	Increase in CO_2 in essentially all environments, although less predictable effect in freshwaters which can be out of equilibrium with the atmosphere	Decreased inorganic carbon affinity with growth at high CO_2; can be a switch to diffusive CO_2 entry in some eukaryotes	No general effect on carbon cost of synthesis of the photosynthetic apparatus
Temper-ature	Increase in temperature in all environments	Prediction of increased CCM expression, orincreased fraction of organisms with CCMs, at higher temperatures as a result of lower CO_2 solubility, is not uniformly supported by the available data.	Inapplicable: temperature (Boltzmann energy) is not resource consumed in the synthesis of the photosynthetic apparatus, in its operation or in its maintenance
PAR	Increase in PAR in pelagic planktonic environments	Decreased inorganic carbonaffinity with growth at low PAR	Possible decreased energy needed in synthesis of photo-synthetic apparatus which uses a CCM. Energy saving in operation of a CCM if there is low CO_2 leakage and a low CO_2 affinity and lowCO_2:O_2 selectivity
Nitrogen	Decrease in combined nitrogen in upper mixed layer of lotic environments	Generally increased inorganic carbon affinity with growth at low NO_3^- One example each of decreased carbon affinity withgrowth at lowest NO_3^- concentration tested, and with growth over entire NH_4^+ range tested.	Decreased nitrogen cost of synthesis of the photosynthetic apparatus incorporating a CCM if the savings in the synthesis of smaller amounts per cell of Rubisco and of the PCOC enzymes are not offset by the nitrogen cost of the synthesis of CCM components No consumption of nitrogen in the operation of the CCM
Phosph-orus	Decrease in phosphate in upper mixed layer of lotic environments	Two examples of increased inorganic carbon affinity, twoexamples of decreased inorganic carbon affinity, with growth at low phosphate	Where there is a decreased protein content in the photosynthetic apparatus there could be a corresponding decrease in the need for phosphorus needed to synthesise the photosynthetic apparatus as a result of decreased requirement for RNA
Iron	Probable decrease in iron in upper mixed layer of lotic environments	One example of increased inorganic carbon affinity with growth at low iron, one example of no effect	Decreased Fe content of the photostynthetic apparatus if the decreased requirement for NADPH in the near absence of Rubisco oxygenase activity and the PCOC, with correspondinly lower content of non-cyclic electron tramsprt components, is not offset by the additional thylakoid components needed for the additional ATP requirement for CCM operation, especially if this additional ATP is made using cyclic photophosphorylation using only Phosystem I with its high Fe content
Zinc	As for iron	No direct measurements	Variable preditions of relative,zinc requirements depending on CCM mechanism
UVA	Increase in UVA in lotic planktonic environments, but decrease with higher concentration of DOC*	No data	Not applicable
UVB	Increase in UVB in lotic planktonic environments, but decrease with higher concentration of DOC*	Variable responses of CCMs with increased UVB flux for growth.	Not applicable

1042 Footnote to Table 2

1043 Effects on CCMs of environmental factors, and the direction of change of these environmental factors in algal
1044 and aquatic plant habitats with global environmental change between icehouse episodes. Modified from [11].
1045 Further details and references are given in the text and in [11, 77-79, 85, 86, 90, 152-160].

1046 Predicted resource costs of synthesising and operating a photosynthetic apparatus using a CCM relative to one
1047 relying on entry of CO_2 by diffusion [42, 44-47, 49, 67, 85, 161].

1048 *DOC = Dissolved Organic Carbon

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