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SI ADVANCES IN PHOTOSYNTHESIS

Evolutionary trends in RuBisCO kinetics and their co-evolution with CO_2 concentrating mechanisms

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SUMMARY

RuBisCO-catalyzed CO_2 fixation is the main source of organic carbon in the biosphere. This enzyme is present in all domains of life in different forms (III, II, and I) and its origin goes back to 3500 Mya, when the atmosphere was anoxygenic. However, the RuBisCO active site also catalyzes oxygenation of ribulose 1,5-bisphosphate, therefore, the development of oxygenic photosynthesis and the subsequent oxygen-rich atmosphere promoted the appearance of CO_2 concentrating mechanisms (CCMs) and/or the evolution of a more CO_2-specific RuBisCO enzyme. The wide variability in RuBisCO kinetic traits of extant organisms reveals a history of adaptation to the prevailing CO_2/O_2 concentrations and the thermal environment throughout evolution. Notable differences in the kinetic parameters are found among the different forms of RuBisCO, but the differences are also associated with the presence and type of CCMs within each form, indicative of co-evolution of RuBisCO and CCMs. Trade-offs between RuBisCO kinetic traits vary among the RuBisCO forms and also among phylogenetic groups within the same form. These results suggest that different biochemical and structural constraints have operated on each type of RuBisCO during evolution, probably reflecting different environmental selective pressures. In a similar way, variations in carbon isotopic fractionation of the enzyme point to significant differences in its relationship to the CO_2 specificity among different RuBisCO forms. A deeper knowledge of the natural variability of RuBisCO catalytic traits and the chemical mechanism of RuBisCO carboxylation and oxygenation reactions raises the possibility of finding unrevealed landscapes in RuBisCO evolution.

Keywords: RuBisCO catalysis, carbon-concentrating mechanisms, CO_2-fixation, photosynthesis, algae, Bacteria, Archaea, plants, carbon isotopic fractionation, autotrophy.

INTRODUCTION

There are at least six different pathways of CO_2 fixation in nature (Berg, 2011). However, the enzyme ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO) mediates the only quantitatively relevant conversion from inorganic carbon to organic carbon, as the key step of the Calvin–Benson–Bassham (CBB) reductive pentose phosphate pathway. The fixation of CO_2 catalyzed by RuBisCO sustains the vast majority of trophic webs, and so, life on Earth. Hence, it is not surprising to have seen the enormous devotion of the scientific community to study this enzyme over the last 60 years. During this time, substantial progress has been made in understanding the structure of the active site and the complete reaction mechanism catalyzed by RuBisCO (Andersson, 2008). Although most of the studies on RuBisCO structural properties and its

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reaction mechanism have been focused on a few model species, and large-scale explorations of RuBisCO catalytic traits have been frequently restricted to angiosperm species (Galmés *et al.*, 2005; Kubien *et al.*, 2008; Hermida-Carrera *et al.*, 2016; Orr *et al.*, 2016; Sharwood *et al.*, 2016a), recent research studies have shed light on the variability of biochemical and molecular RuBisCO traits in previously underreported groups (Satagopan *et al.*, 2014; Galmés *et al.*, 2014a, 2015, 2016; Wilson *et al.*, 2016; Young *et al.*, 2016; Heureux *et al.*, 2017; Valegård *et al.*, 2018; Iñiguez *et al.*, 2018). The increase in the availability of RuBisCO measurements on phylogenetically distant groups have enabled a more profound analysis of RuBisCO fine tuning through evolution (Liu *et al.*, 2017; Young and Hopkinson, 2017; Cummins *et al.*, 2018; Tcherkez *et al.*, 2018).

RuBisCO catalyzes the addition of CO_2 to ribulose 1,5-bisphosphate (RuBP), producing two molecules of 3-phosphoglycerate, and the enzyme is characterized by a relatively low affinity for CO_2 and a slow carboxylation turnover rate (k_{cat}^c; about 1–10 reactions per second). This kinetic behaviour explains the large amounts of RuBisCO required to sustain effective net photosynthetic rates (Evans, 1989), and make RuBisCO one of the most abundant proteins on Earth (Ellis, 1979; Bar-On and Milo, 2019). In addition, RuBisCO not only catalyzes the carboxylation of RuBP, but also its oxygenation. Its reaction with O_2 produces one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate. 2-Phosphoglycolate is a toxic compound that inhibits several enzymes in carbon metabolism (Anderson, 1971; Kelly and Latzco, 1977; Norman and Colman, 1991), and a photorespiratory pathway has evolved for its detoxification and carbon recovery. The photorespiratory pathway requires extra energy investment and provokes a loss of fixed C, leading to a reduction in the net photosynthetic rate (Peterhansel *et al.*, 2010).

The share between RuBP carboxylation and oxygenation by RuBisCO depends on temperature, concentrations of CO_2 and O_2 at the active sites of RuBisCO, and the species-specific kinetic properties for this enzyme. Although all RuBisCOs have catalytic amino acid residues in their primary structures that have been conserved throughout evolution, they often show wide differences in their catalytic properties among diverse phylogenetic groups as a result of different evolutionary histories (Tcherkez *et al.*, 2006; Savir *et al.*, 2010; Galmés *et al.*, 2016). The decrease in CO_2 in the ancient atmosphere and the notable increase in O_2 associated with the development of oxygenic photosynthesis has led to the appearance and diversification of CO_2-concentrating mechanisms (CCMs). CCMs, which are likely to have co-evolved with RuBisCO kinetic traits (Kubien *et al.*, 2008; Raven *et al.*, 2017; Young and Hopkinson, 2017), increase the CO_2 concentration around RuBisCO, allowing the organisms that express these mechanisms both to reduce oxygenation and to enhance carboxylation. Thus, CCMs sustain high CO_2 fixation rates in limiting ambient CO_2 concentrations under different environmental conditions in which they must operate. However, the active rise of CO_2 concentrations around RuBisCO is expensive due to extra energy requirements and, therefore, CCMs have not been developed in all autotrophic organisms, implying potentially stronger selective pressures on RuBisCO catalytic traits.

In the present review, we compile and summarize recent research about the diversity and evolution of the different RuBisCO forms, their kinetic properties, and the environmental factors shaping RuBisCO evolution and the appearance and diversification of CCMs.

THE EVOLUTIONARY HISTORY OF THE DIFFERENT FORMS OF RUBISCO

RuBisCO is present in all domains of life: Bacteria, Archaea and Eukarya. Phylogenetic analyses of genome sequences support the existence of different clades (forms I, II, II/III and III) known as *bona fide* RuBisCOs, as all of these catalyze the carboxylation and oxygenation of RuBP (Tabita *et al.*, 2008; Liu *et al.*, 2017). The most widespread clade is form I RuBisCO, which is subdivided into four subtypes: IA, IB, IC and ID (Tabita *et al.*, 2008). In addition, there is a homologous family of proteins, form IV, known as RuBisCO-like proteins (RLP), which do not catalyze RuBP carboxylation or oxygenation because of key substitutions in many essential active-site residues (Hanson and Tabita, 2001). RLPs have been suggested to be involved in the methionine salvage pathway, sulfur metabolism and D-apiose catabolism in some bacteria and archaea (Ashida *et al.*, 2003; Tabita *et al.*, 2008; Carter *et al.*, 2018). All Rubisco forms, including RLP, have in common the formation of dimers of two large subunits (L_2) of about 50 kDa each, to produce two functional active sites for catalysis (Tabita *et al.*, 2008). Oligomeric forms with $(L_2)_n$ stoichiometry are known to occur in RuBisCO forms II and III, while the structure of form I RuBisCOs also includes small subunits with a molecular mass of *c.* 15 kDa, resulting in L_8S_8 stoichiometry. The small subunits provide structural stability and are required for maximal catalytic activity, but they are not strictly necessary for CO_2 fixation (Spreitzer, 2003; Andersson and Backlund, 2008).

There are different hypotheses for the origin of the RuBisCO superfamily (*bona fide* RuBisCOs and RLP), but the most plausible one is that all of them are of monophyletic origin and that the more complex mechanism of carboxylation function of the *bona fide* RuBisCOs evolved from the simpler mechanism of the RLP family (Ashida *et al.*, 2005, 2008; Erb *et al.*, 2012). However, other studies have suggested that RLP evolved from the ancestral *bona fide* RuBisCO by losing the capacity for RuBP carboxylation (Tabita *et al.*, 2007, 2008).

Form III RuBisCO is believed to be the most ancient form of *bona fide* RuBisCOs, and probably emerged ~3500 million years ago (Mya) (Tabita *et al.*, 2007). This form participates in the assimilation of ribonucleosides in Archaea, where RuBP generated from the ribose moieties of adenosine, guanosine and uridine are metabolized into 3-phosphoglycerate by the addition of CO_2 (Sato *et al.*, 2007; Aono *et al.*, 2015), rather than being involved in an autotrophic carbon assimilation (CBB pathway) as do forms I and II. The intermediate form II/III, found in the archaeon *Methanococcoides burtonii* and in other species of the order Methanosarcinales, also participates in the assimilation of ribonucleosides as does form III, although it is structurally more similar to the bacterial form II (Alonso *et al.*, 2009). Therefore, this intermediate RuBisCO form has been proposed to belong to a new RuBisCO subtype, named form IIIB (Gunn *et al.*, 2017). A recent study has demonstrated the existence of a reductive hexulose pathway (RHP) in some methanogenic archaea; although this new pathway differs somewhat from the CBB cycle, it employs a form III RuBisCO together with phosphoribulokinase (PRK) to regenerate RuBP and fix CO_2 (Kono *et al.*, 2017). The authors speculated that the CBB pathway may have originated from a primitive carbon metabolic pathway utilizing RuBisCO, such as this archaeal RHP pathway. In addition, recent discoveries have indicated that forms III and II/III RuBisCOs are not only present in Archaea but are found in different clades of bacteria (Wrighton *et al.*, 2016; Jaffe *et al.*, 2018); this may be a result of extensive horizontal transfer of the RuBisCO genes among these divergent groups. Some of these bacteria also possess in their genome homologues of PRK, an enzyme critical for the CBB pathway (Jaffe *et al.*, 2018).

Taking into account phylogenetic and metagenomics analyses, it is reasonable to speculate that RuBisCO emerged in a heterotrophic context in methanogenic archaea (Schönheit *et al.*, 2016), and that the common ancestor of Cyanobacteria and Proteobacteria may have acquired fundamental components of carbon metabolism, including the ancestral form III RuBisCO, by lateral gene transfer (Tabita *et al.*, 2008). RuBisCO autotrophic CO_2 fixation via the CBB pathway may have evolved later in Bacteria, as proposed by Schönheit *et al.* (2016) and Erb and Zarzycki (2018), leading to forms II and I RuBisCOs, with eukaryote RuBisCOs being acquired via subsequent endosymbiotic events. This is supported by the fact that form II RuBisCO is only present in Bacteria (except for dinoflagellates, see below), as well as two subtypes of forms I, IA and IC, which are supposed to be the most ancestral RuBisCO I forms. However, recent phylogenetic evidence seems to support an evolutionary scenario in which form II RuBisCO branched earlier from the common RuBisCO ancestor than the extant forms I and III clades (Kacar *et al.*, 2017). Form IC is found in diverse groups of proteobacteria, while form IA is found in some cyanobacteria as well as in proteobacteria. Eukaryotes only possess forms IB and ID RuBisCOs with the exception of peridinin-containing dinoflagellates, in which form I RuBisCO was replaced by a nucleus-encoded, single subunit form II RuBisCO acquired by horizontal gene transfer from a proteobacterium (Morse *et al.*, 1995; Whitney *et al.*, 1995).

Form I RuBisCO evolved through two different lineages, green-type RuBisCOs, that include forms IA and IB, and red-type RuBisCOs, that include forms IC and ID, which might have cyanobacterial and proteobacterial origins, respectively (Tabita *et al.*, 2008). Form IB is found in Cyanobacteria, glaucophytes, euglenozoans, chlorophytes and streptophytes, while form ID is present in non-green algae (rhodophytes, cryptophytes, ochrophytes and haptophytes). The absence of form IB in Proteobacteria might indicate that this RuBisCO subtype evolved more recently in Cyanobacteria from form IA (Badger and Price, 2003). Although the primary acquisition of a cyanobacterial endosymbiont by an eukaryotic heterotrophic host, *c.* 1500 Mya (Yoon *et al.*, 2004), was common to all photosynthetic eukaryotes, the genes for large and small subunits of the ancestral cyanobacterial RuBisCO in the red lineage were apparently replaced by those of a proteobacterium, probably by lateral gene transfer (Assali *et al.*, 1991; Delwiche and Palmer, 1996). This replacement process might have occurred before the secondary and tertiary endosymbiotic events took place, leading to the appearance of the rest of the red lineage eukaryotic photosynthetic groups that dominate the modern oceans (Falkowski *et al.*, 2004). A less probable alternative explanation for the presence of different RuBisCO subtypes in the red and green lineages might be an ancient gene duplication in the common bacterial ancestor coupled later with differential gene loss in both lineages (Delwiche and Palmer, 1996).

Despite being the most abundant forms of RuBisCO in nature and the only ones present in eukaryotes, forms IB and ID display striking differences in their regulation and functioning. Both large (*rbc*L) and small (*rbc*S) subunit genes of the 'red' form ID are included in the chloroplast genome and form a single operon, whereas in the eukaryotic organisms belonging to the green lineage (form IB), the *rbc*S gene was transferred to the nucleus before their diversification (Green, 2011). Efficient assembly of red-type RuBisCOs seems to be strictly mediated by RuBisCO small subunits (Joshi *et al.*, 2015), contrary to green-type RuBisCOs. Likewise, the binding of sugar phosphate inhibitors, as well as its removal by an activase AAA+ protein (Rca in the green lineage versus CbbX in the red lineage), differ significantly between forms IB and ID (Pearce, 2006; Mueller-Cajar *et al.*, 2011; Iñiguez *et al.*, 2018). However, the knowledge on expression, catalysis, and regulation of form ID up to date is very limited in comparison with the well characterized form IB, and future studies on form ID RuBisCOs, as well as other

less studied forms, are required to shed light on the diversity and evolution of the RLP/RuBisCO superfamily.

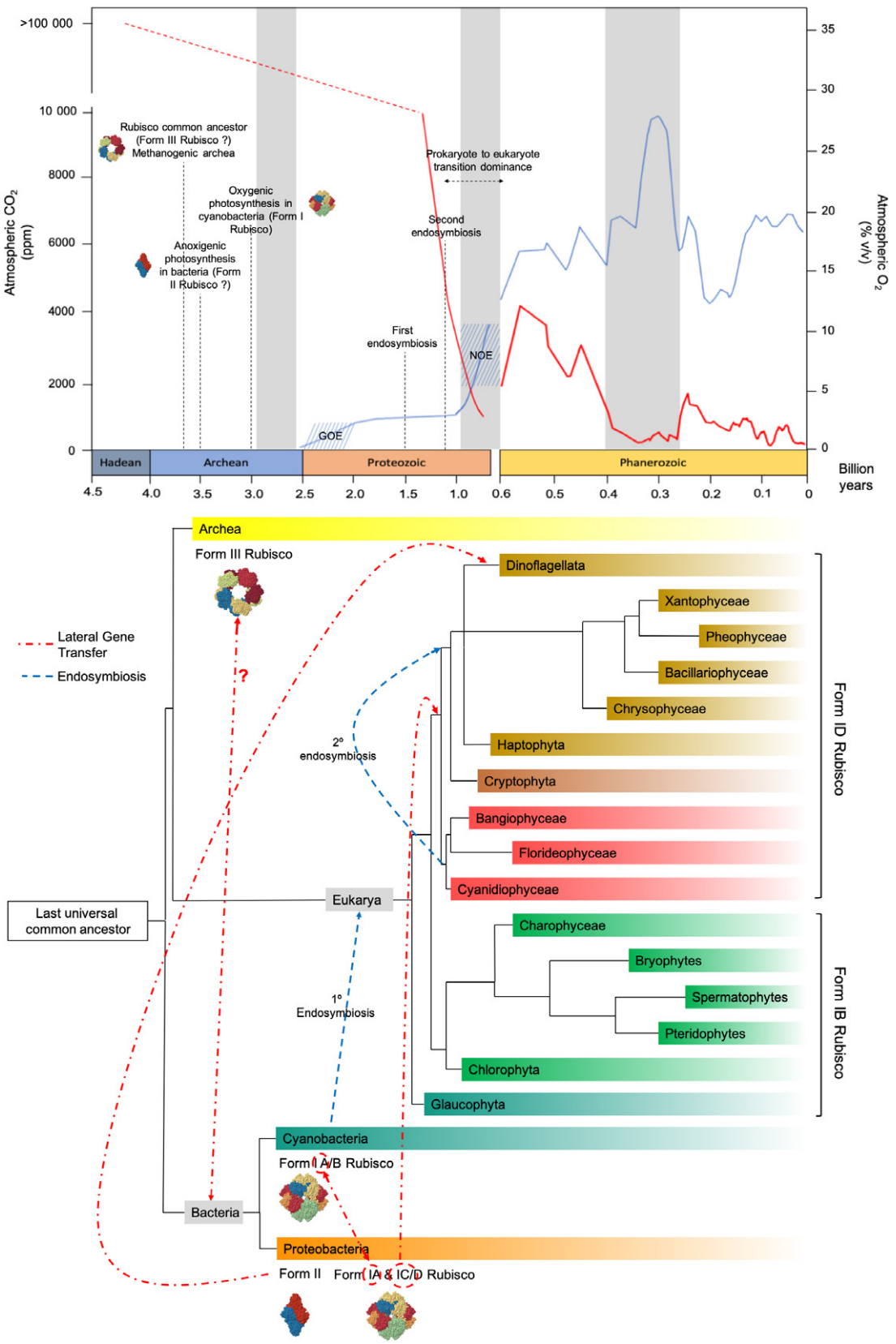
THE DIVERSIFICATION OF PHOTOSYNTHETIC ORGANISMS AND THE ORIGIN OF CO_2 CONCENTRATING MECHANISMS

Figure 1 shows the putative origin of the main RuBisCO forms and the diversification of the different phylogenetic groups expressing *bona fide* RuBisCOs in the context of the predicted changes in atmospheric [CO_2] and [O_2] throughout Earth's history, including the most probable endosymbiotic events and RuBisCO horizontal gene transfers between groups that took place during the evolution. When the first *bona fide* RuBisCO appeared in nature, the Earth's atmosphere was completely anoxygenic and presented very high levels of CO_2, probably between 100–1000 times the current atmospheric [CO_2] (Hessler *et al.*, 2004, see Figure 1). Anoxygenic photosynthesis, which is associated with form II RuBisCO in extant proteobacterial species, is likely to have evolved before the origin of both, oxygenic photosynthesis and form I RuBisCO in the cyanobacterial ancestor (Blankenship, 2010). Recently, Nutman *et al.* (2016) discovered the earliest stromatolites demonstrating that shallow marine carbonate production with biotic CO_2 sequestration was established by 3700 Mya. Fossil-based carbon isotopic measurements of stromatolites provide evidence of autotrophic bacterial activity back to, at least, 3500 Mya (Schopf, 2011, 2014), suggesting a fast radiation and evolution of the common RuBisCO ancestor, likely to be a form III originated in hydrothermal methanogenic archaea (Tabita *et al.*, 2007). However, the origin of oxygen-evolving photosynthesis is still controversial and might have occurred somewhere in the period after the appearance of anoxygenic photosynthesis up to 2500 Mya (Schirrmeister *et al.*, 2016). From that moment, the oxygen content in the atmosphere started to rise as CO_2 was decreasing due to the proliferation and dominance of Cyanobacteria over a billion years, triggering the Great Oxidation Event (GOE; Farquhar *et al.*, 2011) that marked a permanent shift in the atmospheric composition. After the appearance of oxygenic photosynthesis, RuBisCO had to face substantial [O_2] for the first time, and oxygenation of RuBP that produced 2-phosphoglycolate led to the evolution of the photorespiration pathway. Indeed, fine tuning of oxygenic photosynthesis, photorespiration, and evolving mechanisms for detoxifying reactive oxygen species (ROS) has been proposed to have occurred in an anaerobic photosynthesizing cyanobacterial ancestor before the GOE (Hamilton, 2019). This process of co-evolution would have been aided by the prevailing reducing conditions until the organism was able to detoxify 2-phosphoglycolate and ROS. The RuBisCO oxygenation activity might have been beneficial in early anaerobic to microaerobic photosynthesizers in dealing with excess oxygen (Nisbet and Fowler, 1999), which was toxic to all organisms at that time (Raven and Larkum, 2007). Oxygen-evolving photosynthesis allowed the appearance of aerobic respiration, a more energetically efficient process than anaerobic fermentation, leading to the spread and diversification of aerobic life. The evolution of form I RuBisCO (L_8S_8) was strongly linked to the oxygenation of the atmosphere and a unique origin of this form is suggested by the structural and sequence similarity found among all types of RuBisCO small subunit (Spreitzer, 2003). After this event, form I RuBisCO is likely to have evolved independently in Cyanobacteria and Proteobacteria during the GOE, leading to IA/B and IC/D subfamilies, respectively, according to the striking differences between IB and ID RuBisCOs mentioned above.

Lower CO_2/O_2 ratios of aerobic environments forced RuBisCO evolution towards a more CO_2-specific enzyme (Savir *et al.*, 2010), which is reflected in substantial differences in the specificity factor ($S_{c/o}$) of form I versus forms II and III RuBisCOs. CCMs might have first appeared under environmental conditions that caused a significant reduction in the net carbon fixation of certain obligate autotrophic organisms, either by limited CO_2 diffusion to the active site of RuBisCO that resulted in a highly subsaturating CO_2 concentration for the enzyme, and/or by an elevated oxygenase activity (Giordano *et al.*, 2005).

It is widely accepted that CCMs are a clear example of convergent evolution, appearing in diverse phylogenetic groups in different evolutionary moments. Still, there is no consensus on the first origin of a CCM and there are different hypotheses about this origin (see the different possible periods for the origin of CCMs marked with a grey shadow in Figure 1). Raven (1997), followed by Badger *et al.* (2002), proposed that CCMs firstly appeared in Cyanobacteria and algae in the mid-Phanerozoic period, between *c.* 400–300 Mya, when CO_2 dropped to less than 800 ppm and O_2 rose to 35%, imposing a strong selective pressure for CCMs appearance. Riding (2006) proposed that CCM origin would

Figure 1. Changes in atmospheric CO_2 and O_2 concentrations throughout Earth's history, according to Hessler *et al.* (2004), Foster *et al.* (2017), Holland (2006) and Haworth *et al.* (2011) and probable origin of the different RuBisCO forms and photosynthetic metabolisms, along with the primary and secondary endosymbiotic events (upper panel), and the diversification of the main extant autotrophic phyla, including the main endosymbiotic events (marked with a blue line) and RuBisCO horizontal gene transfers (marked with a red line) between the different phylogenetic groups (lower panel). In the upper panel, the different possibilities for the origin of CCMs (as described in the text) are marked with a grey shadow. The divergence times for each phylogenetic group were obtained from the publicly available online *TimeTree* database (http://www.timetree.org; Kumar *et al.*, 2017). Quaternary structures of each RuBisCO form were generated using Jmol software and the crystal structures in the Protein Data Bank for *Spinacia oleracea* (code: 1AUS), *Rhodospirillum rubrum* (code: 5RUB) and *Thermococcus kodakarensis* (code: 1GEH).



have occurred 300 Myr earlier than the Carboniferous period, as [CO_2] declined to levels lower than 10 times the current atmospheric concentration, although [O_2] was still low. Raven and Larkum (2007) have suggested an even earlier origin, as RuBisCO from cyanobacteria forming stromatolites in the Archaean to early Proterozoic period might have faced low CO_2 and high O_2 intracellular concentrations derived from oxygenic photosynthesis due to thick diffusive boundary layers, despite the high CO_2 and low O_2 concentrations of the atmosphere in that moment. Thus, these organisms would have needed a primitive CCM operation to achieve effective photosynthesis. The explanation for the occurrence and diversification of cyanobacteria in stromatolites, despite the low diffusion rates, would be the benefit of UV screening in intertidal habitats exposed to an atmosphere absent of stratospheric O_3 layer (Raven *et al.*, 2008). In fact, it has been speculated that stromatolite formation could have been facilitated by CCMs, as bicarbonate uptake promotes calcium mineralization (Benzerara *et al.*, 2014). This hypothesis might be supported by phylogenomic studies that placed evolution of the basal extant cyanobacterial genus *Gloeobacter*, which possesses CCMs, before the GOE (Sánchez-Baracaldo *et al.*, 2014; Schirrmeister *et al.*, 2016), and by the ability of the resurrected ancestral form I RuBisCOs to be encapsulated in extant carboxysomes (Shih *et al.*, 2016). However, the hypothesis of this early origin of a CCM might be inconsistent with the absence of genes encoding carboxysome proteins (the type of CCMs in Cyanobacteria) in extant photosynthetic eukaryotes, which evolved *c.* 1500 Mya likely by a unique first endosymbiosis of a cyanobacterium. An exception is the presence of photosynthetic organelles, called 'cyanelles', in Glaucophyta resembling bacterial carboxysomes (Burey *et al.*, 2005), which are considered the first divergence among all plastids (Helmchen *et al.*, 1995). This might suggest a CCM origin before the first endosymbiotic event and a later loss of carboxysome genes in the Metabionta (Rhodophyta and Viridiplantae) ancestor, although the evolutionary position of Glaucophyta is still under debate (Deschamps and Moreira, 2009). Therefore, the possibility that CCMs evolved much earlier than the largely accepted Carboniferous origin can be still plausible, despite the high CO_2/O_2 ratio of the ancient atmosphere and the inferred low Archaean seawater pH values (6.5–7; Halevy and Bachan, 2017), that do not point to a strong selective pressure. Although the second endosymbiosis of a rhodophyte alga leading to the rest of the red lineage might have occurred *c.* 1200 Mya (Yoon *et al.*, 2004), chromista algae did not start to diverge until *c.* 700 Mya, when the atmospheric CO_2 probably decreased below 2000 ppm and the oxygen was substantially increasing, during the so-called Neoproterozoic Oxygenation Event (NOE; see Figure 1). After the NOE, the dissolved O_2 and CO_2 levels in the oceans likely reached equimolar concentrations, according to [CO_2] and [O_2] atmosphere estimates and their solubility, which might suppose a strong selective driving force for marine organisms to develop a mechanism that allowed them to boost the CO_2/O_2 ratio at the site of RuBisCO (Griffiths *et al.*, 2017). Furthermore, the rise and diversification of the most abundant extant eukaryotic phytoplankton (diatoms, coccolithophorids and dinoflagellates), all of which are chromophytes and possess an almost ubiquitous presence of CCMs, did not start until the mid-Phanerozoic period, after the worst mass extinction on Earth (Falkowski *et al.*, 2004). This radiation occurred at the end of the period with the lowest atmospheric CO_2/O_2 ratio (see Figure 1), and the ecological success of these eukaryotic groups was probably driven by the strong decline in CO_2, as previously suggested (Lee and Kugrens, 2000). This would support a much more recent origin of CCMs, at least in modern eukaryotic phytoplankton.

DIFFERENT TYPES OF PROKARYOTIC AND EUKARYOTIC CCMS

Most extant aquatic primary producers possess CCMs, which support at least 80% of annual marine net primary production (Field *et al.*, 1998), whereas the majority of terrestrial species do not possess CCMs, and only ~25% of terrestrial primary production is based on CCM operation (Still *et al.*, 2003).

This difference is due to the substantially lower rate of CO_2 diffusion, about four orders of magnitude, in water than in air, leading to boundary layers that could strongly limit photosynthesis in non-agitated waters. In addition, the low solubility of CO_2 in water at ambient temperatures means dissolved CO_2 in equilibrium with atmospheric CO_2 in the range of 10–20 μM, well below the half-saturation constant for CO_2 (K_c) of most aquatic RuBisCOs. Dissolved CO_2 reacts with water and dissociates into bicarbonate and carbonate ions. The equilibrium between the different dissolved inorganic carbon forms will depend on temperature, pH, and ionic strength of the medium. In the oceans, [HCO_3^-] is two orders of magnitude more abundant than dissolved [CO_2]. Furthermore, the rates of interconversion between dissolved CO_2 and HCO_3^- are relatively slow in the absence of enzymes catalyzing this reaction and an intense photosynthetic activity can displace dissolved [CO_2] out of its physicochemical equilibrium. Consequently, many aquatic organisms have developed mechanisms to actively utilize HCO_3^- as part of a CCM.

There is a large variability in the mechanisms that increase CO_2 concentration around RuBisCO in autotrophic organisms, even within the same phylogenetic group. This variability ranges from biochemical processes that involve a first temporal carbon fixation before the definitive RuBisCO-catalyzed carbon fixation (C_4 and crassulacean acid metabolism), to biophysical processes involving active uptake of dissolved inorganic carbon (HCO_3^- and/or CO_2) and/or

localized enhancement of external CO_2 concentration by acidification of the external medium (Giordano *et al.*, 2005).

All Cyanobacteria, the vast majority of eukaryotic algae, some hornworts and many aquatic angiosperms possess CCMs based on biophysical processes. Biophysical CCM components consist on HCO_3^-/CO_2 direct transporters at one or more cellular membranes, and/or proton pumps contributing to the creation of acid zones, coupled with carbonic anhydrases (CAs) located in different cellular compartments (periplasmic space, chloroplast stroma, thylakoid lumen) that accelerate the interconversion between HCO_3^- and CO_2 (Maberly, 1990; Maberly *et al.*, 1992; Beer and Koch, 1996; Larsson and Axelsson, 1999; Sherlock and Raven, 2001; Raven *et al.*, 2002a).

Prokaryotic CCMs, which rely on intracellular proteinaceous structures called carboxysomes, are efficient active inorganic carbon uptake systems that can concentrate CO_2 around RuBisCO up to 100 times the external CO_2 level (Badger and Andrews, 1987). These CCMs are present in all photosynthetically competent cyanobacteria as well as some proteobacteria. A large cytosolic pool of bicarbonate is achieved by direct HCO_3^- transporters and active conversion of passively diffused CO_2 to HCO_3^- mediated by a NADPH dehydrogenase complex located in the thylakoid membrane (reviewed by Price, 2011). Carboxysomes are specialized protein microcompartments that are surrounded by a polyhedral protein shell that seems to restrict CO_2 efflux and O_2 influx, while permitting the transit of HCO_3^-, as well as RuBP and 3-phosphoglycerate (Dou *et al.*, 2008; Cai *et al.*, 2009). The large cytosolic pool of bicarbonate allows its diffusion inside the carboxysomes, where RuBisCO and a carboxysomal CA are encapsulated, and this CA accelerates the dehydration of HCO_3^- to CO_2 that can nearly saturate RuBisCO carboxylation and almost avoid RuBisCO oxygenase reaction under optimal irradiance conditions. The mathematical model of cyanobacterial CCM developed by Mangan *et al.* (2016) demonstrates that both cytosolic pH $\approx$ 8 and CO_2 retention inside the carboxysome, are necessary for an energetically efficient CCM, with a significantly reduced CO_2 leakage out of the cell.

There are two types of carboxysomes, α-carboxysomes and β-carboxysomes, with similar physiological functioning despite intrinsic structural differences (Whitehead *et al.*, 2014). α-Carboxysomes are mainly found in oceanic cyanobacteria as well as in some obligate and facultative photolithotrophic and chemolithotrophic proteobacteria, while β-carboxysomes are predominantly found in freshwater and coastal cyanobacteria (Badger *et al.*, 2002; Badger and Bek, 2008), therefore occupying different ecological niches. These two types of carboxysomes are related to the RuBisCO phylogeny, as form IA RuBisCO is associated with the expression of α-carboxysomes (form IAc), while form IB RuBisCO is associated with the expression of β-carboxysomes (form IBc). A common origin of both types of carboxysomes has been suggested, attending to the sequence homology between their shell proteins and to the ability of resurrected ancestral forms IA and IB RuBisCOs to be encapsulated by extant β-carboxysomes (Shih *et al.*, 2016; Kerfeld and Melnicki, 2016). Conversely, the distinct protein components of both types of carboxysomes along with the wide distribution of α-carboxysomes in phylogenetically distant cyanobacterial and proteobacterial lineages may also suggest a convergent evolution after the divergence of α- and β-cyanobacteria (Rae *et al.*, 2013). The similarity among α-carboxysomes of Cyanobacteria and Proteobacteria points to horizontal gene transfer of the form IA RuBisCO and the cluster of α-carboxysome genes between Proteobacteria and Cyanobacteria (Badger *et al.*, 2002; Badger and Price, 2003; Badger and Bek, 2008). Still, the directionality of this (these) transfer(s) and the evolutionary moment of its (their) occurrence is unclear, depending on where the origin of form IA RuBisCO took place (Cyanobacteria or Proteobacteria). Furthermore, a phylogenetic analysis has revealed that extant α-cyanobacteria acquired the carboxysome operon (including form IA RuBisCO genes) from a *Nitrococcus*-like proteobacterium (Marin *et al.*, 2007), probably displacing the form IB RuBisCO and associated β-carboxysomal proteins (Raven *et al.*, 2012). This is not incompatible with the occurrence of a much more ancient lateral gene transfer of form IA RuBisCO from the cyanobacterial ancestor to a proteobacterium (Delwiche and Palmer, 1996; Badger *et al.*, 2002). In either case, lateral transfer of large gene sets is becoming seen as a major part of bacterial CCM evolution.

In some eukaryotic algae, an apparent analogue proteinaceous structure, the pyrenoid, seems to be intimately related to biophysical CCM functioning (Badger *et al.*, 1998), although it is not an absolute requirement for the presence of biophysical CCMs (Raven *et al.*, 2005). There are also few exceptions for organisms possessing pyrenoids but apparently not expressing CCMs (Badger *et al.*, 1998; Maberly *et al.*, 2009). Pyrenoids are non-membrane-surrounded microcompartments found in the chloroplastic stroma of many eukaryotic algae and some hornworts, formed by densely packed proteins, mainly RuBisCO, that, contrary to carboxysomes, do not have a protein shell restricting gas diffusion. The other proteins conforming pyrenoids, at least in the model green alga *Chlamydomonas reinhardtii*, include RuBisCO activase (McKay and Gibbs, 1991), a carbonic anhydrase (Sinetova *et al.*, 2012) and an essential protein that binds RuBisCO to form the pyrenoid matrix, named EPYC1 (Mackinder *et al.*, 2016). Recently, a proteomic characterization of *C. reinhardtii* pyrenoid revealed that it is composed of 190 distinct proteins. Some of these proteins have unexpected functions such as chlorophyll synthesis or amino acid

metabolism (Zhan *et al.*, 2018), suggesting that the pyrenoid is a hub for metabolism. The pyrenoid matrix has been shown to behave as a liquid (Freeman Rosenzweig *et al.*, 2017), and its surface area is constrained by a starch sheath whose morphology is directly regulated by another RuBisCO-binding protein, SAGA1, affecting pyrenoid number and its CO_2-concentrating mechanism function (Itakura *et al.*, 2019). CCMs in pyrenoid-containing organisms involve active CO_2/HCO_3^- transport across the plasmalemma and/or a plastid envelope, and CAs in the different cellular compartments (plasmalemma, cytosol, chloroplastic stroma). In some cases, when the pyrenoid protrudes inside the thylakoids, as observed in *C. reinhardtii*, the CCM also includes an active symport of bicarbonate and protons to the thylakoid lumen, with later conversion of HCO_3^- to CO_2 accelerated by a luminal CA at the low pH of the thylakoid lumen, and subsequent CO_2 diffusion to RuBisCO active sites in the pyrenoid protrusion (Moroney and Ynalvez, 2007). The confinement of almost all RuBisCO in a relatively reduced space where CO_2 is delivered must minimize CO_2 leakage out of the chloroplast. Despite the existence of biophysical CCMs in eukaryotic algae not possessing pyrenoids, such as some members of the class Phaeophyceae, it is widely accepted that CCMs are more effective in concentrating CO_2 around RuBisCO when pyrenoids are present. Then, pyrenoids might be an evolutionary adaptation enhancing the performance of basal CCMs (Meyer *et al.*, 2017), as demonstrated by modelling (Badger *et al.*, 1998) and by examining the decreased photosynthetic CO_2 affinity and increased CO_2 leakage in *C. reinhardtii* mutants not expressing pyrenoids (Meyer *et al.*, 2012). Indeed, the most efficient eukaryotic phytoplankton groups utilizing inorganic carbon, like diatoms (Hopkinson *et al.*, 2011), possess pyrenoids, and their photosynthetic CO_2 affinity are significantly higher than that found in other ochrophytes, like some phaeophytes lacking pyrenoids (Badger *et al.*, 1998). In the same way, pyrenoid-containing chlorophytes, such as *C. reinhardtii*, are able to accumulate higher CO_2 concentrations than other phylogenetically closer species, such as *Chloromonas*, not possessing pyrenoid but also expressing CCMs (Morita *et al.*, 1998). However, more research is needed on the role of pyrenoid function in CCMs of algal species in addition to the few model organisms analyzed to date.

Biochemical CCMs consist of an additional carboxylation step before that mediated by RuBisCO in which inorganic carbon in the form of HCO_3^- is fixed to a C_3 molecule to form a C_4 intermediate that is subsequently decarboxylated at the RuBisCO active sites (Giordano *et al.*, 2005). In organisms possessing C_4 photosynthetic metabolism, the first carboxylation and decarboxylation reactions are spatially separated, whereas in CAM these reactions are temporally separated (the first carboxylation occurs at night, while decarboxylation at the place of RuBisCO occurs during the day). Biochemical CCMs are mostly restricted to terrestrial vascular plants and to some aquatic angiosperms, while very few exceptions of C_4-like single-cell metabolism in algae have been recognized (Reiskind *et al.*, 1988; Reinfelder *et al.*, 2000; Roberts *et al.*, 2007).

While biochemical CCMs are constitutively expressed in most species, with few exceptions (Keeley and Rundel, 2003), the expression of biophysical CCMs in Bacteria and algae is facultative and it is regulated by a large number of environmental factors, such as inorganic carbon and nutrient availability, irradiance, and temperature conditions (Giordano *et al.*, 2005). Diffusive CO_2 entry in CO_2 limiting environments could involve a greater nitrogen investment in RuBisCO plus photorespiratory enzymes than would be invested in CCM proteins (Raven *et al.*, 2004), and so organisms with CCM would need less N to fix the same quantity of CO_2 than organisms with only diffusive CO_2 entry. However, CCMs have an elevated energetic cost, therefore they could be downregulated due to energetic cellular constraints or under environmental conditions in which RuBisCO is nearly CO_2 saturated. This response will strongly depend on the maximum photosynthetic capacity of each organism, especially its RuBisCO kinetics.

Eukaryotic algal CCMs are usually downregulated at high dissolved CO_2 conditions, decreasing the photosynthetic affinity for inorganic carbon, although CO_2 accumulation can still occur as a low-affinity mechanism in some species (Amoroso *et al.*, 1998). Other eukaryotic algae, like some polar phaeophytes, did not show apparent downregulation of their CCMs at the range of dissolved CO_2 tested (Iñiguez *et al.*, 2016, 2017). In Cyanobacteria, HCO_3^- availability, rather than CO_2, is the inorganic carbon form that regulates CCM expression (Mayo *et al.*, 1986). Low light conditions, relative to those yielding the maximum rate of growth, usually downregulate CCMs due to limitations on energy supply (Beardall and Giordano, 2002). Furthermore, the concentrations of dissolved CO_2 and HCO_3^- in equilibrium are controlled by pH, temperature, and salinity of the medium as explained before, so changes in these variables may also indirectly alter CCM activity.

CCMs in different species have been shown to respond to temperature in different species-specific ways (Beardall and Giordano, 2002), and the specific thermal dependences of RuBisCO kinetics for a certain organism, which have been assayed for a very few cyanobacterial and algal species up to date, must have an important role in the response of CCM operation to temperature (see section 6).

RUBISCO CATALYTIC DIVERSITY AND ITS CO-EVOLUTION WITH CCMS

The available data on RuBisCO kinetic traits from phylogenetically distant groups of organisms reveal substantial differences among the different RuBisCO forms (Figure 2). The most obvious differences are found in the $S_{c/o}$ values

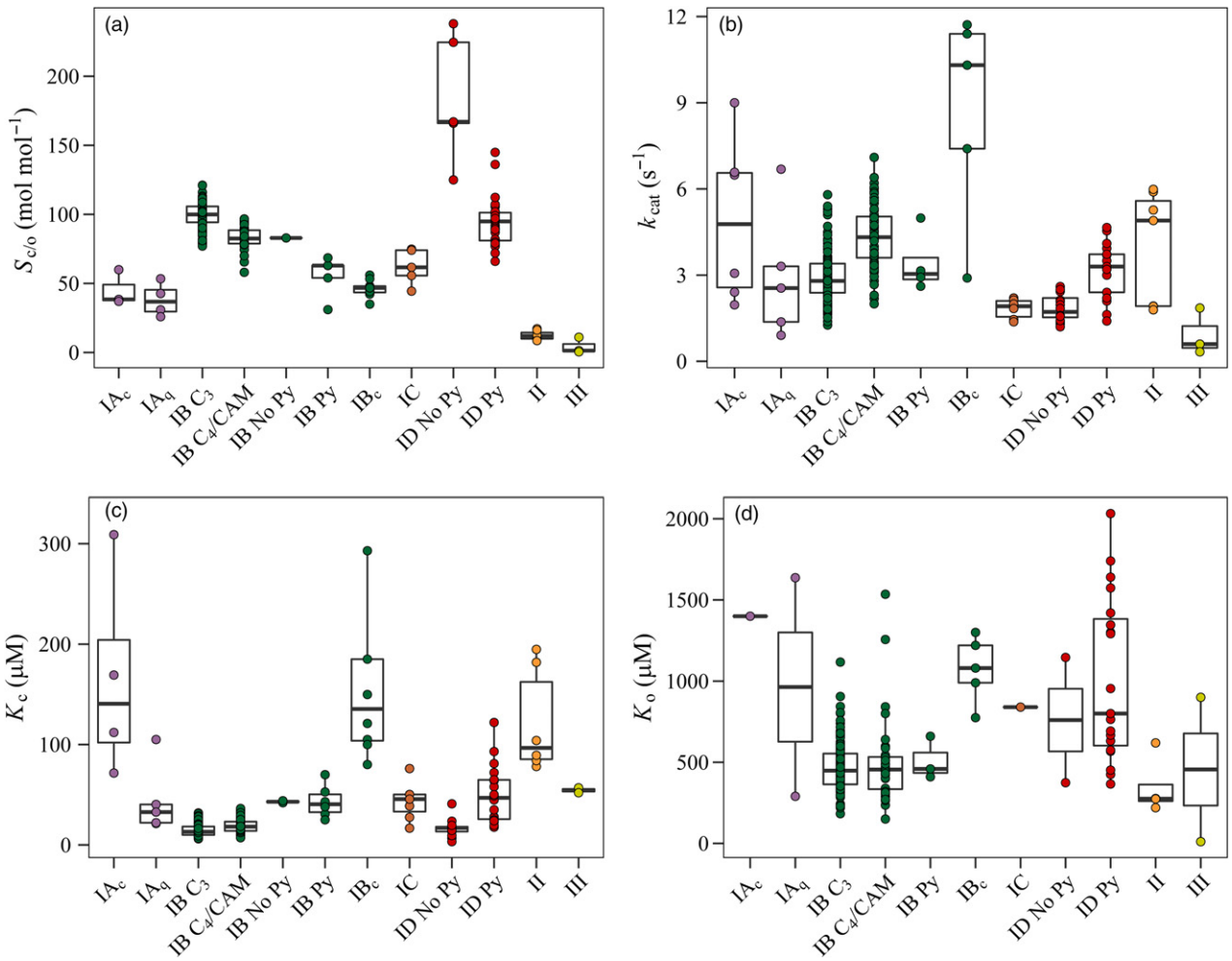


Figure 2. Boxplot depiction for the compiled RuBisCO kinetics (k_{cat}^c, K_c, K_o, $S_{c/o}$) at 25°C (see Data S1) of the different forms of RuBisCO from extant organisms. The points represent an average value for those species (or bacterial strains) reported by more than one study. Only data measured at 23–30°C were considered and those not measured at the standard temperature of 25°C were standardized using the temperature functions described in Galmés *et al.* (2016). *Py* means pyrenoid presence, *No Py* means pyrenoid absence.

of either forms I, II and III, which correlate with changes in the CO_2/O_2 ratios they have experienced throughout evolution. The values for $S_{c/o}$ of form I (in the range 25–240 mol mol^{-1} at 25°C) are higher than those of forms II and III, showing values lower than 15 mol mol^{-1}. This difference reflects the consequence of oxygenic photosynthesis association to form I RuBisCO.

The lowest $S_{c/o}$ values ever reported (0.5–11 mol mol^{-1}), revealing almost no discrimination between CO_2 and O_2, correspond to Archaea (form III or II/III). Form II/III of the archaeon *M. burtonii* has been grouped with form III in Figure 2 due to their functional similarity. The analyzed archaeal species are anaerobic organisms never facing O_2 in their environment, and whose RuBisCO is not involved in autotrophic carbon fixation but in the assimilation of ribonucleosides. Most notably, half-saturation constants for CO_2 (K_c) and O_2 (K_o) for RuBisCOs from the three archaeal species analyzed to date (Kreel and Tabita, 2007, 2015; Wilson *et al.*, 2016) share a common trend not previously observed in any other RuBisCOs. They all possess a stronger RuBisCO affinity for O_2 than for CO_2 at their optimum growth temperature (83°C for the hyperthermophilic species *Thermococcus kodakarensis* and *Archaeoglobus fulgidus* and 25°C for the Antarctic species *M. burtonii*), which might mirror the kinetics of the common ancestor *bona fide* RuBisCO with an anoxygenic and probably heterotrophic origin. By contrast, Yoshida *et al.* (2007) reported a RuBisCO affinity for O_2 more than 10 times lower than the affinity for CO_2 in *T. kodakarensis* at 25°C, which might imply a maximum oxygenase turnover rate (k_{cat}^o) higher than k_{cat}^c, according to the low $S_{c/o}$ values (11 mol mol^{-1}). The apparent discrepancy in the measurements for *T. kodakarensis* between both studies might reflect an effect of the kinetic thermal dependencies of its thermophilic RuBisCO.

The lowest ever reported values of k_{cat}^c and RuBisCO carboxylation efficiency (k_{cat}^c/K_c) at 25°C are also observed

in forms III and II/III, which might also reflect the ancient origin of these forms not being involved in an autotrophic CO_2-fixation metabolism, as well as the absence of selective pressure in the direction of reducing the oxygenation activity throughout evolution. Still, RuBisCO k_{cat}^c at the optimum growth temperature of the hyperthermophilic archaea *T. kodakarensis* and *A. fulgidus* was extremely high, of 17 and 23 s^{-1} respectively (Kreel and Tabita, 2007, 2015).

After Archaea, the lowest $S_{c/o}$ values corresponded to form II RuBisCO, which is expressed under low [O_2] in Proteobacteria, except for peridinin-containing dinoflagellates that possess pyrenoid-based CCMs. Some proteobacteria that possess the gene coding for form II RuBisCO are aerobes, such as *Thiomonas intermedia* and *Halothiobacillus neapolitanus* (Badger and Bek, 2008). Therefore, although Proteobacteria can only perform anoxygenic photosynthesis, evolution of form II RuBisCO might have been shaped under the presence of certain amounts of O_2, in contrast with form III RuBisCO in Archaea. Form II also possesses relatively high k_{cat}^c values and poor affinity for CO_2 (high K_c values), leading to k_{cat}^c/K_c values higher than those from Archaea, but still lower compared with most form I RuBisCOs from eukaryotic organisms. As there is no clear association between the expression of form II RuBisCO with an active CCM in Proteobacteria, this form must be adapted to function in low O_2 and high CO_2 environments. The affinity for O_2 for form II RuBisCO is also lower (higher K_o values) than that from Archaea measured at their optimum growth temperature (Figure 2, note that the K_o of 900 μM for form III RuBisCO was not measured at the organism's optimum growth temperature) reflecting an evolution under less strict anaerobic conditions, while still lower than that for most form I RuBisCOs.

Extant autotrophic proteobacteria present an enormous metabolic flexibility, from facultative chemoautotrophic to obligate photoautotrophic CO_2-fixing lifestyles, being able to perform aerobic or anaerobic respiration and fermentation (Badger and Bek, 2008). Hence, Proteobacteria have the capacity to grow in environments with a broad range of CO_2 and O_2 levels. This ability to perform CO_2 fixation through the CBB cycle in a wide range of environments is due to the unique presence of different copies of the genes coding for different RuBisCO forms within the same genome, reflecting the clearest example in nature of evolutionary diversification of RuBisCO types shaped by different environmental conditions. Proteobacteria can possess the genes coding for forms II, IA and IC RuBisCOs. Furthermore, form IA can be subdivided into two distinct subtypes, IA_c and IA_q, based on differences in the amino acid sequences of the small subunits and gene arrangements in the genome. RuBisCO IA_c genes are clustered together with α-carboxysome genes in a single operon (Cannon *et al.*, 2003), in a similar way to those found in α-cyanobacteria; these α-carboxysome genes are likely to be associated with the formation of a carboxysome structure (Tabita *et al.*, 2008; Badger and Bek, 2008). In contrast, form IA_q RuBisCO do not possess an associated set of carboxysome genes (Tabita *et al.*, 2008).

Differences between RuBisCO kinetics of the compiled values of form IA_c and IA_q in Figure 2 are only clear for K_c, showing lower values for the IA_q form. However, measurements of RuBisCO kinetics from both IA forms expressed within the same organism also revealed higher k_{cat}^c values for form IA_c in comparison with form IA_q (Hayashi *et al.*, 1998). The higher k_{cat}^c and lower affinity for CO_2 of form IA_c relative to IA_q reflect an adaptation of form IA_c to nearly saturating CO_2 levels around RuBisCO as a consequence of being part of a CCM (Dobrinski *et al.*, 2005), enabling proteobacteria to grow in low CO_2 environments. Form IA_q must be expressed in environments with medium-to-high CO_2 and with the presence of O_2 (Badger and Bek, 2008). The very few α-cyanobacterial strains analyzed showed similar RuBisCO catalytic traits to those measured in the few analyzed form IA_c from Proteobacteria, although Cyanobacteria perform oxygenic photosynthesis whereas Proteobacteria perform anoxygenic photosynthesis, which might lead to different intracellular O_2 gradients.

Form IB_c, found in β-cyanobacteria, possess relatively low $S_{c/o}$ and substantially low affinities for CO_2, in a similar way as form IA_c RuBisCOs, but displaying the highest k_{cat}^c values ever reported (7–13 s^{-1}).

Form IC shows higher $S_{c/o}$ than forms IA and IB_c (Figure 2), which might reflect a better adaptation to higher levels of O_2, despite showing similar k_{cat}^c and K_c than those reported for IA_q RuBisCOs. This form is also expressed in environments with medium-to-high [CO_2] but with significant O_2 levels. The difference in $S_{c/o}$ between form IC and form IA/B_c RuBisCOs resembles the differences between form ID and form IB in eukaryotes, suggesting that the ancestor of form C/D already possessed relatively high $S_{c/o}$, as previously proposed (Young *et al.*, 2012; Rickaby and Hubbard, 2019). CCMs in non-green algae might have appeared much more recently than CCMs in Cyanobacteria, and form IC in Proteobacteria has not been found to be associated with the expression of carboxysomes, suggesting that form C/D RuBisCO may have experienced decreasing CO_2/O_2 ratios during the Proterozoic period that led to high $S_{c/o}$ and low K_c values. The succession of phytoplankton in the Phanerozoic oceans from the cyanobacterial and green algal dominance to non-green algal dominance might have been partially driven by the likely higher $S_{c/o}$ values of the latter (Rickaby and Hubbard, 2019). The probable development of CCMs in non-green algae during this period of lowest CO_2/O_2 ratios removed part of the selective pressure directed to enhance the affinity and selectivity for CO_2 and, instead, favoured selection for higher k_{cat}^c. Hence, it is possible to observe in

Figure 2 a reduction in $S_{c/o}$ and rise of K_c in form ID RuBisCOs from species having a pyrenoid compared with those values from species lacking a pyrenoid. Indeed, Young *et al.* (2012) detected positive selection in *rbcL* to occur at the divergence of Rhodophyta, Haptophyta and Bacillariophyta (diatoms), coincident with periods of declining CO_2. Thus, it is logical to conclude that RuBisCO evolution must be strongly linked to the presence of pyrenoids in form ID RuBisCOs, despite the evidence of CCMs in some non-green algae lacking pyrenoids. In fact, k_{cat}^c and K_c values for non-green algal species lacking pyrenoids, but likely to have CCMs (species belonging to the orders Laminariales, Desmarestiales and Palmariales from Iñiguez *et al.*, 2018), are similar to those values from non-green algae that do not express CCMs (Figure 2).

Within the green lineage (form IB RuBisCO), it is possible to observe a clear trend of an increase in $S_{c/o}$ and a decrease in K_c as $[CO_2]$ around RuBisCO has been reduced (Figure 2). In this sense, pyrenoid-containing green algae show higher $S_{c/o}$ and lower K_c than form IB_c RuBisCO that is associated with the expression of the most efficient CCM – the carboxysomes. Species with biochemical CCMs (C_4 and CAM) show higher $S_{c/o}$ and lower K_c values than form IB RuBisCO in pyrenoid-containing green algae, and C_3 spermatophytes possess the highest $S_{c/o}$ and lowest K_c values of the green lineage (Figure 2). k_{cat}^c also suffered a decrease throughout the green lineage of evolution as a side effect of the increased CO_2 affinity and specificity, with C_3 spermatophytes showing the lowest values, while k_{cat}^c/K_c values displayed an opposite trend. Meyer and Griffiths (2013) have proposed that the selection for improvements in the carboxylation catalytic efficiency in form IB RuBisCO has been relaxed under the saturating CO_2 conditions provided by CCMs in proportion to the extent of time that a CCM has been operating, with values for C_4 plants lower than those for Chlorophyta, and values for Chlorophyta lower than those for Cyanobacteria. Interestingly, K_o values were also lower in organisms with lower $[CO_2]$ around RuBisCO (Figure 2). These results indicate that the capacity of RuBisCO for preferable use of CO_2 was not been gained through repression of the oxygenation capacity. Lack of adaptation in K_o might be related to the role of photorespiration as an energy dissipation process in chlorophytes or streptophytes subjected to excessive irradiance conditions, low temperatures or water scarcity (in the case of terrestrial plants), as previously suggested (André, 2011).

According to Savir *et al.* (2010) and Tcherkez *et al.* (2006), optimization of RuBisCO to the intracellular environment has to inevitably deal with biochemical and structural constraints inherent to this enzyme. This confines RuBisCO evolution to a low-dimensional landscape, which is reflected in statistically significant correlations between the RuBisCO kinetic parameters. Within these biochemical and structural constraints, RuBisCO catalytic traits might have suffered an adaptation to the prevailing $[CO_2]/[O_2]$ and temperature environments, leading to nearly optimal net carbon assimilation rates. The main trade-offs highlighted by Savir *et al.* (2010) and Tcherkez *et al.* (2006) are the inverse relationship between RuBisCO affinity for CO_2 ($1/K_c$) and k_{cat}^c, as well as the inverse relationship between k_{cat}^c and $S_{c/o}$, among others. However, both studies were restricted to a few species and almost all of these possessed form I RuBisCO. Even though there were few outliers (cyanobacteria and proteobacteria species) in their restricted analysis, they suggested an optimization process currently in progress in those species, and so a kinetic adjustment to recent changes in the intracellular environment not yet being completed. This is not very feasible in the context of a likely ancient origin of CCMs in Cyanobacteria. Hence, it raises the possibility that the inherent biochemical and structural constraints might differ not only among different types of RuBisCO, but even within the same type (as observed in form IB from Cyanobacteria versus plants), in response to different evolutionary pressures, leading to a higher RuBisCO kinetic plasticity than previously thought (Cummins *et al.*, 2018). The notable increase in RuBisCO kinetics description of a wide range of species in the last few years allowed us to have a broader picture (see Figure 2). Nevertheless, there are still very few measurements on archaeal (forms III and II/III), bacterial (forms II, IA and IC) and algal (forms ID and IB) species.

Most data to date correspond to terrestrial vascular plants, where these described trade-offs have been unequivocally observed (see Figure 3). Tracheophytes started to diverge 400–300 Mya, experiencing the lowest atmospheric CO_2/O_2 ratio on Earth, and C_4 metabolism did not emerge before 65 Mya (Besnard *et al.*, 2009). The strong selective pressure that land plant RuBisCO might have experienced from its diversification could have driven their RuBisCO towards maximum optimality, that is, increasing the affinity and specificity for CO_2 without an equivalent reduction in k_{cat}^c. Among all phylogenetic groups, the highest RuBisCO carboxylation efficiency (k_{cat}^c/K_c) has been reported in land plants, only shared with Cyanidiophyceae, an ancient thermo-acidophile group of red algae (Figure 2). Yet, within the optima defined by the one-dimensional landscape proposed by Savir *et al.* (2010), there may be further fine tuning to the CO_2 availability at the site of carboxylation (C_c) in plants from different environments on a more recent evolutionary timescale. Examples of this fine tuning are RuBisCOs with low K_c, low k_{cat}^c and high $S_{c/o}$ values in plants from arid and saline environments, where C_c is severely restricted due to diffusive limitations (Galmés *et al.*, 2005, 2014b), and RuBisCOs with high K_c, high k_{cat}^c and low $S_{c/o}$ in plants with C_4 mechanism (Sharwood *et al.*, 2016b), where C_c has increased several fold (Ubierna *et al.*, 2013). The plant genus *Flaveria*

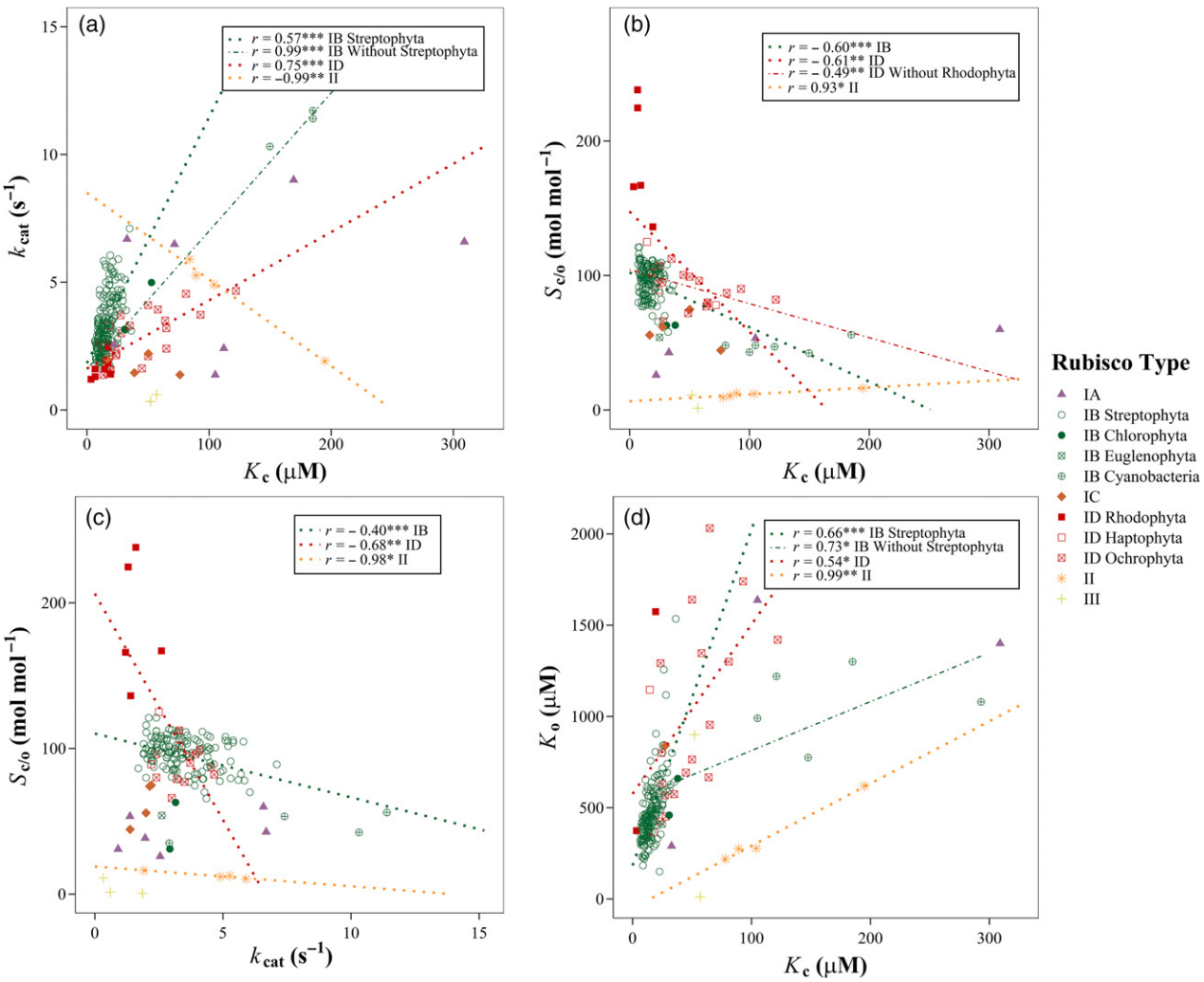


Figure 3. Trade-offs between the RuBisCO kinetic traits at 25°C for the different RuBisCO forms, (a) k_{cat}^{c} versus K_c, (b) $S_{c/o}$ versus K_c, (c) $S_{c/o}$ versus k_{cat}^{c}, (d) K_o versus K_c. Different colours indicate the different RuBisCO forms and different symbols indicate different phyla within the same RuBisCO form. The symbols represent an average value for those species (or bacterial strains) reported by more than one study. Only data measured at 23–30°C were considered and those not measured at the standard temperature of 25°C were standardized using the temperature functions described in Galmés *et al.* (2016). Data are the same as used in Figure 2 (see Data S1). Only statistically significant correlations were drawn. (*p<0.05, **p<0.01, ***p<0.001).

contains C_3, C_4 and C_3–C_4 intermediate members, and adaptation of their RuBisCO kinetics in response to an evolving CCM over a few million years or less has been demonstrated (Kubien *et al.*, 2008; Kapralov *et al.*, 2011; Perdomo *et al.*, 2015). This is a clear example of RuBisCO and CCMs co-evolution. Furthermore, it has been shown that C_3 to C_4 transition in RuBisCO evolution was driven by trade-offs between activity and stability, suggesting that its evolution has been subjected to strong biophysical constraints (Studer *et al.*, 2014).

Nevertheless, in photosynthetic organisms other than vascular plants, these trade-offs are strongly weakened when comparing phylogenetically diverse groups (Galmés *et al.*, 2014a) or even disappear within the same group of organisms, as observed for diatoms (Young *et al.*, 2016), suggesting that they are not universal for all RuBisCOs. However, it must be taken into account that study-to-study differences in the methodologies used for RuBisCO kinetics measurements can weaken the confidence in detecting such trade-offs, and an accurate re-analysis of previously reported data would become necessary to clearly detect RuBisCO phylogenetic evolution and environmental adaptation patterns.

Different trade-offs between the main RuBisCO catalytic traits are found for different RuBisCO forms and among phylogenetically distinct groups (Figure 3). This might be driven by the strength and direction of the selective pressures at which each form of RuBisCO has been exposed during evolution. Archaeal RuBisCO has never been exposed to O_2, so there has been no selective pressure

during evolution to increase its carboxylation efficiency over oxygenation efficiency. Form II RuBisCO is also expressed under anoxygenic or low O_2 conditions and has been exposed to minimal O_2 concentrations throughout its evolution. Moreover, if CCMs in Cyanobacteria have an early origin (see above), their RuBisCOs might never be exposed to such low CO_2 intracellular concentrations as occurred in terrestrial plants. In Cyanobacteria, evolutionary pressure might have led to a selection for higher k_{cat}^c RuBisCOs, instead of selecting those with higher carboxylation efficiency and/or lower oxygenation efficiency. This might explain the striking differences between cyanobacterial RuBisCOs, with the highest k_{cat}^c so far reported but very low carboxylation efficiency, affinity, and specificity for CO_2, and plant RuBisCOs, with the highest carboxylation efficiency, affinity and specificity for CO_2 within form IB RuBisCOs. These differences lead to k_{cat}^c versus K_c and K_c versus K_o trade-offs with a lower slope for Cyanobacteria than for streptophytes (Figure 3a). The existence of contrasting relationships between RuBisCO kinetic parameters observed in Figure 3 may arise from differences in the fundamental mechanisms among the different RuBisCO forms, such as differences in the intrinsic equilibrium of the RuBP enolization reaction (Tcherkez, 2013). Tcherkez (2013) proposed that a deeper knowledge of the associated chemical mechanism of the RuBisCO-mediated reaction is still necessary to better understand the influence of multiple constraints in RuBisCO evolution.

STABLE ISOTOPIC FRACTIONATION AS A TRACER OF RUBISCO EVOLUTION AND OPTIMIZATION

The RuBisCO enzyme imparts a very strong stable isotopic fractionation on fixed carbon. This RuBisCO carbon isotopic fractionation ($\varepsilon_{RuBisCO}$) is empirically determined from the isotopic difference attained between product and substrate when purified, activated RuBisCO is employed *in vitro* to fix dissolved CO_2 (Scott *et al.*, 2011), but it has been determined for a very few number of species. Despite the limited current dataset, the RuBisCO enzymatic fractionation factor is important because it may be indicative of the RuBisCO reaction mechanism (Tcherkez *et al.*, 2006), and because RuBisCO enzymatic fractionation is generally assumed to set the upper limit of *in vivo* expressed photosynthetic isotopic fractionation between a CO_2 substrate and photosynthetically produced biomass (called ε_p; Farquhar *et al.*, 1982; Freeman and Hayes, 1992; Rau *et al.*, 1996). The fixation of CO_2 by RuBisCO occurs through a number of elemental steps: ribulose 1,5-bisphosphate binding, enolization, CO_2 addition and hydration, and cleavage of the intermediate. The use of individual rate constants for these elemental steps, as proposed by Farquhar (1979) and reviewed by Tcherkez (2013), yields mathematical expressions for the usual RuBisCO kinetic traits (k_{cat}^c, K_c, $S_{c/o}$) as well as other chemical parameters such as the $^{12}C/^{13}C$ isotopic fractionation. A key step for both RuBisCO selectivity and RuBisCO carbon isotopic fractionation is the attack of the enzyme-bound enediolized RuBP on a CO_2 molecule to form the carboxyketone intermediate (CKABP). This reaction step is hypothesized to impart selectivity to RuBisCO, based on the degree to which the active site in enzyme-bound enediol captures the bent CO_2 molecule rather than the linear O_2 (Tcherkez *et al.*, 2006). This reaction step is inferred to feature a kinetic activation energy barrier to a transition state (Boyd *et al.*, 2019), which represents the major step in isotopic fractionation by RuBisCO. Carboxylation transition states, which are more specific and similar to the product, correspond to shorter O_2C–C-2 bond lengths. Shorter bond lengths are also higher in energy and vibrational frequency and would therefore entail a greater kinetic isotopic discrimination (Tcherkez *et al.*, 2006; McNevin *et al.*, 2007; Tcherkez *et al.*, 2011). In addition, with greater specificity, the carboxyketone intermediate (CKABP) may be more committed to product formation, resulting in a lower decarboxylation rate.

A positive correlation between $S_{c/o}$ and $\varepsilon_{RuBisCO}$ was previously proposed, largely by the contrast between the high $\varepsilon_{RuBisCO}$ and $S_{c/o}$ of form IB RuBisCO in terrestrial C_3 plants, like *Spinacia oleracea* or *Nicotiana tabacum*, and the lower $\varepsilon_{RuBisCO}$ and $S_{c/o}$ of aquatic prokaryotic photoautotrophs, such as the marine cyanobacteria *Synechococcus* (form IB) and *Prochlorococcus marinus* (form IA; Tcherkez *et al.*, 2006; McNevin *et al.*, 2007). The high $S_{c/o}$ value of terrestrial C_3 plants with RuBisCO IB also coincides with a low K_c, leading to an inverse correlation between $\varepsilon_{RuBisCO}$ and K_c (see Figure 4). The large contrast in kinetic properties among these terrestrial and marine prokaryotic IB forms is consistent with the strong development of CCMs in the marine prokaryotes and its absence in terrestrial C_3 plants, as noted in section 3, a contrast manifest in the RuBisCO fractionation as well.

As $\varepsilon_{RuBisCO}$ determinations have become available from a wider array of organisms, the simple direct linear correlation between RuBisCO specificity and $\varepsilon_{RuBisCO}$ in form IB appears not to be sustained when form ID data from marine eukaryotes and form IC data from Proteobacteria are considered (Figure 4). Two marine non-green algae, with specificity intermediate between C_3 plants and the marine cyanobacteria, exhibit much lower isotopic fractionation. Comparable isotopic fractionation is observed in the soil β-proteobacterium *Ralstonia eutropha*, which is a facultative but not obligate CO_2-fixing autotroph. If the $\varepsilon_{RuBisCO}$ is regulated by the kinetic energy barriers of the carboxylation transition state, the coincidence of widely differing $\varepsilon_{RuBisCO}$ yet similar RuBisCO specificity among marine eukaryotes and marine cyanobacteria may confirm that there is a diversity of mechanistic processes that can yield a similar RuBisCO specificity or K_c, and that a relaxation of RuBisCO specificity in response to existence of CCMs has not been

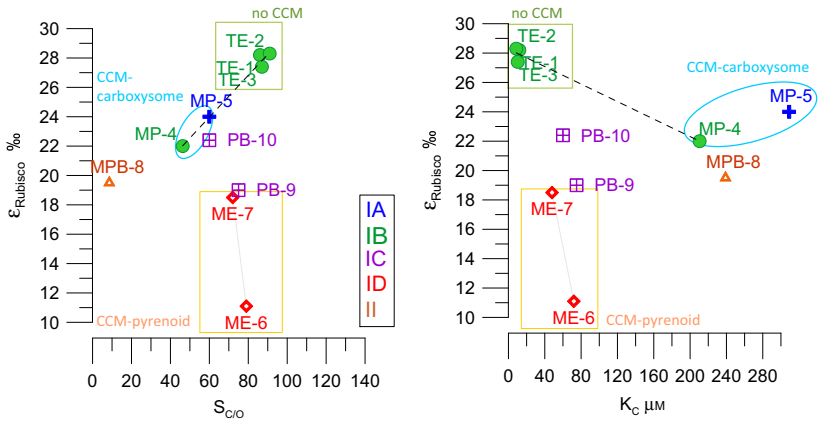


Figure 4. Relationship between RuBisCO *in vitro* carbon isotopic fractionation and the RuBisCO kinetic properties, $S_{c/o}$ and K_c. Colours indicate the RuBisCO form, as shown in the legend. TE (terrestrial eukaryotes, all C_3 in this case), ME (marine eukaryotes), MP (marine cyanobacteria), MPB (marine proteobacteria), PB (non-marine proteobacteria). Compiled organisms and the source of $\varepsilon_{RuBisCO}$ determination are (1) *Spinacia oleracea* (Guy *et al.*, 1993), (2) *Glycine max* (Christeller *et al.*, 1976), (3) *Nicotiana tabacum* (McNevin *et al.*, 2007), (4) *Synechococcus elongates* 6301 (Guy *et al.*, 1993), (5) *Prochlorococcus marinus* MIT9313 (Scott *et al.*, 2007), (6) *Emiliania huxleyi* (Boller *et al.*, 2011), (7) *Skeletonema costatum* (Boller *et al.*, 2015), (8) *Riftia pachyptila* symbiont (Robinson *et al.*, 2003), (9) soil proteobacterium *Ralstonia eutropha* (Thomas *et al.*, 2019), (10) purple non-sulfur proteobacterium *Rhodobacter sphaeroides* (Thomas *et al.*, 2019). Data from $S_{c/o}$ and K_c were obtained from the compiled studies (see Data S1). Rectangles and ellipses highlight the absence of or type of CCMs in obligate photoautotrophs.

driven by a unique process in these organisms. Both the marine prokaryotes and eukaryotic algae in which $\varepsilon_{RuBisCO}$ has been characterized have CCMs, yet there are differences in their CCM architecture.

An additional source of variation in $\varepsilon_{RuBisCO}$ could arise if the carboxylation reaction was partially reversible, as decarboxylation generally fractionates against ^{13}C (O'Leary, 1980). A *N. tabacum* mutant was inferred to possess a very 'loose' transition state for hydrolysis and cleavage and enhanced decarboxylation on the basis of a nearly 10-fold lower k_{cat}^c than wild-type; this mutant also showed much lower RuBisCO fractionation (Mutant L335V; 11.2 ± 1.6‰) than RuBisCO from the wild-type (27.4 ± 0.9‰). The mutant also showed a lower *in vivo* photosynthetic fractionation than the wild-type tobacco (McNevin *et al.*, 2007). Significant decarboxylation reaction in wild-type RuBisCO *in vivo* has been suggested by some models of RuBisCO kinetics (Cummins *et al.*, 2018), but recently debated (Tcherkez *et al.*, 2018) based on estimations on the rate of decarboxylation in *Synechococcus* and *Rhodospirillum* of <5% and <7% of the forward reaction, respectively (Pierce *et al.*, 1986). The possibility of significant decarboxylation under assay conditions and its effect on $\varepsilon_{RuBisCO}$ determinations has not yet been explored.

The *in vivo* $^{13}C/^{12}C$ isotopic fractionation between the external CO_2 source and fixed biomass (ε_p) is expected to approach the RuBisCO enzymatic fractionation ($\varepsilon_{RuBisCO}$) when the ratio of CO_2 supply to RuBisCO is large relative to demand. Conversely, ε_p tends to be lower than $\varepsilon_{RuBisCO}$ as the CO_2 supply diminishes relative to demand. Therefore, ε_p is primarily controlled by the interplay between inorganic carbon supply and demand, that is, CO_2/HCO_3^- acquisition and accumulation mechanisms as well as CO_2 fixation rates and CO_2 leakage out of the cell (Sharkey and Berry, 1985). In some cases, maximum values of *in vivo* ε_p reported has been used to infer $\varepsilon_{RuBisCO}$ (Bidigare *et al.*, 1997). Yet, there is a large difference between *in vitro* $\varepsilon_{RuBisCO}$ of *Emiliania huxleyi* and the maximum *in vivo* whole cell biomass carbon isotopic fractionation of 24‰ observed under high CO_2 conditions, nitrate-limited growth experiments (Bidigare *et al.*, 1997) or 21.5‰ in *E. huxleyi* grown in chemostats (Wilkes *et al.*, 2018), as well as estimates of fractionation from coccolithophores of up to 24‰ from biomarkers isolated from middle-to-late Eocene sediments, a time of presumed high pCO_2 (Pagani *et al.*, 2005). The difference in *in vivo* and *in vitro* fractionation may alternatively result from novel comparable magnitude additional fractionation effects either upstream or downstream of the RuBisCO enzyme in the inorganic carbon utilization mechanisms used by this organism (Tsuji *et al.*, 2008; Wilkes and Pearson, 2019).

The carbon isotopic fractionation of fossilized and lived marine and terrestrial organic matter (ε_p) has long been used to interpret the ecophysiology of marine and terrestrial primary producers and the state of their CO_2-limitation/CCMs operation (Farquhar *et al.*, 1989; Raven *et al.*, 2002a), the latter inference often extended to estimate variations in atmospheric CO_2 in the case of fossilized organisms (Witkowski *et al.*, 2018). These studies have in common the assumption of long-term conservation of $\varepsilon_{RuBisCO}$, as well as a similar $\varepsilon_{RuBisCO}$ among the succession of primary producing communities that have dominated primary production over the Phanerozoic. It remains to be evaluated, whether some proportion of this long-term

variation in *in vivo* carbon isotopic fractionation, may also contain information about the evolution of RuBisCO reaction mechanisms.

TEMPERATURE DEPENDENCE OF RUBISCO KINETIC TRAITS: ADAPTATION TO THE THERMAL ENVIRONMENT AND EFFECTS ON CCM ACTIVITY AND PHOTORESPIRATION

Temperature exerts a direct effect on the velocity of the biochemical reactions, but also modulates the solubility of CO_2 and O_2, their diffusion rates and the equilibrium constants between the different forms of dissolved inorganic carbon.

Despite increased metabolic activity at higher temperatures within the thermal range of each enzyme, RuBisCO oxygenation is favoured over carboxylation at increasing temperatures due to lower dissolved [CO_2] and [CO_2]/[O_2] ratios. This is due to a general decrease in gas solubility and a specific lower solubility of CO_2 than O_2 at warmer temperatures (Skirrow, 1975). Furthermore, higher temperature leads not only to increased k_{cat}^c up to its optimum temperature (usually 50–60°C; Galmés *et al.*, 2015), but also to increased K_c (i.e. lower affinity for CO_2) and reduced $S_{c/o}$ values as universal trends in RuBisCO catalytic behaviour (Jordan and Ogren, 1984; Uemura *et al.*, 1997; Bernacchi *et al.*, 2001; Galmés *et al.*, 2016, 2019). k_{cat}^c seems to be the most responsive parameter to temperature changes (Jordan and Ogren, 1984; Perdomo *et al.*, 2015; Galmés *et al.*, 2015), so the reduction in k_{cat}^c and, consequently, carboxylation efficiency, might be an important issue in extremely cold environments. Nevertheless, data compiled by Galmés *et al.* (2016) revealed that there are phylogenetic groups not analyzed at all for the thermal dependencies for K_c or k_{cat}^c, which precludes generalization to all RuBisCOs. Evidence suggests that the evolution of RuBisCO catalytic constants has also been driven by the prevailing growth temperature (Sage, 2002; Galmés *et al.*, 2005; Tcherkez *et al.*, 2006; Galmés *et al.*, 2016), apart from the CO_2 and O_2 concentrations at the RuBisCO active sites. Within the described temperature general trends, RuBisCO thermal dependencies vary significantly among different organisms, even within closely related species (Galmés *et al.*, 2016; Hermida-Carrera *et al.*, 2016; Orr *et al.*, 2016). When described with an Arrhenius model, the activation energy (ΔH_a) for $S_{c/o}$ varied nearly four-fold, while the ΔH_a for K_c and k_{cat}^c varied three-fold and two-fold, respectively, among the different phylogenetic groups analyzed (Galmés *et al.*, 2015, 2016). However, most of the data came from terrestrial vascular plants and very few species of archaea, bacteria and algae have been investigated so far to draw a conclusive thermal trend outside the spermatophytes. The lowest and highest ΔH_a for $S_{c/o}$ was reported for the cyanobacterium *Synechococcus lividus* and the thermoacidophilic red alga *Galdieria partita*, respectively (Uemura *et al.*, 1997; Zhu *et al.*, 1998). Moreover, the highest value of ΔH_a for $S_{c/o}$ among the only four diatoms analyzed so far (Haslam *et al.*, 2005) was obtained for the one with the highest optimum growth temperature. The same trend was observed for ΔH_a for K_c and for the carboxylation rate of the only two diatoms analyzed for the thermal dependencies of these parameters (Young *et al.*, 2015), with the Arctic diatom showing lower thermal dependencies than the temperate one. The lowest values of ΔH_a for k_{cat}^c ever reported were found for the cyanobacteria and chlorophyte species, which were almost two-fold lower than those reported for terrestrial plants (compiled by Galmés *et al.*, 2015). Although ΔH_a for k_{cat}^c was not correlated with the optimum growth temperature for the few analyzed species (Galmés *et al.*, 2015), a RuBisCO adaptation to the ecological niche of the different phylogenetic groups can be observed in Figure 5. Cyanobacteria are adapted to a wider range of thermal environments than diatoms, and so the lower thermal response of their RuBisCO kinetic traits has led to a narrow variation in the carboxylation rate per active site between 10 and 30°C. Conversely, diatoms dominate cold-temperate to polar oceans and so the carboxylation rate per active site at 10°C saturates with only threefold CCM-mediated increase in [CO_2] at RuBisCO site and photorespiration reached its minimum rate. Still, the maximum carboxylation rate per active site is significantly reduced at 10°C, and so diatoms will require higher RuBisCO concentrations and/or higher activation states to sustain net photosynthetic rates at low temperatures (see explanation below).

Within the same species, RuBisCO thermal adaptation can be also observed between populations from different latitudes. Iñiguez *et al.* (2018) found significantly higher k_{cat}^c and RuBisCO carboxylation efficiency at 4°C in polar non-green seaweed populations compared with their temperate counterparts. Nevertheless, few differences were found in the thermal response of those parameters between temperate and polar populations of a green seaweed (Iñiguez *et al.*, 2018), in a similar way to the results obtained by Devos *et al.* (1998) for psychrophilic and mesophilic green algae.

Regarding terrestrial spermatophytes, a recent compilation of more than 100 species revealed that C_3 plants possess a significantly higher ΔH_a for k_{cat}^c than C_4 plants, although no significant differences regarding the thermal dependencies of any RuBisCO catalytic trait was found between C_3 plants from cold and warm environments (Galmés *et al.*, 2019). However, some differences were found between cool and warm C_3 plants in the average RuBisCO kinetic trait values at discrete temperatures (e.g. higher k_{cat}^c, K_c and $S_{c/o}$ in C_3 species from cold environments relative to those from warm environments). These results indicated that there is an adaptation to the photosynthetic mechanism but limited adaptation to the thermal

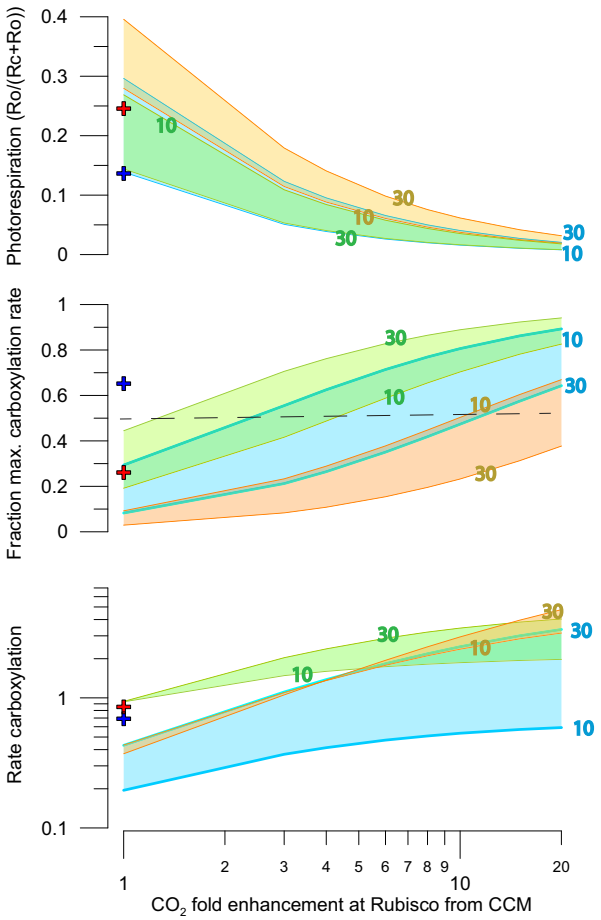


Figure 5. Change in RuBisCO CO_2 assimilation capacity between 10 and 30°C showing CCMs enhancement in the cyanobacterium *Synechococcus* sp. (orange lines), the diatom *Thalassiosira weissflogii* (blue lines) and the C_4 plant *Zea mays* (green lines), using the RuBisCO kinetic traits compiled (Data S1) and the thermal dependencies reported by Galmés *et al.* (2016, 2019). Numbers on or adjacent to each curve indicate the temperature used for the calculation, in °C. For the diatom, data at different temperatures were obtained from Young *et al.* (2015). The thermal dependencies for K_c used for the cyanobacterium are those from *Anabaena variabilis* and for $S_{c/o}$ used for the diatom are those from *Skeletonema costatum*, due to the absence of these measurements for the selected species. The thermal dependencies of K_o for the cyanobacterium and the diatom were assumed to be similar to the thermal dependencies of K_c. pCO_2 at the RuBisCO active site without CCM enhancement was assumed to be 280 ppm and the pO_2 was assumed to be 21%. Dissolved CO_2 and O_2 were calculated for each temperature, assuming an ionic strength of 0.1 M inside the chloroplast. The red (30°C) and blue (10°C) crosses represent the RuBisCO CO_2 assimilation capacity for the C_3 plant *Triticum aestivum*. The upper graph represents the proportion of oxygenation relative to the whole RuBisCO activity (oxygenation rate, R_o, plus carboxylation rate, R_c), the graph in the middle represents the proportion of carboxylation relative to the maximum carboxylation rate, and the lower graph represents the carboxylation rate in mol CO_2 fixed mol^{-1} RuBisCO active site s^{-1} at each temperature. The dashed line in the middle panel highlights 50% of maximum carboxylation rate, to facilitate comparison.

environment. More notably, a positive relationship between ΔH_a for $S_{c/o}$ and ΔH_a for k_{cat}^c indicated that the widely assumed trade-offs between these RuBisCO catalytic traits at 25°C (Figure 3c) vary with temperature, and that contrasting relationships within spermatophytes are in principle possible.

Few studies of RuBisCO temperature sensitivity report data on K_o, and the available data (only for spermatophytes) show contradictory results, with K_o showing no thermal sensitivity, while increasing or even decreasing with temperature in other cases (Badger and Collatz, 1977; Jordan and Ogren, 1984; Boyd *et al.*, 2015, 2019). This lack of consistent trend might be related to the difficulty of directly measuring this parameter, because K_o is usually calculated indirectly from the inhibition of the carboxylase activity at different O_2 concentrations, and this approach might lead to less reliable data.

Additionally, the existence of thermal breakpoints in the temperature response of RuBisCO kinetic traits has been proposed on the basis of a biphasic Arrhenius temperature response mainly in k_{cat}^c (Sage, 2002; Kubien *et al.*, 2003; Perdomo *et al.*, 2015; Sharwood *et al.*, 2016a), but also in K_{cat}^o, K_c and/or $S_{c/o}$ (Badger and Collatz, 1977; Boyd *et al.*, 2019) of plant RuBisCOs, leading to E_a values that differ between temperature ranges. Different explanations for these thermal breakpoints have been proposed, such as changes in the rate-limiting step of the reaction mechanism caused by changes in enzyme conformation (Badger and Collatz, 1977), deactivation of RuBisCO at low temperatures (Sage, 2002; Kubien *et al.*, 2003), or differences in the thermal responses of the elementary rate constants proposed by Farquhar (1979) that describe the reaction mechanism of RuBisCO (Boyd *et al.*, 2019). It is unknown if these thermal breakpoints are universal to all RuBisCOs, as most of the data of thermal dependence of RuBisCO kinetic traits to date was obtained in studies where only three or four temperatures were analyzed (Haslam *et al.*, 2005; Galmés *et al.*, 2005; Hermida-Carrera *et al.*, 2016; Orr *et al.*, 2016). To clearly identify these thermal breakpoints, a minimum of six different temperatures within a broad temperature range is needed.

RuBisCO adaptation to the thermal environment might be also attained by the modification of the enzymatic optimum temperature. The existence of an important variation in the optimum temperature for k_{cat}^c among the different phylogenetic groups has been reported (Galmés *et al.*, 2015), although the limited data do not correlate with the optimum growth temperature of the studied organisms. The highest ever reported RuBisCO optimum temperature, 90°C, corresponds with the thermophilic archaeon *T. kodakarensis* (Ezaki *et al.*, 1999), which agrees well with its optimum growth temperature of 85°C, suggesting either an adaptation to the thermal environment in this species or maybe an ancient optimum temperature for all RuBisCOs that was reduced throughout evolution in the rest of the phylogenetic groups. Within spermatophytes, C_3 plants from warm habitats had significantly higher optimum

temperature for k_{cat}^{c} than C_3 plants from cold habitats (Galmés *et al.*, 2015), revealing that enzymatic optimum temperature fine tuning also occurred relatively recent in the evolutionary timescale.

The universal trend of a lower affinity and specificity for CO_2 at elevated temperatures common to all RuBisCOs, along with a lower dissolved [CO_2] and [CO_2]/[O_2] ratios, which favour oxygenation over carboxylation and hence photorespiration, has been related to the evolution and diversification of C_4 and CAM plants under warmer climate conditions (Ehleringer *et al.*, 1991). The appearance of C_4 and CAM metabolisms enables these plants to maintain high net photosynthetic rates with a reduced investment in RuBisCO synthesis (and so, less nitrogen investment) in those environments. This explains the high abundance of C_4 and CAM plant species in warm environments that do not extend to cold environments (high latitude and altitude ecosystems) due to the energy cost of CCMs, contrary to C_3 plants (Raven *et al.*, 2017).

Following the same premise, low water temperatures would decrease the need for CCM operation in aquatic photosynthetic organisms, due to the higher RuBisCO affinity and specificity for CO_2 and the higher dissolved [CO_2] and [CO_2]/[O_2] ratio in cold waters (Raven *et al.*, 2002b). As shown in Figure 5, reaching 50% saturation of RuBisCO for a diatom requires a 15-fold increase in [CO_2] at RuBisCO active sites at 30°C, whereas at 10°C, it requires only a three-fold increase in [CO_2] while, for cyanobacteria, it requires more than a 30-fold increase in [CO_2] at RuBisCO active sites at 30°C, and only a 10-fold increase in [CO_2] at 10°C. In a C_4 plant, reaching 50% saturation of RuBisCO does not require CCM enhancement at 10°C, while it requires a four-fold increase in [CO_2] at 30°C (Figure 5). As CCM does not improve the saturation of this C_4 RuBisCO at cold temperatures, the C_4 biochemical CCM does not confer an advantage while it has an energy cost; this may explain why C_3 plants outcompete C_4 plants in cold environments. However, all these assumptions are based on potential RuBisCO assimilation capacity and do not take into account temperature-driven changes in the conductance of CO_2 to the RuBisCO active sites, as well as changes in RuBisCO concentration, the activation state of the enzyme, and the contribution of respiratory and photorespiratory CO_2 release. Low temperatures produce a slower CO_2 equilibration between the surface waters and the atmosphere (Raven and Falkowski, 1999) and a slower equilibration between the different dissolved inorganic carbon forms (Egleston *et al.*, 2010). Furthermore, at lower temperatures, CO_2 diffusion within the cell is also slowed down (Boudreau, 1997). These changes induce a strong reduction in [CO_2] around RuBisCO during steady-state photosynthesis relative to the corresponding dissolved CO_2 levels at equilibrium conditions, and would justify the presence of CCMs in many photosynthetic organisms inhabiting cold aquatic environments (Mitchell and Beardall, 1996; Beardall and Roberts, 1999; Iñiguez *et al.*, 2018). Nevertheless, the energy required to achieve near CO_2 saturation conditions around RuBisCO at low temperatures would probably decrease with respect to warmer waters, as previously suggested by Kranz *et al.* (2015) for polar diatoms.

An increase in RuBisCO content and activation state at lower temperatures might be a common acclimation or adaptation response among photosynthetic organisms, as increased affinity and specificity for CO_2 do not compensate for the strong decline in maximum carboxylation activity. For a five-fold CCM-mediated increase in [CO_2] in a diatom, which corresponds with nearly CO_2 saturated carboxylation rate at 10°C, the carboxylation rate at 10°C is still nearly five-fold lower than the carboxylation rate of the same species at 30°C (Figure 5). This implies that a greater cellular quota of RuBisCO active sites would be required at lower temperatures to meet the same cellular carboxylation rates as at warm temperatures, for the same CCM investment.

Significantly higher RuBisCO content has been shown for cold-adapted diatoms, chlorophytes, and land plants in comparison with their temperate counterparts (Devos *et al.*, 1998; Yamori *et al.*, 2005; Young *et al.*, 2015; Jaikumar *et al.*, 2016; Iñiguez *et al.*, 2018). Moreover, the maximum RuBisCO carboxylation rate also depends on the binding of sugar phosphate inhibitors to the catalytic site and RuBisCO activase function. RuBisCO activase promotes the removal of these tightly bound inhibitors from the catalytic sites of both active and inactive forms of RuBisCO by ATP hydrolysis (Parry *et al.*, 2008). Thus, either elevated RuBisCO activase activity or quantity and/or lower affinity for tight binding inhibitors would be required for maintaining high levels of RuBisCO activation state at low temperatures, although this has not yet been corroborated. An almost 100% activation state of RuBisCO has been observed in Antarctic vascular plants (Salvucci and Crafts-Brandner, 2004; Pérez-Torres *et al.*, 2006), and the same response is suspected for polar algae, based on RuBisCO kinetic and quantity measurements at their environmental temperatures, in order to achieve the *in vivo* carbon fixation rates measured for these species (Young *et al.*, 2015; Iñiguez *et al.*, 2018).

CONCLUDING REMARKS

The analysis of a wide range of RuBisCO kinetic traits reveals diverse patterns among the RuBisCO forms and phylogenetic groups as a consequence of the operation of different environmental selective pressures throughout evolution. The main environmental driver is the CO_2/O_2 ratio at the site of carboxylation, which in turn is affected by temperature, ambient CO_2 and O_2 concentrations, the CO_2 and O_2 conductances to the RuBisCO active sites, and the presence or lack of CCMs in the given organism.

Evolutionary pathways that allowed an increase in net CO_2 assimilation throughout the diversification of the species run in two directions, either improvements in the RuBisCO carboxylation efficiency and CO_2 selectivity or the appearance of CCMs. In those organism in which a CCM evolved, relaxation of the selective pressure to improve CO_2 affinity and selectivity allowed the evolutionary selection of RuBisCOs with higher carboxylation rates. The described differences in RuBisCO kinetic traits between organisms expressing or not CCMs are observed among relatively closer phylogenetic groups that have a common RuBisCO evolutionary background previous to the appearance of these CCMs. This cannot be assumed as a universal trend when comparing all organisms with or without CCMs. Moreover, unexpected differences in the trade-offs between the kinetic traits of different RuBisCO forms suggest diverse biochemical and structural constraints, and not a universal low-dimension landscape of optimality as previously suggested.

Still, a much wider range of RuBisCO kinetics measurements, including carbon isotopic fractionation measurements of *in vitro* purified RuBisCO versus *in vivo* biomass, of the underrepresented groups, as well as a more profound knowledge of the chemical mechanism of the RuBisCO-mediated reaction, are necessary to better identify the different constraints shaping RuBisCO evolution.

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AUTHOR CONTRIBUTIONS

JG and UN compiled and standardized the data from previous studies. CI, SC-B, HS and PA-N carried out the analyses and produced the figures. CI wrote most parts of the manuscript with the contribution of all co-authors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data gathered are available in the supporting material and were obtained from the literature cited in this material.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Data S1. Compiled RuBisCO kinetic traits ($S_{c/o}$; k_{cat}^{c}; K_c; K_o) from previously published studies used to produce Figures 2–5.

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