### Title

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# New discoveries expand possibilities for carboxysome engineering

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## Abstract

Carboxysomes are $CO_2$-fixing protein compartments present in all cyanobacteria and some proteobacteria. These structures are attractive candidates for carbon assimilation bioengineering because they concentrate carbon, allowing the fixation reaction to occur near its maximum rate, and because they self-assemble in diverse organisms with a set of standard biological parts. Recent discoveries have expanded our understanding of how the carboxysome assembles, distributes itself, and sustains its metabolism. These studies have already led to substantial advances in engineering the carboxysome and carbon concentrating mechanism into recombinant organisms, with an eye towards establishing the system in industrial microbes and plants. Future studies may also consider the potential of in vitro carboxysomes for both discovery and applied science.

### Keywords

carboxysome; carbon fixation; Self-assembly; bioengineering; Plant engineering; Biotechnology; bacterial microcompartment

## Introduction

All cyanobacteria and many chemoautotrophic proteobacteria use specialized proteinaceous organelles called carboxysomes to facilitate $CO_2$ fixation. Carboxysomes have fascinated researchers and biotechnologists for both their icosahedral structure and ability to enable efficient carbon fixation kinetics. It's estimated that ~10–25% of $CO_2$ fixed globally passes through these compartments annually [1,2]. Since they were first purified in 1973 [3], researchers have sought to both understand and engineer carboxysomes. Although the presence of Rubisco signaled a critical role in $CO_2$ fixation, studies on carboxysomes continue to reveal new and unexpected components, structures, and potential applications.

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Competing interests

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Carboxysomes are icosahedral protein assemblies ranging from 100–500 nm in diameter, depending on the species [4]. They have a proteinaceous shell composed primarily of hexameric proteins and are capped with pentameric proteins at the icosahedral vertices. In general, they enclose Rubisco, carbonic anhydrase (CA), a Rubisco nucleating protein, and, likely, Rubisco activase. There are two lineages of carboxysomes, α and β, which evolved convergently in freshwater and coastal cyanobacteria (β lineage) and marine cyanobacteria and proteobacteria (α lineage) [5]. Remarkably, both lineages arrived at the same general carboxysome structure and function, though they differ in gene organization and protein sequences.

Carboxysomes function within a broader metabolic network called the Carbon Concentrating Mechanism, or CCM (Figure 1a). Inorganic carbon transporters in the cell membrane pump $HCO_3^-$ into the lumen, raising its concentration to about 30x the equilibrium concentration in water [2,6]. The disequilibrium between $HCO_3^-$ and $CO_2$ is advantageous because it stockpiles a charged, and therefore membrane-impermeable, form of carbon in the cell. This preferences the dehydration reaction in the carboxysome, concentrating $CO_2$ near Rubisco. Rubisco is thus poised to operate near its $V_{max}$ when carboxylating ribulose-1,5-bisphosphate (RuBP) to produce two molecules of 3-phosphoglycerate (3-PG) (Figure 1b). In addition to carboxylation, Rubisco can also oxygenate RuBP; the product of this off-target reaction must be recycled via wasteful photorespiration pathways. The high $CO_2$ environment of the carboxysome therefore competitively inhibits oxygenation, and it remains an open question as to whether exclusion of $O_2$ by the shell is necessary for CCM function [2]. Finally, this unique environment enabled Rubisco evolution to maximize for carboxylation activity, and carboxysomal Rubiscos are among some of the fastest known Rubiscos, despite having low specificity for $CO_2$ over $O_2$ [7,8].

Knocking out various components of the CCM renders cells incapable of growing at the atmospheric $CO_2$ concentration (~0.04%), and they must be grown in high $CO_2$ (~1–10%) [9,10]. In particular, carbonic anhydrase must be active only inside of the carboxysome; knocking it out or expressing it in the cytosol destroys the CCM [11]. Carboxysomes must also limit $CO_2$ permeability so that it doesn't diffuse away from Rubisco. Pentamer deletion strains, which produce carboxysomes with pores at the icosahedral vertices, only grow in high $CO_2$ [12]. A precise understanding of how shell proteins limit $CO_2$ diffusion while allowing entry and exit of other intermediates such as 3-PG and RuBP is still not well understood. Despite speculation that the carboxysome shell is selectively permeable, promoting uptake of $HCO_3^-$ while blocking $O_2$, no direct evidence has been experimentally measured. Mathematical models show that the CCM does not require $O_2$ impermeability to function [2,13], though both $O_2$ and $CO_2$ may encounter an increased resistance at the hexamer pore compared to $HCO_3^-$ and 3-PG [13]. In total, these results show that concentrating $CO_2$ near Rubisco by limiting $CO_2$ leakage from the carboxysome is essential to the function of the CCM and is an important principle in the development of biotechnological tools to concentrate $CO_2$.

## From carboxysome structure to functional CCM reconstitution

Reconstituting functional carboxysomes, i.e. those that can concentrate carbon, into an alternative host organism has been a major academic and bioengineering goal. However, it has been difficult to do because structure alone cannot recapitulate the CCM. The first recombinantly produced carboxysomes were α-carboxysomes from the model proteobacterium *Halothiobacillus neapolitanus*, expressed in *E. coli*. Expressing the native 10-gene operon was sufficient to produce wild-type looking carboxysomes [14]. Similarly, a synthetic operon of 12 genes from cyanobacterium *Synechococcus elongatus* PCC7942 produced wild-type looking β-carboxysomes in *E. coli* [15]. The engineered heterologous systems in both studies possessed active Rubisco, but it remained unclear whether recombinant carboxysomes could concentrate carbon, arguably the carboxysome's essential feature. A transposon mutagenesis screen of *H. neapolitanus* under high $CO_2$ vs. low $CO_2$ conditions revealed dozens of new genes responsible for the functioning of the α-carboxysome CCM, including several uncharacterized proteins in a secondary operon [16]. Characterizing unknown hits, as well as undertaking a systematic study in β-carboxysomes beyond the previous screens [9,10], will be crucial to uncovering what components are necessary to fully reconstitute the CCM. The following sections highlight recently discovered proteins and protein activities and how they may translate into using CCMs for enhancing metabolism.

## New discoveries in carboxysome assembly and CCM function

Researchers made early progress in identifying and characterizing major players in the carboxysome and CCM such as Rubisco, CA, shell proteins, and carbon transporters, reviewed in greater detail in references [5,17]. This section reviews proteins discovered or characterized in recent years that have greatly increased our understanding of the carboxysome and CCM, and which are important new entries in the carboxysome biotechnological toolbox.

### CsoS2 & CcmM –

CsoS2, from α-carboxysomes, and CcmM, from β-carboxysomes, are essential for carboxysome assembly and structure. Though they have no sequence or domain homology (Figure 2a & b), they share many striking similarities. Both are conserved, essential proteins located in the core carboxysome locus of their respective lineages [17]. Both are highly abundant in the carboxysome, with numbers roughly equal to Rubisco holoenzyme [5].

Notably, both CsoS2 and CcmM bind Rubisco and facilitate carboxysome nucleation. Both α-and β-carboxysome Rubiscos evolved binding sites that bridge two large subunits while making contacts with the small subunit (Figure 2c) [18,19]. This likely ensures that only the 16-subunit Rubisco holoenzyme is encapsulated during carboxysome assembly. Both CsoS2 and CcmM Rubisco-binding domains contain 3–5 repeat motifs separated by predicted disordered sequences (Figure 2a & b). Despite these shared features, the binding domains differ in their secondary structure. In CsoS2, the Rubisco-binding N-terminal domain (NTD) repeats are alpha helical, while the C-terminal CcmM repeats have structural similarity to the Rubisco small subunit (termed small subunit-like or SSUL) [18–20]. In CsoS2, a single

repeat binds with low affinity, but multivalent interactions could promote high affinity binding across multiple Rubiscos, thus nucleating carboxysome assembly (Figure 2d) [19]. Likewise, the three CcmM repeats together bind Rubisco with micromolar affinity [18,20]. Following a common theme for repetitive, multivalent proteins, both Rubisco-binding domains of CsoS2 and CcmM were shown to undergo liquid-liquid phase separation (LLPS) with Rubisco, though it should be noted both studies required salt concentrations below that of physiological 150 mM [18,19].

CsoS2 and CcmM both have a short and long isoform, and the significance of this is not fully understood (Figure 2a & b). The isoforms are produced in CsoS2 by ribosomal frameshifting and by an internal ribosome entry site (IRES) in CcmM [21,22]. In wild-type α-carboxysomes, both the short (Csos2A) and long (CsoS2B) forms are present at a roughly equimolar ratio [21]. When the frameshifting site is mutated, CsoS2B is sufficient to reconstitute carboxysomes on its own, but CsoS2A cannot [21]. Relatedly, CcmM has a short (M35) and long (M58) form. Both M35 and M58 are needed for functional β-carboxysomes [22].

Both CsoS2 and CcmM may possess redox-regulated intrinsic flexibility. Suggestively, in *Thermosynechococcus elongatus* BP-1, the CcmM C-terminal γ-CA is only active under disulfide-forming oxidizing conditions [23]. Most repeat segments of both CsoS2 and CcmM contain 1–2 cysteines. Cells with β-carboxysomes with mutated CcmM cysteines grew 2–3 times slower than wild-type, and many carboxysomes were irregularly shaped [18]. The effect in α-carboxysomes is not yet known. In biochemical studies, the reduced form of CcmM repeats bound Rubisco with higher affinity but showed less mobility under LLPS conditions [18]. This suggests a model in which the carboxysome nucleates under reducing cytosolic conditions and, upon complete assembly, matures into a liquid-like oxidizing environment (Figure 2d). Microscopy of developing β-carboxysomes using a redox-sensitive GFP suggested that this model may be true in vivo [24]. The effect of redox regulation in these compartments remains an understudied, yet potentially highly significant, aspect of their assembly and function.

### McdA & McdB –

Cells with β-carboxysomes arrange them linearly along a central longitudinal axis throughout growth and equally distribute carboxysomes to daughter cells during division [25]. This organization is driven by a pair of proteins, McdA and McdB [26]. McdA is a ParA-type ATPase that binds the nucleoid and shows a characteristic oscillatory behavior between cell poles. McdB, by analogy to plasmid partitioning systems [27], is thus thought to engage both the carboxysome and McdA. This facilitates an even distribution of carboxysomes, and those that lack either or both proteins show carboxysome clumping at a polar end. Expression of carboxysomes in a strain that lacks the proper positioning and partitioning machinery results in carboxysome aggregation, and loss of carbon fixation function in descendants without carboxysomes [25,28]. However, cells with McdA/B knockouts do not require high $CO_2$ to grow, likely because carboxysome-less cells can simply produce new ones, though their doubling time is significantly longer [25].

### DabA & DabB –

Inorganic carbon ($C_i$) transporters are essential to the CCM as active $C_i$ accumulation powers the downstream action of the carboxysome [2,6]. A review by Price et al. summarizes five of the $C_i$ uptake systems [6]. Recently, the DAB complex joined this list. DabA and DabB form a membrane-bound complex that appears to couple $CO_2$ transport into the cell to a cation gradient [16,29,30].

### Rubisco activases –

Rubisco is prone to inhibition by its substrate, RuBP, and other sugar derivatives. Rubisco activases catalyze release of this inhibitor. These enzymes are essential in plants and algae, but do not appear to be essential in carboxysome-containing bacteria [31,32]. The activases are divergent in the two carboxysomal lineages: α-lineages contain activase CbbQ and associated protein CbbO, while β-lineages contain β-Rca. Through convergent but different mechanisms, both activases bind Rubisco and are likely targeted to the carboxysome [32–35]. Though the biochemistry of these activases is increasingly understood, more research needs to be done to understand their role in carboxysomal carbon fixation.

## Bioengineering the carboxysome and CCM

### Optimizing the bacterial CCM

CCM-enhanced microbes could serve many bioindustrial applications seeking to take advantage of $CO_2$-dependent metabolism. New discoveries suggest optimization could start with Rubisco (Figure 3c). Fixation flux could, in theory, be improved via encapsulation of a faster Rubisco, many of which were recently discovered and characterized [8]. In contrast to carboxysomal Form I Rubiscos, most of the fastest Rubiscos are Form II, and would need to be engineered for carboxysome targeting likely using CsoS2, CcmM, or other encapsulation peptides [36,37]. The carboxysome appears to be sensitive to the type of Rubisco it encapsulates - cells with an orthologous Form Iα Rubisco expressed in an α-carboxysome did not grow well in air, and replacement with a Form II lacked carboxysomes and required high $CO_2$ for growth [38,39]. A recombinant Rubisco may also require its cognate Rubisco activase to be expressed in the carboxysome.

To engineer a heterologous bacterial host to utilize a carboxysomal CCM, more genes are needed than just those in the major carboxysomal operon, which typically contains Rubisco, a carboxysomal nucleating protein, CA, and shells. A complete reconstitution of the *H. neapolitanus* CCM in *E. coli* required expression of a secondary operon alongside the major operon, thus enabling Rubisco-dependent *E. coli* to grow at atmospheric $CO_2$ [40]. This secondary operon included the DAB inorganic carbon transporter, the CbbO and CbbQ Rubisco activase complex, and acRAF, a proposed Rubisco chaperone [41], along with several other unknown ORFs. Of these, both the DAB and acRAF were shown to be essential CCM components in the native organism *H. neapolitanus* [16]. The DAB complex and bicarbonate transporter SbtA are the only transporters that have been demonstrated to be active when expressed in a heterologous system [16,42], making them useful candidates for CCM engineering. This successful reconstitution marks a substantial progression in our knowledge from carboxysome structure to CCM function.

Now that a first-principles study in *E. coli* has shown heterologous reconstitution of a carboxysomal CCM to be possible, expansion into industrial hosts is a logical next step (Figure 3a). Baumgart et al. expressed the *H. neapolitanus* carboxysome operon in the biotechnologically relevant bacterium *Corynebacterium glutamicum*, but carboxysomes were small and malformed [43]. Despite this, there is precedent for recombinant bacterial microcompartments to express in many diverse bacterial species, as was demonstrated with expression of the Pdu compartment in over 6 different hosts [44]. Introducing the CCM into eukaryotic hosts such as yeast may enable efforts to engineer bioindustrial strains that are better equipped to utilize carboxylation as part of a metabolic engineering strategy [45]. In other cases, the $CO_2$-dependent growth behavior of autotrophic strains, such as *C. necator*, could be improved through introduction of a CCM [46]. Finally, lower DNA payloads are advantageous for recombinant CCM engineering, and researchers are testing the limits of minimal carboxysome systems by eliminating unnecessary proteins or creating fusions (Figure 3d). Many of these minimal systems show structural integrity and Rubisco activity [47–50].

## Optimizing the plant CCM

Much effort has gone towards creating bacterial CCMs in plants. Many agriculturally important C3 plants such as wheat and rice lack CCMs, and instead devote ~5% of leaf biomass to Rubisco [51], consuming large amounts of nitrogen in the process. Plants with engineered carboxysomal CCMs could theoretically increase yield while consuming far less nitrogen [52]. Many groups have proposed how to engineer carboxysomal CCMs into plants, and readers are directed to cited papers for more in-depth details [53,54]. In general, the engineering milestones are as follows: (1) Insert bicarbonate transporters into the chloroplast inner membrane to raise the concentration of bicarbonate in the stroma, (2) Express carboxysomes in the chloroplast, and (3) Knock out stromal carbonic anhydrases in order to maintain a high ratio of $HCO_3^-$ to $CO_2$ (Figure 3b).

Initial efforts towards this ultimate goal are already underway. Minimal α- and β-carboxysomes have been expressed in chloroplasts, and studies showed the formation of carboxysome-like structures [49,55,56]. Cyanobacterial Rubiscos expressed in plants maintained kinetic properties equivalent to those of their native host [49,56,57]. As expected due to lack of bicarbonate transporters, plants only grew under high $CO_2$ conditions, though with severe growth deficiencies compared to wild-type. Single-gene bicarbonate transporters BicA and SbtA have been expressed in the chloroplast inner envelope membrane, though it is unclear if they had activity [58,59]. Future efforts will thus need to focus on identifying, characterizing, and testing transporters that are capable of functional heterologous expression. A recent survey of dissolved inorganic carbon transporters in bacteria may provide useful candidates [60]. It is also possible that additional components such as the partitioning proteins McdA and McdB will improve growth by ensuring even carboxysome distribution among dividing chloroplasts in leaf cells.

## Future Directions

The relative simplicity of carboxysomes opens up the possibility of creating in vitro structures capable of performing carbon concentration, fixation, and other activities in order

to understand and engineer function (Figure 3e). Rubisco and CsoS2 or CcmM readily form liquidseparated droplets in vitro, achieving the first step of cargo nucleation [18,19]. A logical next step is to show partitioning of other cargo proteins such as a CA and Rubisco activase into the droplets, followed by shell encapsulation. Experiments to test preferential partitioning of metabolites such as RuBP, $HCO_3^-$, or $CO_2$ into the light or dense phase could probe whether or not LLPS plays a role in metabolite transfer and $CO_2$ concentration.

Recent insights on carboxysome structure and assembly are now enabling them to be re-engineered for alternative metabolisms, a domain which has mostly been limited to other types of bacterial microcompartments (Figure 3f) [61]. Recently, Li et al. expressed an [FeFe]-hydrogenase and ferredoxin in the α-carboxysome shell in *E. coli* to enhance $H_2$ production while shielding the hydrogenase from inactivating $O_2$. They observed an increase in $H_2$ in an aerobic environment compared to unencapsulated enzyme [37]. This kind of study opens doors for exciting new biotechnological applications of carboxysomes and structures engineered from them, while continuing to shed light on basic carboxysome biology. In particular, this study suggests that the carboxysome is an $O_2$-excluding environment, a theory which has generated significant discussion [2,13]. In addition, shell protein pore engineering (Figure 3g) may continue to further enable novel metabolism, including even redox-based reactions, while also providing exciting new insights into how carboxysomes permit entry of substrates, exit of products, and restrict loss of intermediates.

## Conclusion

Carboxysomes are unique among protein microcompartments for their ability to concentrate $CO_2$ and turn it into a useful cellular product. They are a biotechnologist's dream: they self-assemble in diverse organisms with a set of standard biological parts. They are, however, deceptively simple structures. Recent studies reveal that we are still discovering many of the proteins necessary to build functional carboxysome-based $CO_2$-concentrating systems. These and future discoveries will prove crucial to making meaningful engineering advances.

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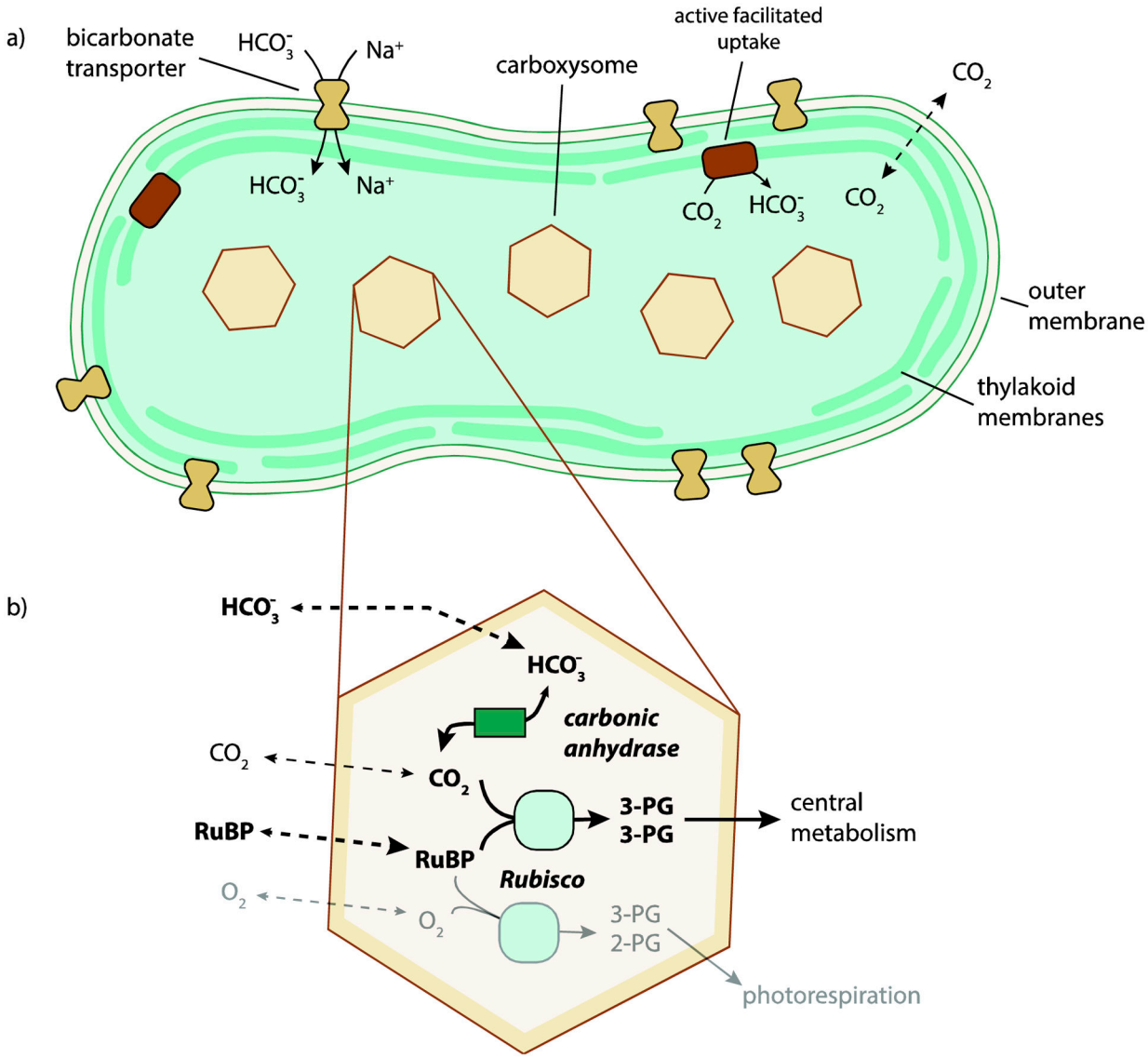


**Figure 1.**

a) The CCM in a cyanobacterial cell. Bicarbonate transporters and facilitated $CO_2$ uptake proteins raise the intracellular $HCO_3^-$ concentration while $CO_2$ flows freely across the plasma membrane. b) Carboxysome metabolism. $HCO_3^-$ enters the carboxysome along its concentration gradient, where it is converted to $CO_2$ via a carbonic anhydrase. $CO_2$ and RuBP serve as substrates for Rubisco, which produces two molecules of 3-PG. $O_2$ may occasionally serve as a Rubisco substrate, though at a minimal level.

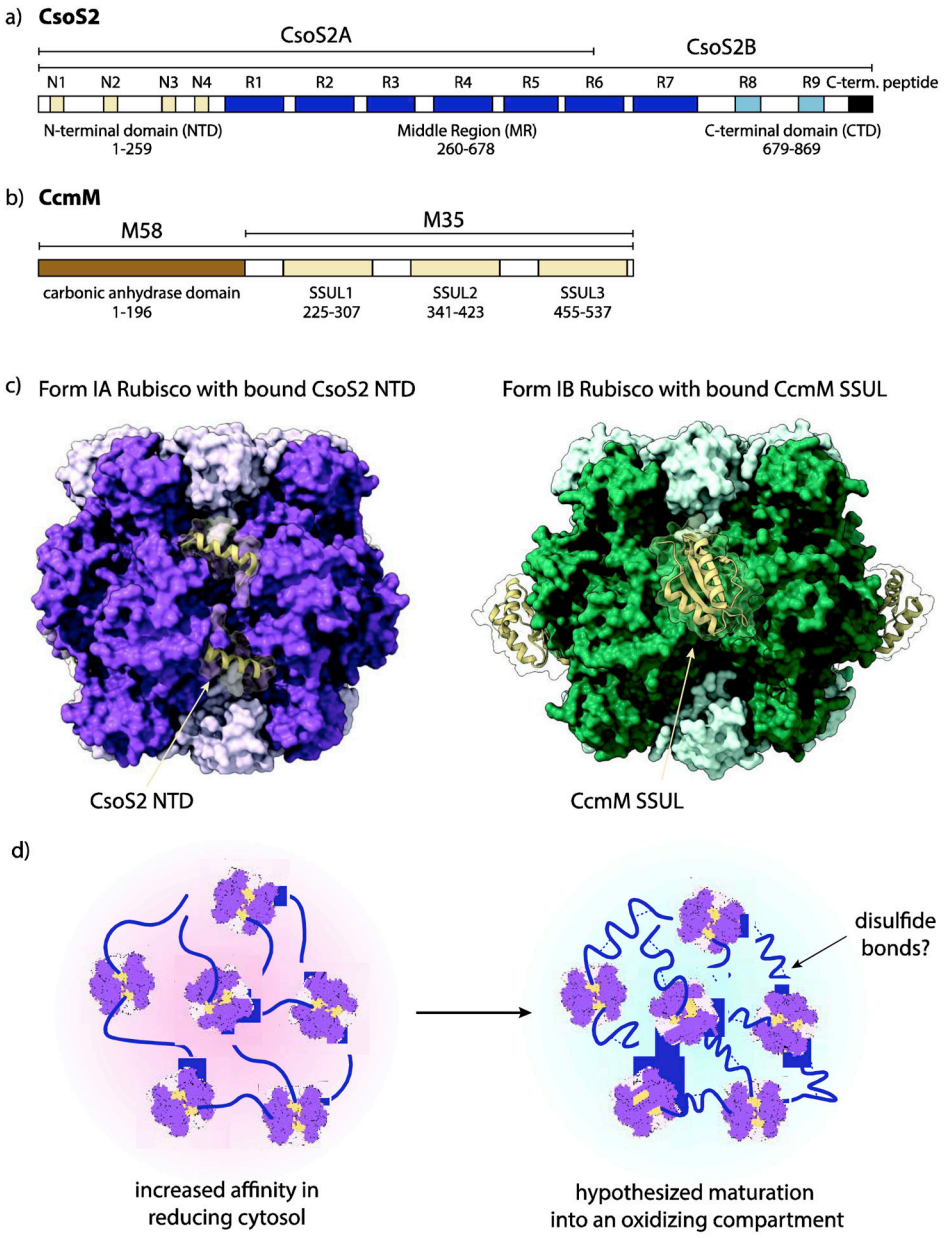


**Figure 2.**

a) Domain structure of CsoS2 from *H. neapolitanus* (uniprot ID: O85041; CSOS2_HALNC), with marked short (CsoS2A) and long (CsoS2B) forms. b) Domain structure of CcmM from *S. elongatus* PCC7942 (uniprot ID: Q03513; CCMM_SYNE7), with marked short (M35) and long (M58) forms. SSUL stands for “small subunit-like” domain. c) Structures of the CsoS2 NTD bound to Form 1A Rubisco (PDB: 6UEW) and CcmM SSUL bound to Form 1B Rubisco (PDB: 6HBC). Structures were rendered in ChimeraX. d) Hypothesized model of carboxysome nucleation. The carboxysome nucleating protein binds Rubisco with high avidity and affinity in the reducing cytosol. Maturation may involve oxidation (or exclusion of reducing agents) and disulfide-bond induced conformational changes.

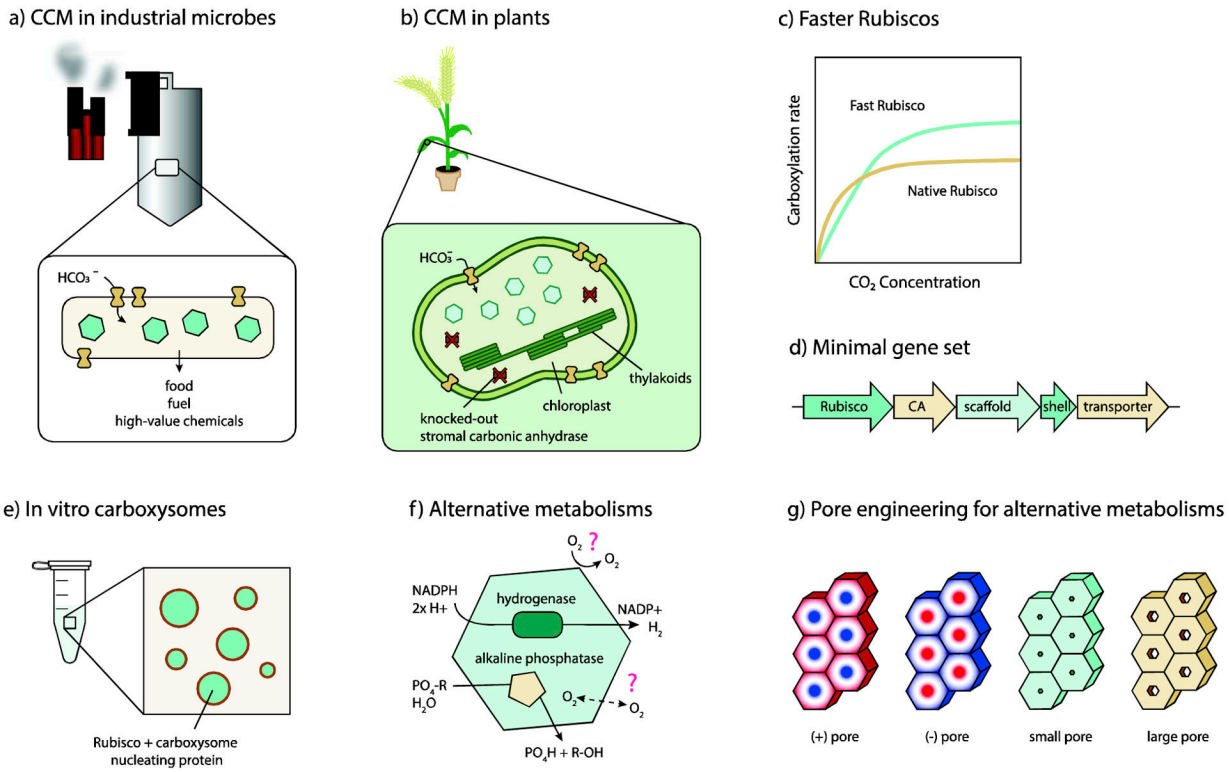


**Figure 3.**

a) Engineering a carboxysomal CCM into industrial microbes could convert atmospheric $CO_2$ into high value products. b) Engineering a carboxysomal CCM into plants could increase plant $CO_2$ efficiency and promote growth and biomass yield. c) Faster Rubiscos could enable more efficient carbon fixation. d) A minimal gene set lowers the DNA payload when engineering the CCM into new host organisms. e) In vitro carboxysomes are a novel platform to study carboxysome assembly, and could act as in vitro catalytic reactors. f) Carboxysomes can be repurposed for alternative metabolisms. Enzymatic activity may depend on whether or not the carboxysome is an oxygen privileged environment, which remains unknown. g) Pore engineering, such as changing the charge or size of the pore, may aid development of alternative metabolisms.