Primer

The biology of carbon dioxide

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Carbon dioxide is the substrate for the central carbon-fixing enzyme of photosynthesis, ribulose bisphosphate carboxylase-oxygenase (Rubisco), and is the form of inorganic carbon produced in respiration. The CO_2 concentration in the atmosphere is increasing as a result of additional inputs from the burning of fossil fuels and from deforestation in excess of what can be removed by additional photosynthesis on land and solution in the ocean. This has implications not only for photosynthesis, but also for global climate: CO_2 is the second most important greenhouse gas, after H_2O vapour, in the atmosphere.

This continuing increase in atmospheric CO_2, from ~280 μmol per mol in the mid eighteenth century to ~370 μmol per mol today, has many precedents in the time since the first O_2 evolving photosynthetic organisms occurred (Figure 1). In order to understand the means by which photosynthetic organisms respond to the current increase in CO_2 we first consider the variations in CO_2 during the evolution of photosynthetic organisms, as well as how these organisms have responded to, and helped to cause, these changes.

The history of atmospheric CO_2

In the absence of anthropogenic influences the major inputs of CO_2 to the atmosphere today is biosphere respiration, and the major output is biosphere photosynthesis. These fluxes in respiration and gross photosynthesis are about 150 Pg (petagrams, x10^{15}) carbon per year and determine the atmospheric CO_2 in the (geologically) short term of tens to hundred of years. Over rather longer time periods of the order of hundreds of thousands of years — such as Pleistocene glacial–interglacial cycles — equilibration with the ocean is a major controller of atmospheric CO_2. Finally, over times of millions of years and more, the rock cycle regulates atmospheric CO_2, even though the fluxes of subduction of sedimented carbon and the release of carbon from volcanoes as CO_2 is only some 0.2 Pg carbon per year.

The outcome of these processes is seen in the changes in atmospheric CO_2 over geological time (Box 1). The further back in Earth history, the less constraint there is on estimates of atmospheric CO_2. For the last ~700,000 years, the gases in polar ice give very accurate and precise estimates of atmospheric CO_2, which varies from as high as 280 μmol per mol in interglacial periods to as little as 180 μmol per mol at the glacial maxima.

For most of the rest of the Phanerozoic, back to ~500 million years ago, atmospheric CO_2 has been estimated from biogeochemical modelling, and for the last ~400 million years, from the natural abundance of stable carbon isotopes in palaeosol carbonates and from the density of stomata in terrestrial embryophytic plants. Such studies reveal a period of low CO_2, similar to today's values, during a glacial episode in the Carboniferous–Permian periods some 300 million years ago. For the rest of the Phanerozoic the CO_2 levels were higher than today's values, with perhaps 20 times today's value about 400 million years ago.

For the Precambrian period there are even less clear constraints on estimates of atmospheric CO_2. One constraint is that of the occurrence of liquid water on the Earth in the context of the greenhouse effect and the increasing radiant energy output of the sun, which rose by some 25% over the last 4.5 billion years. A problem here is that CO_2 is not the only possible greenhouse gas apart from H_2O vapour: especially before the oxygenation of the atmosphere, beginning some 2.3 billion years ago, CH_4 was probably more important than it has been for most of the time that the atmosphere has been oxygenated. Another constraint is the occurrence of the iron mineral siderite. It is probable that the atmospheric CO_2 level before 500 million years ago was generally significantly higher than it is today, but could have been similar to today's value about 2000 million years ago, and especially at ~700 and ~600 million years ago during glacial episodes.

The evolution of photosynthetic organisms and atmospheric CO_2

Two lines of evidence as to the occurrence of photosynthetic organisms 3.45–3.8 billion years ago have come under critical scrutiny, and as a result we can no longer be sure that there were photosynthetic organisms, and especially O_2-evolvers, before perhaps 2.7 billion years or, even, very conservatively, 1.9 billion years ago.

The chemical fossil evidence for cyanobacteria goes back to 2.7 billion years ago, while there is

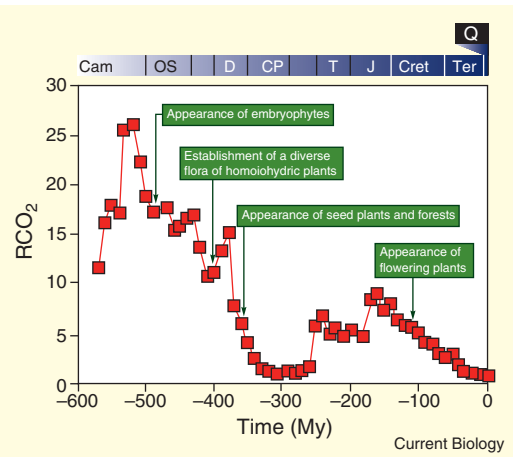

Figure 1. Variation in atmospheric CO_2 with time.

CO_2 content of the atmosphere relative to the present content (RCO_2) through the Phanerozoic according to Berner and Kothavala's Geocarb III model. Time plotted as million years (My) ago from present. Three significant events in the evolution of terrestrial vegetation are indicated. Key to time axis: Cam, Cambrian; O, Ordovician; S, Silurian; D, Devonian; C, Carboniferous; P, Permian; T, Triassic; J, Jurassic; Cret, Cretaceous; Ter, Tertiary; Q, Quaternary. Reproduced with permission from American Journal of Science.

macrofossil evidence of a red alga from about 1.2 billion years ago. Other marine eukaryotic photosynthetic organisms are known from later in the Proterozoic, and a diversity of marine photosynthetic organisms is known from the Cambrian (from 540 million years ago) onward. Terrestrial photosynthetic organisms belonging to the embryophytes are known from about 490 million years ago, and organisms with stomata and other features of homiohydry were diverse by 400 million years ago, with forests of *Archaeopteris* by 360 million years ago.

Taxonomically and structurally diverse forests, including seed plants, were widespread by the time of the low CO_2 episode ~300 million years ago. The decrease in atmospheric CO_2 between ~380 and ~320 million years is related to the increased land area occupied by plants of increasing stature with deeper root systems, with CO_2 removal from the atmosphere as increased organic carbon burial and, especially, increased weathering of carbonate and silicate rocks. Flowering plants originated about 120 million years ago.

There has been a general downward trend in atmospheric CO_2 since O_2-producing organisms evolved, albeit with significant fluctuations. Rubisco apparently evolved as a carboxylase at a time of higher atmospheric (and oceanic) CO_2 so that a relatively low affinity for CO_2 would not be a problem. As Rubisco apparently originated before global oxygenation, the oxygenase activity was not expressed. As CO_2 decreased and O_2 increased, the kinetic characteristics of Rubisco became an impediment to achieving high rates of photosynthesis, and in some clades the kinetic characteristics of Rubisco changed in evolution so that photosynthesis per unit enzyme became higher at relatively low CO_2 and high O_2 concentrations.

However, there are limits to the extent of change in these kinetic parameters, and many photosynthetic organisms in aquatic environments today have inorganic carbon concentrating mechanisms (CCMs). CCMs overcome the kinetic 'deficiencies' of Rubisco by increasing the CO_2 concentration, and the CO_2:O_2 ratio, around Rubisco by energetically uphill transport of one or more of the species CO_2, HCO_3^- and H^+, or by energized biochemical reactions resembling C_4 metabolism in some higher plants. CCMs are clearly polyphyletic.

These CCMs presumably evolved when CO_2 concentrations in the surface ocean were low — at any of the times mentioned above as having major glaciations. Earlier evolution of CCMs would mean greater problems of maintaining CCMs in intervening higher-CO_2 episodes, except as small populations in low-CO_2 refugia. While comparisons of the fossil-calibrated molecular phylogenies of the organisms with the molecular phylogeny of the CCM components and the time of low CO_2 episodes should help to distinguish among possible times of evolution of CCMs, there are relatively few known molecular

Box 1. Terminology.

CCMs (inorganic carbon concentrating mechanisms): CCMs are a range of energy-requiring mechanisms which ensure that the CO_2 concentration around Rubisco during steady-state photosynthesis is higher than that in the environment. The environment is taken as the surrounding water for aquatic organisms, and water in equilibrium with the atmosphere for land plants.

Embryophyte: embryophytes are often termed 'higher plants'. These evolutionary descendants of certain green algae include the seed-bearing plants, such as flowering plants, and the ferns and their relatives. These are all vascular plants with xylem, tissue, and are by far the predominant primary producers on land. The embryophytes also include the hornworts liverworts and mosses, which lack xylem.

Euphyllophyte leaves: the euphyllophytes are the predominant vascular plants on Earth today, including the ferns and horsetails as well as the seed plants. One of their characteristics is their leaf structure. The other extant vascular plants are the lycopods, which have a different evolutionary origin of their leaves.

ECM (extracellular matrix): in plants the ECM refers the material external to the outer face of the plasma membrane. This includes the cellulose, hemicellulose and pectin components of the cell wall, various cell-wall-associated proteins, the pectin rich middle lamella and in the cells of the leaf epidermis the cuticle.

Homoiohydry: homoiohydry is the typical condition of vascular plants, with the capacity to greatly restrict the rate of water loss (at the expense of also greatly reducing the rate of photosynthesis) when water supply from the soil is not adequate to meet the evaporative demands of the atmosphere. The reduction in the rate of gas exchange involves the closure of stomata in the plant shoot, increasing a variable resistance in parallel with the large fixed resistance of the cuticle. Photosynthesis, with evaporative water loss, occurs when the stomata are open, allowing gas exchange between the atmosphere and intercellular gas spaces. The water lost in evaporation is replaced by soil water, the water lost being replaced by soil water moving up the plant in the xylem.

KCS (β-ketoacyl-CoA synthase): KCS is part of the fatty acid elongase complex responsible for the synthesis of very long chain fatty acids.

Phanerozoic: the time of 'apparent or evident life' characterized by the occurrence of fossils of mineralized invertebrates and, later, invertebrates. The Phanerozoic began about 540 million years ago.

Precambrian: the time before about 540 million years ago; fossils are much less abundant than during the Phanerozoic.

Rock cycle: the rock cycle is a part of the carbon cycle which takes carbonate minerals and particulate organic carbon which has been deposited on the ocean floor and incorporates them into the Earth's crust. After, on average, millions of years the carbon reappears at the Earth's surface in vulcanism and mountain building, with CO_2 released to the atmosphere.

Rubisco (ribulose bisphosphate carboxylase-oxygenase): this enzyme catalyses two major reactions, the carboxylase reaction which adds carbon dioxide to an organic molecule, and an oxygenase which adds oxygen to an organic molecule. Rubisco is the most abundant protein, in terms of mass, on Earth, and is involved in the assimilation of about 150 Pg carbon from CO_2 each year.

markers for the core components of CCMs. Such markers are available for a major clade of cyanobacteria, but even that does not clearly indicate the time of evolution of these CCMs. Horizontal gene transfer is, as ever, a complicating possibility for such analyses.

One point that is clear about CCMs in marine primary producers is that the expression of the CCM, as indicated by the inorganic carbon affinity, is a function of the inorganic carbon concentration, and specifically the CO_2 concentration, used for growth of the organism. We discuss below what is known of the sensory pathway for this control mechanism.

For most embryophytic land plants, stomata are important for regulating photosynthesis relative to water vapour loss in transpiration. CO_2 supply to the surface of photosynthesising plants on land is, other things being equal, favoured relative to that in aquatic plants, as a result of differences in diffusion coefficient and diffusion boundary layer thickness in the two fluid media. But for land plants there is necessarily water loss in transpiration whenever atmospheric CO_2 is taken up by cells. For the great majority of land plants, which are desiccation-intolerant, the cuticle-stomata-gas space homoiohydric system is crucial for allowing plants to photosynthesise when water is available, but conserve water at the expense of photosynthesis when water supply is limited relative to the potential evaporative demand.

The stomatal index — the fraction of epidermal cells that are stomatal guard cells — and density — the number of stomata per unit area of photosynthetic organ — are very generally a phenotypic function of the CO_2 concentration at which a plant is grown. This effect is also seen in the fossil record, which shows a very significant increase in stomatal density as CO_2 decreased between 400 and 300 million years ago, along with an increase in size of euphyllophyte leaves, although this is likely to be largely a genotypic effect. Subsequently in this paper we examine the signalling pathways involved in regulation of the CO_2-dependent changes in stomatal density and some associated anatomical changes.

Elevated concentrations of CO_2 promote biomass accumulation

The numerous studies that have been carried out on the effects of elevated CO_2 – typically double current ambient concentrations – on plants have shown that, in general, compared to plants grown under ambient concentrations of CO_2, growth at elevated CO_2 results in the production of bigger plants with fatter leaves. The ability of elevated CO_2 to promote growth and the accumulation of biomass has been recognized by the horticultural industry for many years where it is common practice to grow glasshouse crops under conditions of enriched CO_2.

Although the effects of elevated CO_2 on plant growth and morphology are well documented, we know much less about the fundamental molecular details of how elevated CO_2 controls plant development. For example, even though we know that elevated CO_2 induces increased cell division in some species while inducing cell expansion in others, we know rather little about the signalling pathway(s) responsible for coupling CO_2 to such responses.

Control of guard cell development by CO_2

Perhaps the best-studied example of a CO_2-regulated developmental response in plants is the effect of elevated CO_2 on stomatal development. Almost 20 years ago, Woodward observed that there is a correlation between the density of stomata found on the leaf surface and atmospheric CO_2 concentration. Subsequent work revealed that stomatal density in many, but not all, species declines in response to increasing concentrations of CO_2. Interestingly, the correlation is not absolute, and even within a single species there is variability in the response.

The ecological significance, if any, of this variability has yet to be explained. Of considerable relevance to our understanding of CO_2 signalling was the demonstration that the mature leaves control the number of stomata that form on newly developing leaves. This tells us two important things about CO_2 signalling in plants: first, there is a systemic long-distance signalling system in operation for detecting CO_2 levels and sending this information to developing leaves; and second, that, at least as far as stomatal development is concerned, newly developing leaves are incapable of detecting changes in atmospheric CO_2, so the CO_2 sensor for this signalling pathway must reside in the mature leaves.

CO_2 sensing

How are CO_2 levels detected in plants? It seems possible that there may be more than one mechanism. Long and colleagues suggest that Rubisco is the primary CO_2 sensor, and that because of the presence of the relatively gas impermeable waxy cuticle, the sites of CO_2 detection must be the guard cells or the underlying mesophyll cells.

If Rubisco does act as a CO_2 sensor, then a likely candidate for a long-distance CO_2 signalling molecule would be a product of photosynthesis, such as sucrose. In fact, given the emerging signalling profile of sucrose, this molecule is an attractive candidate master regulator of many of the developmental and growth responses to elevated CO_2. However, this possibility is somewhat tempered by the results of von Caemmerer and colleagues, who observed similar stomatal densities in wild-type tobacco plants and transgenic plants with just 10–15% of the normal levels of Rubisco.

Downstream from CO_2 perception

At present we only know the identity of a single component in the signalling pathway by which elevated CO_2 controls stomatal development — the product of the *HIC* gene. *Arabidopsis hic* mutant plants develop up to 25% more stomata than wild-type when grown at elevated CO_2. *HIC* encodes a putative β keto acyl CoA synthase (KCS) which is part of the

fatty acid elongase complex responsible for the synthesis of very long chain fatty acids (VLCFAs).

Among other things, VLCFAs are used in the biosynthesis of the wax components of the plant cuticle and extracellular matrix (ECM). *Arabidopsis* has a large KCS gene family and mutants in other family members, such as *FIDDLEHEAD*, show extreme developmental phenotypes, including organ fusions. How might the *HIC* gene product negatively regulate stomatal development at elevated CO_2?

Current ideas are focussed on the ECM, specifically its role in the control of cell fate. In the case of HIC, the current hypothesis is that the altered wax composition of the *hic* mutant ECM permits more stomata to form when the plants are grown at elevated CO_2. Whether the (presumed) altered wax composition affects the physical properties of the ECM such that the diffusion of an unidentified morphogen controlling epidermal cell fate is affected, or whether ECM components are directly responsible for the control of guard cell development, is not yet known.

We also do not know how the HIC signalling pathway interfaces with and modulates the primary signalling pathway controlling stomatal development, which is believed to involve a subtilisin like protease (SDD1), a leucine rich repeat receptor like protein (TMM) and a MAP kinase kinase kinase (YODA). Understanding how environmental signals modulate this pathway is obviously important. But the picture is likely to be complex, as it is known that the magnitude of the CO_2-induced effect on stomatal development is itself modulated by light.

Another CO_2 signalling pathway in the epidermis

CO_2 also controls the aperture of the stomatal pore. When stomata are exposed to elevated CO_2 there is a rapid decrease in guard cell turgor resulting in a reduction in the aperture of the stomatal pore. Currently, we do not know the identity of the guard cell CO_2 sensor, although it seems unlikely that it will be Rubisco as the transgenic tobacco plants with reduced Rubisco mentioned above display wild-type stomatal responses to CO_2.

Whatever the nature of the guard cell CO_2 sensor the fact that *hic* mutant stomata close in response to elevated CO_2 suggests that this is a totally separate pathway. Current evidence implicates malate and the intracellular second messengers calcium and pH in CO_2-induced stomatal closure. There is also evidence that the CO_2 signalling pathway interacts with the guard cell abscisic acid (ABA) signalling pathway, as the *Arabidopsis abi1-1* mutant, which is insensitive to ABA, also fails to respond to elevated CO_2.

Not only are we in the dark about the nature of the guard cell CO_2 sensor, but we also do not know the identity of the molecular species of inorganic carbon that guard cells detect. Presumably the receptor will be located either inside of, or at the surface of, the guard cell, in which case it will detect CO_2 dissolved in the apoplasm. Accordingly it is possible that the receptor could detect either dissolved CO_2 or bicarbonate. Are there any clues concerning the operation of CO_2 signalling pathways emerging from studies of other organisms?

CO_2 signalling in cyanobacteria and Chlamydomonas

Cyanobacteria appear to sense inorganic carbon supply in regulation of the CCMs through the intracellular inorganic carbon concentration, or its metabolic outcome in terms of photorespiration. Tempting as it may be to invoke the bicarbonate regulation of adenylyl cyclase (a soluble non-G protein activated form of the enzyme), and the PKA signalling pathway, in inorganic carbon sensing in cyanobacteria, a lot more work is needed. Furthermore, there is no evidence that this system is found in higher plants (although interestingly bicarbonate-activated adenylyl cyclase is found in other bacteria, the slime mould *Dictyostelium*, arthropods and mammals).

Freshwater flagellates such as *Chlamydomonas* and *Euglena* also detect and respond to CO_2. Experiments on chemotactic behaviour in *Chlamydomonas* have revealed that they respond to dissolved CO_2 rather than bicarbonate. *Chlamydomonas* also responds to low CO_2 availability through the induction of a carbon-concentrating mechanism (CCM) discussed above. Underlying this process is a signal transduction system linking CO_2 perception with the induction of the genes involved in the CCM.

The master regulator in the low CO_2 induction of CCM genes is a zinc finger transcriptional regulator known as CCM1 (also known as CIA5). Among the genes induced by low CO_2 through the CCM1 are *Lcr1*, which encodes a Myb-related protein and a carbonic anhydrase (*Cah1*). In the current model for induction of *Cah1* by low CO_2, LCR1 amplifies the extent of CCM1-induced *Cah1* expression. The observation that CCM1 is constituently expressed suggests that it must be activated by an as yet unidentified low CO_2 signal in order to regulate CCM gene expression. Although the activation mechanism is currently unknown posttranslational modification is a likely candidate. Finally, as in higher plants, the nature of the *Chlamydomonas* CO_2 sensor(s) remains open to speculation. Interestingly, expression profiling has revealed the existence of several genes that could encode candidate bicarbonate transporters. Perhaps one or more of these gene products could function as a CO_2 sensor?

Conclusions

Our musings about the timing and selection pressures on the evolution of CO_2 signal transduction pathways and mechanisms of CCMs are unlikely to progress further until we have identified and characterized at the molecular genetic level more of the key components involved in these two processes. Towards this aim we are in the process of using infrared thermal imaging to identify *Arabidopsis* mutants that display aberrant stomatal behaviour in response to elevated concentrations of CO_2. In addition to these molecular data we also

require better proxies for past atmospheric CO_2 concentrations, especially in the Precambrian.

Further reading

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The PKD protein qilin undergoes intraflagellar transport

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Cilia play diverse roles in motility and sensory reception, and defects in their formation and function underlie cilia-related human diseases [1]. One such disease is polycystic kidney disease (PKD), a heritable nephropathy associated with defects in the formation and function of sensory (also known as primary) cilia within renal tubules of the kidney [2]. Because the assembly and maintenance of these sensory cilia depends upon the intraflagellar transport (IFT) of axoneme and ciliary membrane components, such as polycystins [3], defective IFT is one of the factors that can contribute to PKD. Qilin is a ciliary, PKD-associated protein of as yet unknown function; here, we show that qilin does in fact undergo IFT to build sensory cilia on *Caenorhabditis elegans* neurons.

Qilin was identified recently in a forward genetic screen for PKD-related genes in zebrafish [4]. *qilin* mutants can assemble sensory cilia, suggesting that qilin is associated with PKD via an IFT-independent pathway [4]. This interpretation is complicated, however, because several zebrafish IFT particle proteins are maternally loaded and may support ciliogenesis even in the presence of loss-of-function mutations in the corresponding gene [4]. Subsequent genomic analysis uncovered qilin homologs in the human ciliary proteome and in the model flagellate *Chlamydomonas* [5]. *Chlamydomonas* qilin did not match any of the IFT particle polypeptides so far identified biochemically [4], but genome-wide transcriptome analysis revealed that it is upregulated during flagellar regeneration, suggesting that it has a role in flagellar assembly [6]. It therefore remains possible that qilin is associated with PKD because it participates in IFT.

To address this possibility, we have used time-lapse microscopy and genetics to determine whether qilin participates in IFT in the nematode *C. elegans* [7], which lacks a bona fide kidney yet has emerged as a valuable model for studying the basic cilia-related mechanisms associated with PKD [8]. First, we identified a qilin homolog in *C. elegans* as C04C3.5 (*E* value $2.1e^{-46}$; identity = 35%; positives = 57%). We asked if any of the *C. elegans* chemosensory ciliary mutants (defective in *che*motaxis; *osm*otic avoidance; *da*uer larva *f*ormation, and *dy*e-*f*illing [9]) contain a molecular lesion within C04C3.5 and found that such a mutant, *dyf-3*, had, in fact, recently been cloned [10]. Careful fluorescence and electron microscopy of sensory ciliary structure in the *dyf-3* mutant had revealed a complete loss of distal segments and truncated middle segments of the cilia [10]. This morphology phenocopies mutations in IFT subcomplex B polypeptides, but is distinct from *osm-3* and *bbs-7/bbs-8* mutants (which have fully intact middle segments), indicating that *C. elegans* qilin/DYF-3 contributes to the assembly of both middle and distal ciliary segments as a component of IFT particle B (Figure 1A,B). In the residual truncated middle segment, no IFT can be detected using time-lapse fluorescence microscopy of the IFT particle subunit OSM-6::GFP in a *dyf-3* mutant background, suggesting that qilin is required for normal IFT within these sensory cilia. This finding is distinct from *osm-3* and *bbs-7/bbs-8* mutants, which support IFT along the middle segments, but leaves open the question of how the middle segment is maintained within *dyf-3* mutants in the absence of IFT.