Rubisco proton production can drive the elevation of CO₂ within condensates and carboxysomes

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Membraneless organelles containing the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) are a common feature of organisms utilizing CO₂ concentrating mechanisms to enhance photosynthetic carbon acquisition. In cyanobacteria and proteobacteria, the Rubisco condensate is encapsulated in a proteainaceous shell, collectively termed a carboxysome, while some algae and hornworts have evolved Rubisco condensates known as pyrenoids. In both cases, CO₂ fixation is enhanced compared with the free enzyme. Previous mathematical models have attributed the improved function of carboxysomes to the generation of elevated CO₂ within the organelle via a colocalized carbonic anhydrase (CA) and inwardly diffusing HCO₃⁻, which have accumulated in the cytoplasm via dedicated transporters. Here, we present a concept in which we consider the net of two protons produced in every Rubisco carboxylase reaction. We evaluate this in a reaction–diffusion compartment model to investigate functional advantages these protons may provide Rubisco condensates and carboxysomes, prior to the evolution of HCO₃⁻ accumulation. Our model highlights that diffusional resistance to reaction species within a condensate allows Rubisco-derived protons to drive the conversion of HCO₃⁻ to CO₂ via colocalized CA, enhancing both condensate CO₂ and Rubisco rate. Protonation of Rubisco substrate (RuBP) and product (phosphoglyceraldehyde) plays an important role in modulating internal pH and CO₂ generation. Application of the model to putative evolutionary ancestors, prior to contemporary cellular HCO₃⁻ accumulation, revealed photosynthetic enhancements along a logical sequence of advancements, via Rubisco condensation, to fully formed carboxysomes. Our model suggests that evolution of Rubisco condensation could be favored under low CO₂ and low light environments.

Rubisco | carboxysomes | pyrenoids | protons | protein condensates

Carbon dioxide (CO₂) fixation into the biosphere has been primarily dependent upon action of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) over geological timescales. Rubisco is distinguished by the competitive inhibition of its carboxylation activity by the alternative substrate molecular oxygen (O₂), leading to loss of CO₂ and metabolic energy via a photosynthetic pathway in most phototrophs (1). Almost certainly the most abundant enzyme on the planet (2), Rubisco’s competing catalytic activities have required evolution of the enzyme, and/or its associated machinery, to maintain capture of sufficient carbon into organic molecules to drive life on Earth. In concert with geological weathering, the evolution of oxygeen photosynthesis ~2.4 billion years ago has transformed the atmosphere from one rich in CO₂ and low in O₂ to one in which the relative abundances of these gases has overturned (3). Under these conditions, the Rubisco oxygenation reaction has increased, to the detriment of CO₂ capture. This catalytic paradox has led to different adaptive solutions to ensure effective rates of photosynthetic CO₂ fixation including the evolution of the kinetic properties of the enzyme (4), increases in Rubisco abundance in the leaves of many terrestrial C₃ plants (5), and the evolution of diverse and complex CO₂ concentrating mechanisms (CCMs) in many cyanobacteria, algae, and more recently hornworts, CAM, and C₄ plants (6, 7).

A defining characteristic of contemporary cyanobacteria is the encapsulation of their Rubisco enzymes within specialized, protein-encased microcompartments called carboxysomes (8). These microbodies are central to the cyanobacterial CCM, in which cellular bicarbonate ([HCO₃⁻]) is elevated by a combination of membrane-associated HCO₃⁻ pumps and CO₂-to-HCO₃⁻ converting complexes (9–11), to drive CO₂ production within the carboxysome by an internal carbonic anhydrase (CA; 12, 13). This process results in enhanced CO₂ fixation, with a concomitant decrease in oxygenation, and is a proposed evolutionary adaptation to a low CO₂ atmosphere (14, 15).

An analogous CCM operates in many algal and hornwort species, which contain chloroplastic Rubisco condensates called pyrenoids (16, 17). Pyrenoids are liquid–liquid phase separated Rubisco aggregates, which lack the protein shell of a carboxysome (18). These CCMs accumulate HCO₃⁻ and convert it to CO₂ within the pyrenoid to maximize CO₂ fixation. Common to cyanobacterial and algal systems is the presence of unique Rubisco-binding proteins, enabling condensation of Rubisco from the bulk cytoplasm (18–25). Condensation of proteins to form aggregates within the cell is increasingly recognized as a means by which cellular processes can be segregated and organized, across a broad range of biological systems (26–28). The commonality of pyrenoid and carboxysome function (29) despite their disparate evolutionary

Significance

Rubisco is arguably the most abundant protein on Earth, and its catalytic action is responsible for the bulk of organic carbon in the biosphere. Its function has been the focus of study for many decades, but recent discoveries highlight that in a broad array of organisms, it undergoes liquid–liquid phase separation to form membraneless organelles, known as pyrenoids and carboxysomes, that enhance CO₂ acquisition. We assess the benefit of these condensate compartments to Rubisco function using a mathematical model. Our model shows that proton production via Rubisco reactions, and those carried by protonated reaction species, can enable the elevation of condensate CO₂ to enhance carboxylation. Application of this theory provides insights into pyrenoid and carboxysome evolution.

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histories (6), suggests a convergence of function driven by Rubisco condensation. In addition, dependency of functional CCMs on their pyrenoids or carboxysomes (30, 31) has led to the speculation that the evolution of Rubisco organization into membraneless organelles likely preceded systems which enabled elevated cellular HCO$_3^-$ (14), raising the possibility that Rubisco condensation and encapsulation may have been the first steps in modern aquatic CCM evolution.

We consider here that, in a primordial model system without active HCO$_3^-$ accumulation, co-condensation of Rubisco and CA enzymes is beneficial for the elevation of internal CO$_2$ because Rubisco carboxylation produces a net of two protons for each CO$_2$ reacted, raising the possibility that Rubisco condensation and encapsulation may have been the first steps in modern aquatic CCM evolution.

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Results

The Modeling of Free Rubisco and Rubisco Compartments. To demonstrate the feasibility of our proposal, we initially consider a model of free Rubisco, a Rubisco condensate, and a carboxysome based on a set of compartmentalized reactions described in Fig. 1 and associated tables of parameters (Tables 1 and 2 and SI Appendix, Methods). We present data for the tobacco Rubisco enzyme as an exemplar, noting that evaluations of other Rubisco enzymes in the model (Table 2) provide comparative outcomes. We assume a system with fixed external inorganic carbon (Ci (CO$_2$ and HCO$_3^-$) supply in the absence of a functional CCM, simulating a primordial evolutionary state prior to the development of HCO$_3^-$ accumulation in unicellular photosynthetic organisms (14).

Our model consists of three nested compartments with a specialized Rubisco compartment (which can be described as a Rubisco condensate or carboxysome by modifying the compartment boundary permeabilities) at the center (Fig. 1). This compartment is surrounded by an unstirred boundary layer, which we assume has diffusive resistance to substrate movement, and is bounded by an external compartment, at pH 8.0, which supplies reaction substrates. We contain Rubisco reactions within the central compartment but allow the protonation and deprotonation of reaction species (ribulose-1,5-bisphosphate [RuBP] and phosphoglycerate [PGA]) to occur in all compartments. We include the competing Rubisco substrates, O$_2$ and CO$_2$, and enable the latter to be interconverted with the more abundant HCO$_3^-$ species through pH control and the interaction of CA, whose position in the model we manipulate. Specific details of the model and its parameterization are provided in Table 1, Methods, and SI Appendix, Methods.

Previous models consider the function of carboxysomes, for example, within cells capable of active accumulation of HCO$_3^-$ in chemical disequilibrium with CO$_2$, and apply diffusional resistances to Rubisco reactants and products within a modeled reaction compartment (9, 34, 35, 39, 44–49). We also apply diffusional resistances to all substrates in our model but consider cytoplasmic CO$_2$ and HCO$_3^-$ supply to be in chemical equilibrium, as would occur in the absence of an active CCM, in order to address any beneficial role of Rubisco compartmentation alone. The key aspects of this model are as follows: a chemical equilibrium of CO$_2$ and HCO$_3^-$ in the compartment surrounding Rubisco, the inclusion of proton production by the Rubisco carboxylation and oxygenation reactions (and their equilibration across the carboxysome shell by diffusion), and proton movement via protonated RuBP and PGA species. Application of the model to existing experimental data (SI Appendix, Fig. S2) provides a good estimation of the differential function of both the free Rubisco enzyme and carboxysomes isolated from the cyanobacterium Cyanobium (41), thus providing confidence in the model.

Carboxysome and Condensate Proton permeability. An important assumption in our model is that there is some resistance to substrate movement across Rubisco compartment boundaries, including protons. The diffusion of protons across the carboxysome envelope has been considered previously (50) but within the context that pH stabilization is entirely dependent upon free diffusion through the shell and in the absence of Rubisco activity, which could lead to internally produced protons. In that study, pH-dependent fluorescent protein inside the carboxysome responded within millisecond time scales to changes in the external pH, resulting in the conclusion that protons entered or exited the carboxysome freely. However, this result is also consistent with some level of diffusional resistance to protons, since considerable restriction to proton permeability can yield internal pH equilibration within even faster time frames (SI Appendix, Fig. S3). Indeed, these previous findings have been shown to be consistent with a steady-state ΔpH across the carboxysome shell, where the relative rates of internal proton production and leakage across the shell can maintain an acidic interior (39). We therefore assume permeabilities to protons, which are consistent with existing data, yet enable some restriction on proton movement. Molecular simulations suggest that pores in the carboxysome shell favor negatively charged ions such as HCO$_3^-$, RuBP, and PGA (51), and it is unlikely that the H$_3$O$^+$ ion will easily traverse the protein shell. For the diffusion of H$_3$O$^+$ in water, it is considered to have a higher diffusion rate than other ions in solution due to its
participation in a proton wire system in collaboration with water (52).

For modeling purposes here, we have assumed that negatively charged ions have a permeability of $10^{-6}$ m/s across the carboxysome shell while $\text{H}_2\text{O}^+$ has a higher value of $10^{-4}$ m/s (SI Appendix, Methods). The data in Fig. 2 show that proton permeability values greater than $10^{-3}$ m/s for the carboxysome shell leads to low carboxysome $\text{[CO}_2\text{]}$ and lower Rubisco carboxylation turnover (under subsaturating substrate supply).

Unlike the carboxysomes, Rubisco condensate proton permeability does not appear to affect Rubisco carboxylation in our model under subsaturating substrate conditions (Fig. 2). However, the condensate $\text{[CO}_2\text{]}$ does appear to correlate with modeled changes in internal pH, suggesting a role for protons in determining condensate $\text{[CO}_2\text{]}$ and carboxylation rates (SI Appendix, Fig. S4). In the case of a condensate, RuBP$^{6-}$ is able to carry protons from outside to inside and therefore provides

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### Table 1. Typical initial values used in the COPASI biochemical compartment model in this study

| Model compartment | Parameter | External (e) | Unstirred layer (u) | Condensate (c) |
|-------------------|-----------|--------------|-------------------|---------------|
| Rubisco sites (mol/m$^3$)$^*$ | Absent | Absent | 10 |
| Substrate permeabilities (m/s)$^1$ | | | |
| Free Rubisco scenario | All substrates | n/a | 1 | 1 |
| Condensate scenario | H$^+$ | 1 $\times$ 10$^{-2}$ | 1 $\times$ 10$^{-2}$ | |
| CO$_2$, HCO$_3^-$, RuBP, and PGA | n/a | 1 $\times$ 10$^{-4}$ | 1 $\times$ 10$^{-4}$ | |
| Carboxysome scenario | H$^+$ | 1 $\times$ 10$^{-2}$ | 1 $\times$ 10$^{-2}$ | |
| CO$_2$, HCO$_3^-$, RuBP, and PGA | n/a | 1 $\times$ 10$^{-4}$ | 1 $\times$ 10$^{-6}$ | |
| CA catalysis factor$^2$ | Internal CA | Constant ratio$^5$ | 1 | 1 $\times$ 10$^5$ |
| Unstirred layer CA | Constant ratio$^5$ | 1 $\times$ 10$^5$ | 1 |
| Internal and unstirred layer CA | Constant ratio$^5$ | 1 $\times$ 10$^5$ | 1 $\times$ 10$^5$ | |
| CA rate constants (1/s)$^6$ | CO$_2$ $\rightarrow$ HCO$_3^-$ $+ H^+$ | n/a | 0.05 | 0.05 |
| HCO$_3^-$ $+ H^+$ $\rightarrow$ CO$_2$ | n/a | 100 | 100 | |
| Compartment volume (m$^3$)$^7$ | 1 | 9.95 $\times$ 10$^{-18}$ | 4.19 $\times$ 10$^{-18}$ | |
| Species concentrations (mol/m$^3$) | HCO$_3^-$ | 0.01 to 25$^i$ | $\approx$ | $\approx$ |
| CO$_2$ (Rubisco substrate) | 0.001 $\times$ [HCO$_3^-$] | $\approx$ | $\approx$ |
| O$_2$ (Rubisco substrate) | 0.25 to 0.36$^{**}$ | $\approx$ | $\approx$ |
| H$^+$ (Rubisco product) | 1 $\times$ 10$^{-5}$ (pH 8.0)$^{11}$ | $\approx$ | $\approx$ |
| RuBP$^{6-}$ (Rubisco substrate) | 1 $\times$ 10$^{-2}$ to 10$^i$ | $\approx$ | $\approx$ |
| RuBP$^{5-}$ | $\approx$ | $\approx$ | $\approx$ |
| PGA$^{6-}$ (Rubisco product) | $\approx$ | $\approx$ | $\approx$ |
| PGA$^{5-}$ | $\approx$ | $\approx$ | $\approx$ |

### Notes:

$n/a$, not applicable.

$^*$Rubisco active-site concentration is set at 10 mol/m$^3$ as an upper bound of likely concentrations, which would allow for movement of holoenzymes within the compartment and both small molecule and activation chaperone passage (SI Appendix, Methods).

$^1$Permeabilities of the unstirred layer and condensate compartments to H$^+$, CO$_2$, HCO$_3^-$, RuBP, and PGA are varied to simulate the free-enzyme, condensate, or carboxysome scenarios. A detailed description of the specific values utilized in the model is provided in SI Appendix, Methods.

$^2$CA catalysis factor > 1 indicates presence of CA in that compartment. Here, we use the value of 1 $\times$ 10$^5$ as used by Reinhold, Kosloff, and Kaplan (34).

$^3$A constant HCO$_3^-$:CO$_2$ ratio of 100:1 was used in the external compartment, assuming negligible effects of a single Rubisco compartment on the bulk external C$_i$ species, regardless of CA level.

$^4$CA rate constants were kept the same in internal and unstirred layer compartments. Values of 0.05 and 100 (1/s) allow for the attainment of a HCO$_3^-$:CO$_2$ ratio of 100:1 (the approximate proportion, assuming the uncatalyzed interconversion of each species, at chemical equilibrium when pH is 8.0).

$^5$External compartment volume is 1 m$^3$, and the volumes of the unstirred and condensate are determined from the condensate radius, here set to 1 $\times$ 10$^5$ m for most scenarios or to 5 $\times$ 10$^{-3}$ when modeling small carboxysomes (SI Appendix, Methods).

$^6$The ranges of HCO$_3^-$ and RuBP concentrations used here are typical ranges for these substrates in cyanobacterial and microalgal cells (35–37).

$^7$Concentrations of O$_2$ in water at 25 °C under current atmosphere (20% O$_2$ vol/vol) and atmospheres where CCMs may have evolved (30% vol/vol; refs. 7 and 38).

$^{11}$A pH of 8.0 in the external compartment approximates that of a typical cyanobacterial cell (39) and is fixed in modeled scenarios.

$^{11}$Values determined as output from the model.

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Within the model (of protons, we assess model responses under subsaturating substrate supply, 10^4 to 10^6-fold smaller than those driving CO_2, HCO_3^−, proton exchange across the carboxysomal shell, for example, are < one proton and means that the diffusional driving forces for condensate, and carboxysomes to [RuBP] under subsaturating absence of a compartment with associated diffusional limitations. Rubisco enzyme is unaffected by proton production due to the condensate permeabilities to protons and RuBP shows that mass action within a diffusion-limited compartment leads to decreased rate of CO_2 efflux from the carboxysome by altering the rate of CO_2 efflux (Fig. 5) as described above. For a condensate, CO_2 efflux plays a significant role in pH balance (Fig. 5) and 40.0‡ 60 0.042 (15, 41)

**Table 2. Rubisco catalytic parameters used in competition modeling**

| Rubisco source | k_cat^C (1/s) | k_cat^O (1/s) | K_M^CO_2 (μM) | K_M^O_2 (μM) | K_M^RuBP (μM) | S_CIO2 (μM) | (k_cat^C/K_M^CO_2) (1/s/μM) | References |
|----------------|--------------|--------------|---------------|-------------|---------------|-------------|--------------------------|------------|
| Tobacco        | 3.40         | 1.14         | 10.7          | 295         | 18.0          | 82          | 0.318                    | (67)       |
| Synechococcus  | 14.4         | 1.22*        | 172           | 585*        | 69.9          | 40          | 0.084                    | (36, 40)   |
| Cyanobium      | 9.40         | 1.42†        | 169           | 1,400†      | 40.0†         | 55          | 0.056                    | (15, 41)   |
| Ancestral F1A  | 4.77         | 1.42         | 113           | 2,010       | 40.0‡         | 61          | 0.039                    | (15, 36)   |
| Ancestral F1B  | 4.72         | 0.50         | 120           | 641         | 69.9†         | 50          | 0.039                    | (15, 36)   |
| Chlamydomonas  | 2.91         | 0.63         | 33.0          | 422         | 19.0‡         | 61          | 0.088                    | (42, 43)   |

*The k_cat^C and K_M^O_2 for Synechococcus Rubisco are from Occhialini, Lin, Andralojc, Hanson, and Parry (40).
†The K_M^RuBP for the Cyanobium enzyme was estimated using the Prochlorococcus K_M^O_2 (15), and the published values of k_cat^C, K_M^CO_2 and S_CIO2 for Cyanobium (41) using k_cat^O = (k_cat^C × K_M^CO_2)/S_CIO2.
‡K_M^RuBP values for Ancestral F1A and F1B enzymes are those of Cyanobium and Synechococcus, respectively, from the references highlighted.
*The K_M^RuBP value for Chlamydomonas is from Zhu and Spreitzer (42) while all other values are from Spreitzer, Peddi, and Satagopan (43).

In considering why these results are obtained, it is important to note that at pH 8.0, which we set for the bulk medium in our model, free [H^+] is only 10 nM. In the Rubisco compartment volume utilized in our model (~5 × 10^-15 m^3), this represents less than one proton and means that the diffusional driving forces for proton exchange across the carboxysomal shell, for example, are 10^4 to 10^6-fold smaller than those driving CO_2, HCO_3^−, PGA, and RuBP diffusion (which are in μM and mM ranges). Hence, inward H^+ diffusion will be rate limiting depending on the permeability of the compartment to protons. Therefore, other proton sources must provide substrate for the CA/HCO_3^− dehydoration reaction. The net outcome is that proton production by Rubisco carboxylation within a diffusion-limited compartment leads to decreased pH, elevated CO_2, and improvement in carboxylation turnover, compared with the free enzyme (SI Appendix, Fig. S7).

The model also shows an increase in carboxylation turnover resulting from the protons arising through oxygenation within a carboxysome, although this appears negligible in a Rubisco condensate (SI Appendix, Fig. S8). Notably, like the model of Mangan, Flamholz, Hood, Milo, and Savage (39), we find that carboxysome function does not require specific diffusional limitation to O_2 influx in order to reduce oxygenation, due to competitive inhibition by the increase in CO_2.

**Roles for RuBP, PGA, and CO_2 in Rubisco Compartment pH.** When we run the full model, including net proton production by Rubisco, carboxysome pH becomes acidic at limiting substrate concentrations as a result of limited proton efflux (Fig. 4). As external substrate increases and internal CO_2 rises, the pH approaches that of the bulk external medium (which we set at pH 8.0 with the H^+ concentration). Here, increasing CO_2 efflux from the carboxysome is able to effectively dissipate protons from the Rubisco compartment, since there is a net loss of CO_2 that would otherwise be used for proton production by the CA hydration reaction (CO_2 + H_2O ↔ HCO_3^− + H^+). This can be seen when we modify the rate of CO_2 efflux from the carboxysome by altering compartment CO_2 permeability. Slow CO_2 efflux leads to increased free proton concentrations within the compartment, and fast efflux enables a return to approximately external pH as substrate supply increases (Fig. 4).

When we consider reaction species’ fluxes across the Rubisco compartment boundary, we find that both RuBP^3− and PGA^2− can play a role in carrying protons out of both condensates and carboxysomes (Fig. 5 and SI Appendix, Fig. S9), contributing to the stabilization of internal pH. In our model of carboxysomes, RuBP^3− and PGA^2− efflux plays a significant role in pH balance at very low HCO_3^−. However, as external HCO_3^− rises, carboxysome CO_2 efflux replaces RuBP and PGA as the major proton carrier (Fig. 5) as described above. For a condensate, CO_2 efflux is the primary means of stabilizing internal pH within the model over a range of HCO_3^− concentrations (SI Appendix, Fig. S9).

Similar responses can be seen over a range of RuBP concentrations, where CO_2 efflux also plays a dominant role as a proton efflux carrier in the condensate, while RuBP^3− and PGA^2− efflux are the major contributors to shuttling protons out of the carboxysome (Fig. 5 and SI Appendix, Fig. S9). These results highlight that the inclusion of RuBP and PGA as proton carriers is...
out of the carboxysome. While those models consider functional
compartment pH (\(\text{HCO}_3^-\)) and either 35, 50, 87, or 1,300
\(\mu\text{M} \text{RuBP}\) generating CO\(_2\) via the CA enzyme (\(\text{SI Appendix}\), Fig. S13). This dependency appears to be much less in a
compartment due to our assumption of much higher permeabilities of RuBP\(^{3-}\) and CO\(_2\) to the condensate interior (\(\text{SI Appendix}\), Fig. S11) and the low \(K_M\text{CO}_2\) of the tobacco Rubisco modeled in this scenario.

Carboxysome Evolution via Rubisco Condensation. Our model shows that Rubisco cocondensed with CA gives improved function over its free enzyme (Fig. 3). Given that Rubisco condensation underpins carboxysome biogenesis (20, 25), we considered that the model may provide insights into carboxysome evolution via intermediate states utilizing Rubisco condensates, prior to HCO\(_3^-\) accumulation in the cell through inorganic carbon (C\(_3\)) transport.

To investigate this proposal, we analyzed the performance of potential evolutionary intermediates in a series of hypothesized pathways from free Rubisco to carboxysomes, in the absence of functional C\(_3\) (assumed this was a later evolutionary progression; ref. 14). We first assumed a photosynthetic loss of 1/2 mol of CO\(_2\) for every mol of O\(_2\) fixed within the model (while carboxylation yields two molecules of PGA from one CO\(_2\), oxygenation yields only one PGA, and one CO\(_2\) is lost via photorespiration) and by calculating average net carboxylation rates by each hypothesized evolutionary intermediate under different atmospheric conditions (see Methods). This allowed relative fitness comparisons of proposed evolutionary states under what might reasonably be considered low or high atmospheric CO\(_2\), as may have been experienced under atmospheres where CCMs arose (7, 38).

We assume here that a greater average net carboxylation rate for a particular evolutionary intermediate (over a given range of HCO\(_3^-\)) would result in greater relative fitness and, therefore, evolutionary advantage. We apply this concept over HCO\(_3^-\) ranges within the model, rather than single-point comparisons, to provide a clearer view of Rubisco responses resulting from variations in the observed response curves and to assess responses under different atmospheric CO\(_2\) compositions (\(\text{SI Appendix}\), Fig. S14).

We made these fitness calculations at both 20 and 30% (vol/vol) O\(_2\), to simulate alternative atmospheres under which CCMs may have arisen (7, 38). We calculated the average net carboxylation rates for the defined HCO\(_3^-\) ranges (see Methods) and generated phenotype matrices to allow comparison of possible evolutionary states (Fig. 6 and Datasets S1 and S2) (54). In evolutionary state comparisons, those states which lead to larger average net carboxylation rates were considered to have greater fitness.

The relative fitness of form I Rubisco enzymes (55) from a variety of sources were compared within the model, over all proposed evolutionary intermediates, to observe any differences resulting from varying Rubisco catalytic parameters (Table 2). A complete analysis of each Rubisco source and its performance at each proposed evolutionary step, under varied HCO\(_3^-\) and O\(_2\) conditions, is supplied in the Datasets S1 and S2 (54).

Regardless of the type of Rubisco used in this analysis, the same pattern of potential evolutionary augmentations was favored (Figs. 6 and 7 and Datasets S1 and S2) (54). In all cases, condensation of Rubisco in the absence of a CA enzyme, as an initial evolutionary step (“Rubisco – CA”) to “Condensate – internal/external CA”; Fig. 6), resulted in a decrease in fitness, emphasizing that the starting point for the evolution of Rubisco condensates and carboxysomes likely began with a cellular CA present (“Rubisco + CA”). Again,
we emphasize here that our modeling assumes no active C_4 accumulation as observed in modern aquatic CCMs where the occurrence of a cellular CA, outside a carboxysome for example, dissipates an accumulated HCO_3^- pool as membrane-permeable CO_2 and leads to a high-CO_2–requiring phenotype (49). In Fig. 6, we provide an example evolutionary matrix for hypothesized progressions from a free Rubisco enzyme to a contemporary carboxysome. As a primary evolution, we speculated that condensation of Rubisco could have occurred either with or without cocondensation of CA (“Condensate + internal CA” or “Condensate + external CA”) or an alternate evolution where some CA was cocondensed and some remained external to the condensate in the unstirred layer (“Condensate + internal/external CA”). All three possibilities gave rise to condensates with improved fitness over the free enzyme (Figs. 6 and 7 and Datasets S1 and S2) (54). While the greatest improvement was calculated for a condensate with both internal and external CA

Fig. 3. Modeled responses of free Rubisco, condensate, and carboxysome to external substrate supply. Modeled responses of free Rubisco, condensate, and carboxysome to external HCO_3^- (A) and RuBP (B) supply. Carboxylation turnover rates (Top) and the ratio of Rubisco compartment CO_2 to external CO_2 (Lower) for the free enzyme, condensate, and carboxysomes with zero, one, two, or three protons being produced as products of the carboxylation reaction at subsaturating RuBP (A) or HCO_3^- (B). Variation in permeabilities to RuBP between the free enzyme, condensate, and carboxysomes within the model result in increases in the apparent K_M^RuBP as permeabilities decline (SI Appendix, Fig. S6). For HCO_3^- responses, the RuBP concentration used in each scenario is indicated. For RuBP responses, the HCO_3^- supply is set at 1 mM (0.01 mM CO_2). External CO_2 is set to 1/100 × external HCO_3^- . Net proton production by Rubisco carboxylation is theoretically two protons (Fig. 1). In all cases, modeled rates include CA activity only in the Rubisco compartment. Rubisco turnover in a carboxysome producing three protons per carboxylation drops below that of the two-proton system due a proton-driven shift in the RuBP_3^-:RuBP_4^- ratio, decreasing the concentration of the Rubisco substrate species, RuBP_4^- . Maximum carboxylation turnover rate (k_cat, 3.4 [1/s]) of the tobacco Rubisco used in this example (Table 2) is indicated by the dashed line (Upper). The COPASI (66) model was run in parameter scan mode, achieving steady-state values over the range of HCO_3^- concentrations indicated. Proton number was manufactured by modifying the Rubisco carboxylation reaction stoichiometry to produce zero, one, two, or three protons in the model.
maximally decreased (SI Appendix, Fig. S4), we assessed the relative fitness of condensates and carboxysomes at saturat- ing RuBP (50 μM) over HCO₃⁻ ranges in the model. Low cellular RuBP can generally be attributed to light-limited RuBP regeneration via the Calvin cycle in photoautotrophs (56). A concentration of 50 μM RuBP is approximately three times the K₉₆RuBP of the tobacco Rubisco used here (Table 2) and supports ~63% of the CO₂-saturated rate for a condensed Rubisco (SI Appendix, Fig. S6). At 50 μM RuBP, we observe enhanced net carboxylation turnover in the condensate compared with the free enzyme, especially at low HCO₃⁻ (SI Appendix, Fig. S15). An additional benefit can be observed for very small carboxysomes since changes in the apparent K₉₆RuBP are size dependent (SI Appendix, Figs. S6 and S15). However, 50 μM RuBP is insufficient to support appreciable carboxylation in a modeled large carboxysome due to decreased substrate permeability (SI Appendix, Figs. S6 and S15).

Discussion

The Functional Advantages of a Condensate/Carboxysome. The modeling of both Rubisco condensates and carboxysomes in this study demonstrates a number of factors, which we predict play a key role in the function and evolution of these Rubisco “organelles.” First, the formation of a Rubisco condensate creates a localized environment in which HCO₃⁻ can be converted to CO₂ in the presence of CA. CO₂ can be elevated relative to the external environment by the creation of a viscous unstirred protein-solution boundary layer. The presence of condensates or carboxysomes in prokaryotic cells or chloroplasts, where protein concentrations are high, would favor this (57, 58).

Fig. 5. Proton carriers help maintain compartment pH. Diffusional flux of chemical species across the condensate/carboxysome boundary over a range of HCO₃⁻ and RuBP concentrations in the model. Protons are carried by RuBP⁺, PGA⁺, and CO₂ (as the substrate required for free proton release via the CA hydration reaction). Net free H⁺ fluxes are extremely small, and contributions to internal pH primarily arise through net fluxes of proton-carrier substrates (solid circles). The deprotonated RuBP⁺ and PGA⁺ are the substrate and product of Rubisco carboxylation, respectively, within the model. Positive flux values indicate net influx into the compartment and negative values indicate net efflux. The COPASI (66) model was run in parameter scan mode, achieving steady state at each substrate concentration. Data presented are for the tobacco Rubisco (Table 2).

Low Light May Drive Condensate and Carboxysome Evolution. Observing increased relative responses of compartment CO₂ to low RuBP concentrations in the model, where condensate pH is

Fig. 4. Carboxysome proton concentration is modulated by CO₂ efflux. Carboxysome-free proton concentration (A and B) and carboxysome pH (C and D) indicate that a functional carboxysome compartment undergoes net acidification at limiting HCO₃⁻ and RuBP supply (SI Appendix, Fig. S4). Plotted are proton concentration and pH over a range of [HCO₃⁻] and [RuBP] for modeled carboxysomes with an internal CA, allowing for two protons to be produced per carboxylation reaction and under typical modeled CO₂ permeability within the model (10⁻⁶ m/s; solid pink lines). If CO₂ efflux were rapid and unimpeded (CO₂ permeability 1 m/s; dashed green lines), pH rapidly returns to ~8 (black dashed line, panels C and D) as external limiting substrate supply increases. Slow CO₂ efflux (CO₂ permeability 10⁻⁹ m/s; dashed orange lines) does not allow for dissipation of protons. Efflux of CO₂ from the carboxysome contributes to pH maintenance as it represents the loss of substrate for the CA hydration reaction (CO₂ + H₂O ↔ HCO₃⁻ + H⁺), which would otherwise lead to free proton release. Each dataset was modeled with an initial [RuBP] of 5 mM for HCO₃⁻ response curves and 20 mM HCO₃⁻ in the case of RuBP response curves, and CA activity is confined only to the carboxysome compartment. All other permeabilities under these conditions are set to 10⁻⁶ m/s as for a carboxysome (Table 1). The COPASI (66) model was run in parameter scan mode, achieving steady-state values over a range of substrate concentrations. Data presented are for the tobacco Rubisco (Table 2).

(“Condensate + internal/external CA”), this state showed a negative transition in evolving to a condensate with only internalized CA (“Condensate + internal CA”).

Following initial condensate formation, we propose that the next probable advancement would be the acquisition of bacterial microcompartment proteins (8) to form a shell with enhanced diffusional resistance. Within the model, only condensates with internalized CA enzymes (“Condensate + internal CA” and “Condensate + internal/external CA”) displayed an improved CO₂ fixation phenotype during a single step acquisition of a carboxysome shell (Figs. 6 and 7 and Datasets S1 and S2) (54). There was no difference in fitness phenotype between carboxysomes with internal CA (“Carboxysome + internal CA”) and those with CA both internal and external in the unstirred layer (“Carboxysome + internal/external CA”) in the model. Further improvements could be made by the addition of HCO₃⁻ transporters, the loss of unstirred layer CA, the acquisition of specific internal CA enzymes, and evolution of Rubisco kinetic properties.

Rubisco proton production can drive the elevation of CO₂ within condensates and carboxysomes

Fig. 5. Proton carriers help maintain compartment pH. Diffusional flux of chemical species across the condensate/carboxysome boundary over a range of HCO₃⁻ and RuBP concentrations in the model. Protons are carried by RuBP⁺, PGA⁺, and CO₂ (as the substrate required for free proton release via the CA hydration reaction). Net free H⁺ fluxes are extremely small, and contributions to internal pH primarily arise through net fluxes of proton-carrier substrates (solid circles). The deprotonated RuBP⁺ and PGA⁺ are the substrate and product of Rubisco carboxylation, respectively, within the model. Positive flux values indicate net influx into the compartment and negative values indicate net efflux. The COPASI (66) model was run in parameter scan mode, achieving steady state at each substrate concentration. For HCO₃⁻ response curves, RuBP was set to 50 μM for a condensate and 1.3 mM for a carboxysome based on changes in apparent K₉₆RuBP values arising from diffusional resistance (Fig. 3 and SI Appendix, Fig. S6). External [HCO₃⁻] was set to 1 mM for the generation of RuBP response curves. Data presented are for the tobacco Rubisco (Table 2), and model parameters for a condensate or a carboxysome are indicated in Table 1. These data are summarized in SI Appendix, Fig. S9.
The condensation of Rubisco results in the colocalization of Rubisco-reaction protons. This enhances the potential to elevate CO$_2$ by driving both the conversion of HCO$_3^−$ to CO$_2$ in the Rubisco compartment and decreasing compartment pH under certain conditions (SI Appendix, Fig. S4). This role for protons is seen most clearly in carboxysomes where both CA and Rubisco activity are highly dependent on proton production by the Rubisco reaction (Fig. 3 and SI Appendix, Fig. S13). Rubisco condensates show a smaller enhancement by Rubisco proton production due to greater permeability to protonated RuBP and PGA, but under conditions of low RubBP and low HCO$_3^−$ condensate pH can be lowered and CO$_2$ elevated (Fig. 3 and SI Appendix, Fig. S4). In addition, the advantages of condensate formation are enhanced when the ratio of external HCO$_3^−$ to CO$_2$ is increased (SI Appendix, Fig. S12) as would occur at higher cytoplasmic pH and in the presence of active HCO$_3^−$ accumulation and subsequent transfer of CA from the cytoplasm to the Rubisco compartment.

The modeling emphasizes that the exchange of protons between internal and external environments is probably independent of free-proton or H$_3$O$^+$ diffusion. Instead, the exchange is dominated by the movement of protonated RuBP and PGA (with $pK_a$'s around 6.6) and efflux of CO$_2$, which consumes a proton internally around 6.6) and efflux of CO$_2$, which consumes a proton internally.

The Effects of Rubisco Compartment pH and the Relevance of Sugar Phosphate Proton Carriers. By accounting for the $pK_a$ of physiologically relevant phosphate groups on RuBP and PGA (SI Appendix, Fig. S1), the model reveals that these species allow for sufficient ingress of protons into a Rubisco compartment to drive higher rates of carboxylation. This is because the concentration gradients for RuBP and PGA are significantly high (in the mM range), compared with protons at pH 8.0 (in the nM range), enabling them to act as proton carriers in physiologically relevant concentrations. The sophisticated model of Mangan, Flamholz, Hood, Milo, and Savage (39) also considers the net production of a proton by the CA dehydration reaction and the generation of two protons by Rubisco carboxylation. They calculate that a relatively acidic carboxysome is possible under steady-state conditions, depending on proton permeability. However, analysis of viral capsid shells, which have some similarity to the carboxysome icosahedral shell, suggests proton transfer may need to be mediated by specific channels (59, 60). It is important to establish the real permeability of the carboxysome shell to the hydronium ion and whether high rates of exchange are mediated by channels, proton wires, or shuttles linked to protonated sugar phosphates suggested here.

Drivers of Rubisco Compartment Evolution. Our evolution analysis suggests that specificity, which Rubisco has for CO$_2$ over O$_2$ ($S_{CO2}$), is a likely key driver in determining fitness for the free Rubisco enzyme under low CO$_2$ atmospheres (SI Appendix, Results and Fig S16). Indeed, the tobacco enzyme, having the greatest $S_{CO2}$ (Table 2), displayed the best performance under all low-CO$_2$ scenarios (Fig. 5). Rubisco carboxylation efficiency (i.e., its carboxylation rate constant; $k_{cat}/K_M$CO$_2$) is also an important driver in determining relative fitness under low O$_2$ atmospheres for both the free enzyme and condensates (SI Appendix, Figs. S16–S19). Furthermore, this analysis suggests that under increased atmospheric O$_2$ (as would have occurred some 300 Mya, when levels of O$_2$ rose and CO$_2$ fell; ref. 14) there is selective pressure to reduce $k_{out}$ of the free enzyme (SI Appendix, Fig. S16). This suggests that ancestral cyanobacterial Rubisco may have had kinetics similar to the tobacco form IB enzyme, implying relatively high photorespiration rates in pre-CCM cyanobacteria. Notably,
by any Rubisco catalytic parameter at elevated CO₂ (SI Appendix, Fig. S21). Changes in atmospheric O₂ may have led to the selection of enzymes with better specificity, catalytic efficiency, and $K_m$RuBP during intermediate stages of Rubisco condensate evolution (SI Appendix, Figs. S17–S19).

The relative response of Rubisco condensates and small carboxysomes to low RuBP supply suggests that low light may also provide conditions conducive to condensate and carboxysome evolution. Low light generally leads to low RuBP (56). In the model, such conditions lead to greater advantage of Rubisco condensates over large carboxysomes (SI Appendix, Fig. S15). This highlights that elaborations on simple Rubisco condensation can be afforded through facilitated substrate supply or potential diffusion barriers other than carboxysome formation. The association between pyrenoids and thylakoids, for example, suggests that luminal protons can possibly contribute toward the conversion of $\text{HCO}_3^-$ to CO₂ (62) and allows the possibility that the pyrenoid function may be enhanced by the combined action of both Rubisco and thylakoid-generated protons. This result highlights the alternative evolution of Rubisco compartments such as pyrenoids, which would provide enhancements exclusive of shell formation.

Limitations of the Model. The model is designed as an idealized Rubisco compartment within a static environment. We use a single “condensate” of 1 μm in radius for the demonstration of condensate function, approximating a large pyrenoid (63). Notably, both carboxysomes and pyrenoids range significantly in size (8, 64), and the effect of compartment size in the model is addressed in SI Appendix, Methods and Fig. S22. It does not include an “extracellular” compartment from which the cytoplasmic compartment can receive $\text{Ci}$ via either diffusion or specific $\text{Ci}$ pumps. We do not model the system as a functional CCM, holding a static equilibrium between $\text{Ci}$ species in the system rather than a disequilibrium, which occurs in cyanobacterial cells (65). In the absence of active $\text{HCO}_3^-$ pumps or a cytoplasmic CA, preferential diffusion of CO₂ through the cell membrane, coupled with its drawdown via carboxylation by Rubisco, would lead to relatively low cytoplasmic [C] with a $\text{HCO}_3^-$:CO₂ ratio possibly favoring CO₂ (SI Appendix, Fig. S12). However, notably there is no fitness benefit to Rubisco condensate in the absence of a CA (Fig. 6), and scenarios where the $\text{HCO}_3^-$:CO₂ ratio lean heavily toward CO₂ are less likely.

We do not apply a pH sensitivity to Rubisco catalysis in the model and omit unknown contributors to cellular buffering. This is both for simplicity and to highlight that without pH modulation in the model we would see dramatic changes in pH. Previous models (39) apply the pH dependency of Rubisco as established by Badger (53), as would be applicable in this instance. We would expect to see a decrease in Rubisco activity in our model resulting from acidification or alkalinization of the Rubisco compartment. However, we do not currently know how a condensate or carboxysome might modulate pH changes in reality and how these might affect the enzymes within it. We also do not currently know how a condensate or carboxysome might modulate pH changes in reality and how these might affect the enzymes within it. We also do not currently know how a condensate or carboxysome might modulate pH changes in reality and how these might affect the enzymes within it. We also do not currently know how a condensate or carboxysome might modulate pH changes in reality and how these might affect the enzymes within it. We also do not currently know how a condensate or carboxysome might modulate pH changes in reality and how these might affect the enzymes within it.
of Rubisco condensates, while increases in atmospheric O2 may
substrate and inhibitor concentrations within the Rubisco compartment (Menten rate equations were applied to Rubisco catalysis as dependent upon elongatus
Table S3).

**Methods**

**Mathematical Modeling.** Modeling of Rubisco compartment scenarios and data output were carried out using the biochemical network simulation program COPASI (copasi.org), described by Hoops et al. (66). COPASI (version 4.25, build 207) was used to simulate reaction time courses achieving steady-state conditions in a three-compartment model where reaction species are linked in a biochemical network (Fig. 1). For standard modeling conditions, catalytic parameters of the tobacco Rubisco were used, while those of Chlamydomonas reinhardtii, Cyanobium marinus PCC7001, Synechococcus elongatus PCC7942, and predicted ancestral IA and IB enzymes (15) were also used in evolutionary fitness analysis (Table 2). Michaelis–Menten rate equations were applied to Rubisco catalysis as dependent upon substrate and inhibitor concentrations within the Rubisco compartment (SI Appendix, Methods). O2 was applied as an inhibitor of the carboxylation reaction and CO2 an inhibitor of oxygenation. Greater detail of model parameterization is provided in SI Appendix, Methods. The ordinary differential equations describing the model components can be found in SI Appendix, Methods. Reactions, reaction species, and model parameters can be found in SI Appendix, Tables S1–S3, respectively.

Variation in compartment types (i.e., the free enzyme, a condensate, or a carboxysome) was simulated in the model by varying unstirred boundary and condensate permeabilities to all reaction species (Table 1 and SI Appendix, Methods). The permeabilities to RuBP and PGA were assumed to be the same, as were those for PGA and RuBP, and a single permeability value applied to either RuBP or PGA species. Proton production by carboxylation and oxygenation reactions was varied by adjusting the proton stoichiometry for each reaction (SI Appendix, Equations and Table S1). Protonation and deprotonation of RuBP and PGA in each compartment was enabled by assigning reaction rates equivalent to their \(^{\frac{\text{pK}_a}{\text{vol}}}_{\text{HCO}_3^-}\) values (SI Appendix, Table S3).

The size of the external compartment was set to 1 m3 within the model and both the Rubisco compartment and unstirred boundary layer volumes determined by setting the Rubisco compartment radius. For standard modeling procedures, we used a spherical Rubisco compartment radius of \(1 \times 10^{-5} \text{ m}^3\), and the unstirred boundary layer volume was determined as a simple multiplier of the Rubisco compartment radius. The Rubisco compartment radius used in our modeling generates a large condensate or carboxysome, akin to contemporary pyrenoids; however, variation in the compartment size has little effect on the conclusions of the modeled outcomes since even small carboxysomes display higher Rubisco turnover rates than large condensates in the model (SI Appendix, Fig. S22). Extremely small carboxysomes have lower amplitude responses to proton and RuBP permeabilities (Fig. 2), and size may have led to favorable Rubisco kinetics during evolution to modern carboxysomes (SI Appendix, Fig. S21 and Dataset S2) (54).

CO2 concentration in the external compartment was set to 0.01 \times \text{external } [\text{HCO}_3^-] (SI Appendix, Equations), assuming negligible effects of a single Rubisco compartment on the bulk external C species. Intercalation between CO2 and HCO3- was allowed to proceed in the unstirred and Rubisco compartments with rate constants of 0.05 for the forward reaction (\(\text{CO}_2 + \text{HCO}_3^- \rightarrow \text{CO}_3^- + \text{H}_2\text{O}\)) and 0.00 for the back reaction (\(\text{CO}_3^- + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{HCO}_3^-\)). CA contribution was enabled by applying a multiplying factor to each rate, such that a factor of 1 simulates the absence of CA. Typical CA multiplying factors for each type of modeled compartment are listed in Table 1. CA function external to a Rubisco compartment was modeled by modifying CA in the unstirred layer (Table 1). O2 concentration in the model was typically set at a contemporary atmospheric level of 20% (vol/vol) by assigning a concentration of 0.25 mM and the water-soluble concentration at 25 °C. For simulations at 30% (vol/vol) O2 (an estimated volumetric concentration in the atmosphere when it is proposed CCMs arose), a concentration of 0.36 mM was used.

Steady-state reactions were initialized by setting the reactive concentrations in the external compartment and running the model to achieve steady state. External pH was set at 8.0 using a compartment [\(\text{H}^+\)] of \(1 \times 10^{-5} \text{ mol}\) and saturating Rubisco substrate concentrations. An initial [\(\text{HCO}_3^-\)] was set to 20 mM and [\(\text{RuBP}\)] was set to 5 mM. Interconversion of RuBP and RuBP at \(pK_a 6.7\) and pH 8.0 results in \(-95%\) of all RuBP as the RuBP species.

Rubisco site concentrations were typically set to 10 mM. This value is similar to that calculated for \(\alpha\) and \(\beta\)-carboxysomes (36) although higher than that estimated for pyrenoids (18). It is nonetheless a reasonable upper limit for the purposes of examining system responses within the model.

**Evolution Analysis.** Hypothesized free enzyme, condensate, or carboxysome evolutionary intermediates (Fig. 6) were generated and analyzed within the model using the parameters in Table 1, applying the catalytic parameters of each Rubisco enzyme in Table 2. For each scenario, the model was run over a range of [\(\text{HCO}_3^-\)] from 0.01 to 25 mM, at saturating RuBP (5 mM). Both carboxylation and oxygenation rates were output for each scenario and converted to turnover numbers by accounting for active-site concentrations within the model. Net carboxylation turnover rates (1/\(\alpha\)) were calculated by assuming a photosynthetic cost of 1/2 mol of \(\text{CO}_2\) loss for each mole \(\text{O}_2\) fixed (SI Appendix, Fig. S14). Modeling was carried out at both 20 and 30% O2 (vol/vol, Table 1). Performance comparisons of each hypothesized evolutionary state were made for both low CO2 (0.01 to 1 mM HCO3-) and high CO2 (1 to 10 mM HCO3-) ranges by calculating the average net carboxylation rates for each scenario at each CO2 range and O2 concentration (SI Appendix, Fig. S14). Fitness comparisons were determined from the absolute differences in average net carboxylation rates between each modeled scenario (Fig. 6 and Datasets S1 and S2) (54). An additional dataset was calculated for
the tobacco Rubisco enzyme at 20% (vol/vol) O2 and 50 μM RuBP to determine compartment-type performance under conditions simulating low light.

Correlations between average net carboxylation rates (over specified modeled atmospheres) and Rubisco catalytic parameters (Table 2) for each proposed evolutionary state (Fig. 6 and SI Appendix, Figs. S16–S21) were calculated as Pearson correlation statistics, with P values calculated as two-tailed distribution of calculated t-statistics (SI Appendix, Results and Datasets S1 and S2) (54). In our results, we highlight correlations where P < 0.05. For plotting purposes (SI Appendix, Results and Figs. S16–S21), Rubisco catalytic parameters (Table 2) were normalized to the largest value in each parameter set and plotted against average net carboxylation rates.

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Data Availability. The data that support the findings of this study are publicly available in Mendeley Data at https://doi.org/10.17632/5ck2m373v4.v4 (54) and in the SI Appendix.

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