Programmed ribosomal frameshifting mediates expression of the α -carboxysome

Thawatchai Chaijarasphong¹, Robert J. Nichols², Kaitlyn E. Kortright², Charlotte F. Nixon², Poh K. Teng², Luke M. Oltrogge², and David F. Savage^{1,2,3, *}

¹Department of Chemistry, UC Berkeley, Berkeley, CA, 94720, USA

²Department of Molecular & Cell Biology, UC Berkeley, Berkeley, CA, 94720, USA

³Energy Biosciences Institute, UC Berkeley, Berkeley, CA 94720, USA

* To whom correspondence should be addressed. Email: savage@berkeley.edu.

13 **Abbreviations**

14 CCM, Carbon dioxide concentrating mechanism; PRF, programmed ribosomal frameshifting;
15 CB, carboxysomes; BMC, bacterial microcompartment; HnCB, *H. neapolitanus* carboxysomes;
16 RuBisCO, Ribulose-1,5- biphosphate Carboxylase/ Oxygenase; MW, molecular weight; PAGE,
17 polyacrylamide gel electrophoresis; EM, electron microscopy; MS, mass spectrometry; MS/MS,
18 tandem mass spectrometry; MALDI-TOF, Matrix assisted laser desorption/ionization – time of
19 flight; GFP, Green fluorescent protein; RFP, Red fluorescent protein; FMDV, Foot-and-Mouth-
20 Disease Virus; IRES, Internal ribosome entry site

21 **Keywords**

22 Bacterial microcompartments; CsoS2; co-translational regulation; carboxysome

Abstract

Many bacteria employ a protein organelle, the carboxysome, to catalyze carbon dioxide fixation in the Calvin Cycle. Only ten genes from *H. neapolitanus* are sufficient for heterologous expression of carboxysomes in *E. coli*, opening the door to detailed mechanistic analysis of the assembly process of this 200+ MDa complex. One of these genes, *csoS2*, has been implicated in assembly but ascribing a molecular function is confounded by the observation that the single *csoS2* gene yields expression of two gene products and both display an apparent molecular weight incongruent with the predicted amino acid sequence. Here, we elucidate the co-translational mechanism responsible for the expression of the two protein isoforms. Specifically, *csoS2* was found to possess -1 frameshifting elements that lead to the production of the full-length protein, CsoS2B, and a truncated protein, CsoS2A, which possesses a C-terminus translated from the alternate frame. The frameshifting elements comprise both a ribosomal slippery sequence and 3' secondary structure, and ablation of either sequence is sufficient to eliminate the slip. Using these mutants, we investigated the individual roles of CsoS2B and CsoS2A on carboxysome formation. In this *in vivo* formation assay, cells expressing only the CsoS2B isoform were capable of producing intact carboxysomes, while those with only CsoS2A were not. Thus, we have answered a long-standing question about the nature of CsoS2 in this model microcompartment and demonstrate that CsoS2B is functionally distinct from CsoS2A in the assembly of α -carboxysomes.

Introduction

Organisms that aerobically fix CO₂ through the Calvin-Benson-Bassham Cycle face a fundamental biochemical problem. The principal enzyme of this cycle, Ribulose-1,5-bisphosphate Carboxylase / Oxygenase (RuBisCO), has both a low affinity for CO₂ and promiscuous activity for the competing O₂ substrate [1]. Cyanobacteria and chemolithoautotrophic bacteria overcome this problem using an integrated physiological strategy known as the Carbon Dioxide Concentrating Mechanism (CCM) [2]. The CCM is a multicomponent mechanism - composed of inorganic carbon transporters and an organelle-like structure called the carboxysome - that function together to selectively deliver high concentrations of CO₂ directly to RuBisCO in order to facilitate carbon fixation.

The carboxysome is the centerpiece of the CCM. The carboxysome is an icosahedral protein complex of 80 – 500 nm composed of thousands of protomers [3-5]. There are two distinct evolutionary lineages of the carboxysome, α and β forms, which differ in their protein composition but share two major structural and functional features: i) a protein shell composed of thousands of hexameric and 60 pentameric capsomers and ii) a lumen possessing numerous copies of the cargo enzymes RuBisCO and carbonic anhydrase [6]. In concert with active transport producing high levels of cytosolic bicarbonate, this structural arrangement leads to the increased concentration of CO₂ within the lumen, allowing RuBisCO to catalyze carbon fixation with both high rate and specificity [7].

Recent work on the cyanobacterial β -carboxysome has identified a series of molecular interactions that non-covalently target holo-RuBisCO (8 large subunits and 8 small subunits), the major carboxysome component by molecular weight (MW), to the lumen of the shell. Specifically, the protein CcmM contains three domains homologous to the small subunit of RuBisCO and is capable of bridging multiple RuBisCO complexes together [8,9]. CcmM has been shown to interact with a second factor, CcmN, which in turn interacts with the hexameric shell protein CcmK2 [10]. Thus, RuBisCO is thought to form a nucleate-like aggregate in the cytosol, facilitated by CcmM, and then matures into a complete carboxysome by the recruitment of additional shell proteins by CcmN [11,12].

A significant variation between α and β carboxysomes occurs at the level of the intermediary factors bridging cargo and shell [4,6,13]. Specifically, the α -carboxysome does not

contain a CcmM or CcmN homolog and the assembly process is less characterized. Biochemical and genetic analysis by Heinhorst and colleagues have implicated ten genes in the production of α -carboxysomes in *Halothiobacillus neapolitanus*, and it has recently been shown that these genes are sufficient for carboxysome expression in the non-native host *E. coli* [5,14-16]. Notably, nine of these ten genes have an obvious function: six are unambiguously shell proteins and encode for either bacterial microcompartment (BMC) domain-containing proteins (*csoS1ABCD*; pfam00936) or vertex-forming pentamers (*csoS4AB*; pfam03319) while three encode the cargo enzymes carbonic anhydrase (*csoS3*) and RuBisCO (*cbbLS*) (Heinhorst 2006). The remaining gene, *csoS2*, has a less obvious functional role in the carboxysome. Early electron microscopy (EM) studies localized the CsoS2 protein to the shell, but more recent two-hybrid, gel-shift, and pull-down experiments suggest CsoS2 may also interact with a number of components including the shell proteins and RuBisCO [17-19]. Moreover, deletion of *csoS2* abolishes carboxysome formation [18]. Thus, one possibility is that CsoS2 plays a role in carboxysome assembly and may be functionally analogous to CcmM and CcmN.

Much of the uncertainty in CsoS2 function lies in an unexpected observation: carboxysomes from *H. neapolitanus* possess two different forms of CsoS2 [17]. The predicted molecular weight of the *csos2* gene product is 92 kDa but purified carboxysomes possess a long isoform, CsoS2B, and a short isoform, CsoS2A, with apparent MWs of ~120 and ~70 kDa, respectively, as analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) analysis. Previous work has shown that CsoS2A and CsoS2B share the same N-terminus but may be post-translationally modified via glycosylation [17], thus offering an explanation for the discrepancy between predicted and observed MWs, although other evidence suggests the proteins may not be modified [19,20].

Elucidating a clear functional role for CsoS2 requires understanding its specific molecular interactions with other carboxysome components and, therefore, the precise definition of primary amino-acid sequence structure for both CsoS2B and CsoS2A. Here, using a heterologous *E. coli* expression system, we show that differential expression of the two isoforms is encoded by ribosomal frameshifting *cis* elements present on the *csoS2* mRNA that are capable of encoding roughly 1:1 stoichiometric expression of CsoS2A and CsoS2B. CsoS2B is 869 amino acids long and produced from full length expression of the 0 frame of the gene. Frameshifting

104 specifically generates the CsoS2A protein possessing a C-terminus from the -1 frame and results
105 in a truncated protein 570 amino acids in length. Surprisingly, in carboxysome formation assays,
106 CsoS2B by itself leads to the formation of assemblies structurally similar to carboxysomes, while
107 CsoS2A alone is clearly incapable of forming carboxysomes. Thus, we have defined the sequence
108 of each CsoS2 isoform, a mechanism of their formation, and provide initial evidence towards
109 their functional importance.

110

111

Results

Recent evidence suggests CsoS2 is crucial to the assembly of the carboxysome, but its precise molecular function remains unclear due to the ambiguous relationship between CsoS2A and CsoS2B [18]. To resolve this uncertainty, we sought to delineate the primary structure of these two proteins and their relative functional importance.

The primary DNA sequence of *csoS2* from *H. neapolitanus* encodes a 92.0 kDa protein. Inspection of this amino-acid sequence reveals a protein with three regions, an N-terminal, Middle, and C-terminal Region (NR, MR, and CR) (Fig. 1A). Previous bioinformatic analysis suggested the NR possesses four homologous repeats with alpha helical propensity and that the MR possesses six homologous repeats of roughly ~50 amino acids in length each possessing three canonical (V/I)(T/S)G motifs (R1-R6; Fig. 1A) [18]. Interestingly, inspection of the primary sequence reveals the possibility of three additional repeats in the CR (Fig. S1A). These repeats (R7-R9) possess extended interstitial amino acids compared to repeats in the MR. CsoS2 also possesses an extremely conserved terminus, which we term the C-terminal Peptide (CTP) (Fig. S1B). Finally, CsoS2 is also predicted to be highly intrinsically disordered, which has been experimentally validated in solution scattering experiments [18].

In order to link this sequence with function, we set out to understand how one such gene can generate two gene products. Heterologous expression of CsoS2 indicated that the mechanism of differential expression used to generate CsoS2A and CsoS2B is conserved between the gamma proteobacteria *E. coli* and *H. neapolitanus*. Carboxysomes purified from either *H. neapolitanus* (HnCB) or *E. coli* (EcCB; see Bonacci et al. 2012) produce bands at the characteristic, anomalous MWs of 120 kDa and 70 kDa and with roughly 1:1 stoichiometry (Fig. 1B). Likewise, expression of CsoS2 protein bearing an N-terminal 6x polyhistidine tag (EcS2) also produced both the A and B form, with a preference for CsoS2A possibly due to higher degradation of CsoS2B or selective enrichment of CsoS2A during the purification procedure. Previous results indicate differential expression may arise from post-translational processing [17] but attempts to verify glycosylation or proteolytic activation were negative (Fig. S2).

Intrinsically disordered proteins often run at larger apparent MWs in denaturing PAGE, offering a partial explanation for the observed anomalous masses [21]. We next verified that the

EcS2 mass matched that of the theoretical gene product using Matrix-assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF) Mass Spectrometry (MS). This revealed two peaks with large m/z , corresponding to masses of 60.8 kDa and 92.3 kDa (Fig. 1C). The latter is an identical (within 0.01%) match to the predicted mass of full-length EcS2 and is expected to be CsoS2B. We therefore concluded that the 60.8 kDa species corresponded to CsoS2A and sought to identify, using this MW as a guide, the mechanism of CsoS2A expression.

The lack of post-translational processing led us to ask whether an alternative mechanism, such as co-translational regulation via frameshifting, could explain the presence of CsoS2A. Programmed ribosomal frameshifting (PRF) is a process whereby an mRNA encodes a specific signal to shift the ribosome from the usual translated 0 frame to either the +1 or -1 frame [22]. This can result in the production of two or more different polypeptides from a single mRNA. While pervasive in viruses, PRF is much less common in prokaryotes and eukaryotes. Only a handful of proteins in *E. coli*, most notably the gamma subunit of DNA polymerase III, are known to be generated by PRF [23].

Based on the MALDI-TOF data, we hypothesized that the terminal stop codon for CsoS2A was likely to be in the latter half of the gene, due to its mass of 60.8 kDa. To probe for PRF, we designed a construct, EcCsoS2-NonStop, possessing synonymous mutations ablating the 22 stop codons in the -1 and +1 frames of the latter half of the gene. The construct also contained stop codons in the -1 and +1 frames just downstream of the normal 0 frame stop. Strikingly, expression of EcCsoS2-NonStop generated only a single band with MW similar to CsoS2B (Fig. 2A). Therefore, expression of a synthetic CsoS2 revealed that frameshifting is likely responsible for the differential expression of the two isoforms.

Further MS analysis confirmed this hypothesis and identified the site of frameshifting. CsoS2A and CsoS2B were isolated from PAGE bands of purified EcS2, trypsinized, and analyzed using MS/MS. Masses from this experiment were matched to a theoretical MW library generated from the CsoS2 sequence in order to identify peptides specific to each isoform. CsoS2A and CsoS2B shared a large number of peptides in the first 2/3 of the protein sequence, but there was a dramatic reduction in CsoS2A peptides beyond amino acid 570 (Fig. 2B). The recent work of Cai *et al.* noted a similar observation [18].

PRF generates proteins possessing an N-terminus from the 0 frame and a C-terminus from either the -1 and +1 frames. We therefore repeated the peptide identification analysis but instead used a theoretical frameshifting-specific library, containing every possible frameshifted polypeptide from the *csos2* gene. Surprisingly, this analysis revealed a unique peptide – HAPKDVR – containing a fusion of amino acids from the 0 and -1 frames (Fig. 2C). The theoretical mass of a CsoS2A protein ending in this sequence exactly matches the experimental value of 60.8 kDa from Fig. 1C, thus providing additional confirmation.

The model system for -1 PRF is the gene *dnaX* from *E. coli*, which generates the gene products tau and gamma of the DNA polymerase III complex [23,24]. A large body of biochemical and biophysical data on this system have defined the signal for frameshifting: i) a 5' ribosomal binding site upstream of the frameshifting site which stalls the ribosome, ii) a slippery sequence of A-AAA-AAG where the ribosome changes frame, and iii) significant secondary structure downstream of the slippery sequence to further impede ribosome translation (Fig. 3B) [22]. These signals cause the ribosome slipping from a 0 frame codon back to the -1 frame codon. This process is enabled by the degeneracy of the genetic code and allowance of mismatches at the wobble codon position. As a consequence, Kornberg et al. have shown a sequence matching the nucleotide motif X-XXY-YYZ has the potential to allow PRF [23].

Strikingly, the *csos2* gene contains similar *cis* elements to *dnaX* at the gene sequence neighboring the sequence encoding the HAPKDVR peptide (Fig. 3B). Most importantly, *csos2* contains a putative slippery sequence, C-CCA-AAG, matching the X-XXY-YYZ motif, directly where there is ambiguity between the 0 frame and -1 frame in the MS/MS data (arrow in Fig. 2C). In addition, RNAstructure analysis [25] of the primary nucleotide sequence indicates a potential stem-loop structure with significant stability (25.7 kcal/mol), downstream of the slippery sequence. This loop contains the C-terminus of the putative HAPKDVR peptide and a stop codon immediately following the arginine codon, suggesting this is the terminus of CsoS2A.

This model was tested by generating constructs deficient in PRF. First, *csos2* was recoded to end with the HAPKDVR-stop sequence in the 0 frame (EcS2A). Expression of this construct was equal in size to CsoS2A and confirmed its anomalous PAGE behavior (Fig. 3C). To test the role of the slippery sequence and secondary structure elements, we next designed constructs with a series of synonymous mutations to ablate either the slippery sequence (NoSlip) or the stem-loop

(NoStem) and perturb PRF (orange and red circles in Fig. 3B). As with EcCsoS2-NonStop, these constructs expressed a long peptide matching the MW of CsoS2B (Fig. 3C). Thus, both the slippery sequence and the downstream secondary structure are required for -1 PRF.

To unambiguously assay the function of the PRF elements, we tested for frameshifting in a completely synthetic context. A region of the *csoS2* gene containing the complete PRF elements and additional 15 base pairs downstream of the stem-loop was cloned between a green fluorescent protein (GFP) and mCherry red fluorescent protein (RFP) reporter such that the 0 frame of the PRF elements would yield a full length GFP-RFP fusion (Fig. S3). Expression of this construct generated two fluorescent species – the expected full-length protein displaying both GFP and RFP fluorescence, and a short form containing only GFP – thus confirming frameshifting (Fig. 3D). Interestingly, the ratio of this construct favors the short form while the ratio of CsoS2A and CsoS2B is roughly 1:1 from the carboxysome operon. Regardless, this result demonstrates the PRF elements are robust to context, and are sufficient to recode the expression of a translating message. Taken together, the above experiments suggest *csoS2* undergoes a programmed ribosomal frameshift to yield the different forms of CsoS2. More specifically, we conclude that CsoS2A is a 570 amino acid protein whose terminal amino acids are encoded within the -1 frame of the gene.

We next investigated the conservation of -1 PRF in CsoS2 homologs from other organisms. The -1 PRF prediction server KnotInFrame [26], with additional criteria (see Materials and Methods), was used to search for -1 PRF candidates among known CsoS2-bearing species (Fig. S5). Intriguingly, *csoS2* genes from 79 out of 162 species were predicted to undergo frameshifting and the distribution of PRF across the phylogenetic tree appears to be somewhat clustered, suggesting that this mechanism is widely used and conserved among the species with closely related CsoS2 homologs. Although further experimental data are needed to validate each prediction, we found that a number of them matched previously reported observations. For instance, *H. neapolitanus* CsoS2 is predicted to frameshift at the location we experimentally confirmed in this study and the predictions matched previous unexplained observations of carboxysomes possessing either one or two CsoS2 isoforms in three different species (*T. intermedia*, *T. crunogena* and *P. marinus* str. MED4) [16,27]. Closer inspection of each organism's characteristics, habitat, and CCM function may shed light on why -1 PRF exists in

some bacteria but not in others.

Finally, we sought to determine the significance of each isoform for carboxysome assembly. Mapping the location of PRF onto the primary sequence of CsoS2 shows that the site is within Repeat 6 at the end of the MR. Thus, CsoS2A contains the NR and five complete repeats within the MR (asterisk Fig. 1A), while CsoS2B contains the entire CR, including the highly conserved CTP. We therefore hypothesized this variation in sequence could create a functional difference between CsoS2A and CsoS2B.

The importance of these sequences was assayed using heterologous carboxysome expression in *E. coli*. It has been shown previously that ten genes from two *H. neapolitanus* operons can be refactored into a single isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible operon and expressed in *E. coli* (EcCBs) [14]. EcCBs are generally well-formed and are functional for CO₂ fixation but, in contrast to native HnCBs, are somewhat more heterogeneous in morphology and can possess broken shells. Regardless, this provides a simple platform to assay an *in vivo* reconstitution of carboxysome assembly as a function of CsoS2 sequence.

Expression of EcCBs in this assay revealed a critical functional difference between CsoS2A and CsoS2B. The EcCB expression plasmid was modified to encode either CsoS2A, CsoS2B (with ablated slippery sequence), or wild-type (WT) CsoS2, and carboxysomes were expressed and analyzed for formation by sucrose gradient purification and EM. In an SDS-PAGE analysis, WT and CsoS2B-containing carboxysomes produced similar gradient fractionation patterns, but CsoS2A yielded no fractions characteristic of carboxysomes (Fig. 4A and S4). EM of WT and CsoS2B showed the formation of carboxysome-like structures while the CsoS2A-derived fraction lacked any significant assembly (Fig. 4B). Thus, CsoS2A, in the absence of CsoS2B, is incapable of forming carboxysomes while CsoS2B by itself is sufficient for shell assembly around the lumen and form carboxysome-like structures.

Discussion

Frameshifting and the two forms of CsoS2. Since the initial biochemical characterization of α -carboxysomes, the anomalous behavior of CsoS2 expression has confounded the determination of its molecular function. Here, we show that -1 programmed ribosomal frameshifting is responsible for the genesis of the two forms of CsoS2.

-1 PRF occurs when the ribosome is stalled during translation by 3' secondary structure and, in certain cases, a 5' internal ribosomal binding site-like sequence. A slippery sequence of the mRNA allows the tRNAs to shift backward to wobble base-pair with the codons in the -1 frame. Slipped ribosomes that continue translating in the -1 frame can then encounter a stop codon and lead to early protein truncation (Fig. 3A). Inspection of the *csoS2* nucleotide sequence revealed that it possesses both a potential slippery sequence and a significant predicted 3' stem-loop. A relatively purine-rich sequence (GGCCAA) is observed 14 nucleotides upstream of the slippery sequence. However, since the sequence is significantly different from the consensus, it is uncertain whether this sequence could act like a ribosome-binding site and contribute to PRF. Removal of either the slippery sequence or stem-loop abolishes CsoS2A, suggesting that these elements are necessary for frameshifting. In addition, PRF function is robust to sequence context, as frameshifting can occur when the *csoS2* gene is expressed by itself in *E. coli* or completely out of context, as seen in the synthetic fluorescent reporter containing only the slippery sequence and the stem-loop. Therefore, these elements are sufficient to explain the generation of two CsoS2 isoforms.

The efficiency of known -1 PRF elements can range from a few percent to as high as 80% [22]. The CsoS2 PRF is 50% and is therefore near the upper range of characterized examples across all kingdoms of life. The dearth of model frameshifting systems in prokaryotes has made the prediction of efficiency difficult, so CsoS2 therefore provides a novel example to better inform this understanding. Clearly, extensive Watson-Crick base-pairing and high GC content in the stem-loop (Fig. 3B) is important for efficiency, but the overall efficiency is likely to be multivariate and context-dependent [28].

While we portray the -1 PRF of CsoS2 as entering the -1 frame when CCA and AAG codons are at the P and A sites of the ribosome (Fig. 3A), respectively, a recent study has shown that PRF can operate through multiple pathways simultaneously [29]. Specifically, the translation

apparatus can enter the -1 frame from several codons along the slippery sequence and can also slip by -4 or +2 nucleotides. Therefore, it is formally possible that CsoS2A in its native context could be the product of not one, but several, translational pathways. Further mass spectrometry studies, by probing for peptides from multiple frames, could elucidate the exact slip events that occur during CsoS2 translation.

Finally, it is not known what advantages are conferred by using -1 PRF as opposed to other strategies in generating isoforms. As -1 PRF is known to result in tight stoichiometric control [30], it is possible that α -carboxysome assembly benefits from having a fixed ratio between CsoS2AB. Notably, in β -carboxysomes, CcmM has two isoforms, the short 35-kDa (M35) and long 58-kDa (M58) proteins, the former generated via an internal ribosome entry site (IRES) [8,31]. Despite different mechanisms, both -1 PRF and IRES involve mRNA secondary structure, but direct comparison between the biological advantages of PRF and IRES is unclear. One possibility is that size heterogeneity differences between α and β -carboxysomes [32] is derived from the differences in function between CsoS2 and CcmM/CcmN and the regulation of their expression by PRF and IRES elements, respectively.

Frameshifting and the mechanism of carboxysome assembly. Several lines of evidence hint at the possibility that CsoS2 may play a central role in organizing carboxysome assembly. However, since it was previously not possible to individually express each form of CsoS2, the functional difference between CsoS2A and CsoS2B has not been elucidated. Here, we use our findings on PRF to specifically produce carboxysomes with either CsoS2A or CsoS2B in *E. coli*. Surprisingly, we find that CsoS2B is necessary and, at least to the resolution of our experiments, is sufficient for the assembly of carboxysomes similar to those possessing both isoforms. Since CsoS2A is not required in this process, it is perplexing that this isoform is produced in a significant amount in the native organism and that the frameshifting elements are widely conserved across clades in the phylogeny of CsoS2. Future experiments, probing the structure and function of carboxysome in a more fine-grained fashion, may reveal a role for the CsoS2A isoform.

While we have clarified the molecular identity of each CsoS2 isoform, their exact functions remain unclear. We speculate on their involvement in α -carboxysome assembly by

analogy to the CcmMN system. M35 has been shown to nucleate RuBisCO, and M58 can connect the RuBisCO aggregate to the shell [8,31,33]. Along these lines, CsoS2A may interact with RuBisCO in the core of the lumen, while CsoS2B binds RuBisCO close to the periphery and uses the C-terminus to anchor them to the shell. Recent results place the C-terminus of CsoS2B either bound to the shell or outside it, which supports this hypothesis. Strikingly, EM images and *in silico* modeling suggested that the ratio of RuBisCO located immediately underneath the shell to those distributed in the remainder of the lumen is close to 1:1, matching the ratio of CsoS2A to CsoS2B in a carboxysome [34]. It is therefore possible that CsoS2A and CsoS2B, driven by the latter's localization to the shell, reside in different zones within the carboxysome (Fig. 4C). Further protein-protein interaction studies and higher resolution studies of α -carboxysome assembly are needed to test this hypothesis.

A major difference between CsoS2 and CcmM lies in the necessity of the isoforms. In β -carboxysomes, both CcmM isoforms are necessary for the assembly process [31]. RuBisCO nucleation, mediated by M35, is required as the first step in the assembly [11]. On the other hand, the formation of α -carboxysomes likely proceeds without RuBisCO nucleation or at least in concert with it, as *H. neapolitanus* carboxysomes can assemble in the absence of luminal RuBisCO [35,36]. The lack of requirement for CsoS2A, therefore, may indicate a different assembly pathway.

Combining our findings above with previous work [18,20,32], we arrive at a potential model describing assembly of the α -carboxysome. Given previous results, CsoS2B is localized near the shell and may facilitate RuBisCO's previously observed tight packing [34] in this region, while CsoS2A primarily organizes RuBisCO in the remainder of the carboxysomal lumen (Fig. 4C). Several unresolved questions in this model warrant further experimental investigation. First, the molecular recognition elements facilitating interaction between CsoS2AB and the other proteins remain unknown. M35, having three RuBisCO-like repeats, can organize RuBisCO into the previously observed aggregate. Repeats in CsoS2AB share no sequence similarity to RuBisCO, so the outcome and quality of any such aggregation may be different. In addition, there is a strong conservation of the last 30 amino acids of CsoS2B (i.e. the CTP), suggesting functional importance, but a specific binding partner has not been identified. Second, while we portray CsoS2A as residing with RuBisCO in the core of a carboxysome, we cannot rule out the

possibility that some CsoS2A could exist in the subshell layer. Third, besides RuBisCO and shell proteins, CsoS2 may also interact with carbonic anhydrase. Finally, due to a number of conserved Cys residues, the two isoforms of CsoS2 may covalently interact with one another [18].

Evolutionary aspects of CsoS2. Although there is no direct evidence for a viral origin to carboxysomes and other BMCs, their similarities are striking. Both types of particles are polyhedral, proteinaceous, and self-assembling. CsoS2 also shares additional characteristics of viral proteins: it is highly intrinsically disordered, and undergoes PRF. The latter is common among viral proteins as viral constraints necessitate tight stoichiometric regulation and genetic economy. For example, a functionally similar protein to CsoS2, VP4, is present in Foot-and-Mouth-Disease virus. Like CsoS2, VP4 is an intrinsically disordered protein, binds to the interior side of the capsid shell, and is necessary for assembly of the viral capsid [37,38]. It remains to be seen whether these functional properties are, in fact, viral in origin or simply the result of the functionality required to facilitate the structural self-assembly of protein capsids.

While α - and β -carboxysomes appear to be evolutionarily independent, they both employ two protein isoforms and repeating units to mediate the assembly. Such a strategy might provide a number of advantages. It has been reported that a multivalent protein can mediate the aggregation of its partner in a switch-like manner [39]. Repeats in CsoS2 and CcmM may provide multivalency for a similar mechanism. Moreover, in both classes of carboxysomes, the shell interaction appears to be mediated by the long form of these proteins yet a shorter, multivalent species is often present. These observations may hint at the nature and rules governing self-assembly of these protein complexes and therefore serve as the basis for future experiments interrogating the design principles of carboxysome assembly and function in the cytosolic milieu.

Materials and methods

Preparation of plasmids

Plasmids were constructed using Golden-Gate Assembly and Phusion polymerase (New England Biolabs). Primers were designed to contain BsaI restriction sites and Golden-Gate recombination sites appropriate for the assembly of the desired construct. In those constructs with an affinity tag, the sequence expressing the tag was also included in a primer. Expression vectors were modified in our laboratory to be compatible with Golden-Gate cloning (Table S1).

The open reading frame for *H. neapolitanus* CsoS2 was amplified from the pHnCB10 plasmid containing nine genes of the *cso* operon and *csoSID* (Bonacci 2012). For the construct containing *csoS2* without stop codons in alternative reading frames, gBlocks containing synonymous mutations that remove stop codons in alternative frames, starting at the 1278th nucleotide of *csoS2*, were ordered from Integrated DNA Technologies. The gBlocks were amplified by PCR to generate BsaI restriction sites and then incorporated into the pET14-based destination vector using Golden-Gate assembly.

For the construct expressing only CsoS2B, a set of primers was designed to either 1) change the slippery sequence from 5'-C-CCA-AAG-3' to 5'-A-CCT-AAG-3' or 2) synonymously mutate several nucleotides in the downstream hairpin to reduce hairpin formation. The sequence of the mutated hairpin region is shown in Fig. 3.

To make the frameshift reporter construct, PCR was employed to introduce the BsmBI sites to the *EGFP* gene, the slippery sequence and stem-loop region in *csoS2*, and the *mCherry* gene. A TEV protease cleavage site was also introduced between the stem-loop and *mCherry*. These amplified products were assembled simultaneously into the pET14-based destination vector via Golden-Gate assembly. The control construct, with the slippery sequence and stem-loop replaced by 5'-GCGGCGGGCGGTGCAGGAGCT-3' (encoding Ala-Ala-Gly-Gly-Ala-Gly-Ala linker), was prepared in a similar manner.

Heterologous expression and purification of His-tagged *H. neapolitanus* CsoS2

Chemically competent *E.coli* BL21(AI) transformed with pBz13 were grown in 1 L LB media. At OD600 of 0.3-0.5, the culture was induced with 0.1% arabinose and grown at 22 °C 175 RPM for 16 hours. Cells were subsequently harvested by centrifugation at 4000xg for 20

minutes. The pellets were resuspended in Buffer A (20 mM Tris 300 mM NaCl pH 7.5) with cOmplete protease inhibitor (Roche), 0.1 mg/ml lysozyme (Sigma-Aldrich), and 25 U/ul benzonase (EMD Millipore). Cell lysis was performed by passing the cell suspension through an Avestin EmulsiFlex-C3 homogenizer three times. Crude lysate was centrifuged at 15,000xg for 20 minutes to remove cell debris. Clarified lysate was incubated with Ni-NTA resin for 1 hour and transferred to a gravity-flow column. The resin was washed with buffer A plus 30 mM imidazole and 1 mM phenylmethylsulfonyl fluoride. The protein was eluted with buffer A plus 300 mM imidazole and 1 mM phenylmethylsulfonyl fluoride. Eluted protein was passed through 10DG desalting column (Bio-Rad) to exchange into buffer B (50 mM Tris 150 mM NaCl pH 7.4) plus cOmplete EDTA-free protease inhibitor cocktail (Roche) and stored at -80 °C until further use.

Expression and visualization of EGFP-mCherry frameshift reporter

BL21(AI) transformed with pRJN-FS or pRJN-control were grown in LB media (10 ml) At OD600 of 0.3-0.5, the cultures were induced with 0.1% arabinose and further grown at 22 °C 175 RPM for 16 hours. Cells were harvested by centrifugation at 4000xg for 20 minutes and subsequently chemically lysed with SoluLyse-Tris (Genlantis). The lysate was clarified by centrifuging at 15000xg for 20 minutes. The clarified lysate was mixed with SDS-PAGE sample loading buffer. Without heating, gel samples were loaded on a 4-20% TGX Criterion gel (Bio-Rad). SDS-PAGE was performed at 150 V with Laemmli electrophoresis running buffer (25 mM, Tris 192 mM Glycine, 1% SDS). The gel was subsequently imaged with ChemiDoc imager (Bio-Rad), using GFP and RFP filters to detect EGFP and mCherry signals, respectively.

Western blotting

Clarified lysates of BL21(AI) expressing the desired protein were mixed with SDS-PAGE sample loading buffer and heated at 95 °C for 10 minutes. Gel samples were electrophoresed on a 4-20% TGX Criterion gel (Bio-Rad) at 200 V with Laemmli electrophoresis running buffer and blotted to polyvinylidene fluoride membrane using a Trans-Blot Turbo Transfer System (Bio-Rad). Proteins were subsequently probed with His-probe Antibody (H-3)

HRP (Santa Cruz Biotechnology) and detected through chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific)

Purification of carboxysomes from *Escherichia coli*

Tuner competent cells (Novagen) were transformed with a plasmid (pHnCB10LC) expressing carboxysomes. Cells were grown in 1 L of Terrific Broth at 37 °C to an OD600 of 0.3-0.5 at which point IPTG was added to a final concentration of 1 mM. The culture was induced for 16 hours at 20 °C and harvested by centrifugation at 5000xg for 20 minutes.

The cell pellet was resuspended in 50 ml Buffer TEMB (10 mM Tris 10 mM MgCl₂ 1 mM EDTA 20 mM NaHCO₃ pH 8.4) with 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml lysozyme (Sigma-Aldrich), and 25 U/ul benzonase (EMD Millipore). Cell lysis was performed by passing the cell suspension through an Avestin EmulsiFlex-C3 homogenizer three times. The crude cell extract was centrifuged at 12,000xg for 30 minutes to remove cell debris. The supernatant was subsequently centrifuged at 40,000xg for 30 minutes to pellet the carboxysomes. The pellet was then resuspended in 20 ml of 33% cellLytic B (Sigma-Aldrich) in TEMB buffer in order to remove lipid contaminants and centrifuged again at 40,000xg for 30 minutes. The pellet was resuspended in three ml TEMB and centrifuged at 3000xg. The supernatant was then applied to 25 ml sucrose gradient made from 10, 20, 30, 40, and 50% sucrose. The gradient was centrifuged at 105,000 xg for 30 minutes. Fractions of one ml were collected and analyzed for the presence of carboxysomes via SDS-PAGE gel. The fractions that contained carboxysomes were pooled and centrifuged at 105,000xg for 90 minutes to pellet the carboxysomes. Carboxysomes were then resuspended in 1 ml TEMB and stored at 4 °C

MALDI-TOF analysis

The matrix was prepared by dissolving 15 mg of sinapic acid (Sigma-Aldrich) in 1 ml of 70:30 water:acetonitrile with 1% TFA. Purified EcS2 was mixed with the matrix in 1:10 v/v ratio. A volume of 2 µl of protein-matrix mixture was spotted on a well in 384-well plate (DE1580TA, Axima) and desiccated. Another 2 µl of protein-matrix was spotted on the same well and desiccated once more. MALDI-TOF was performed in Axima Performance (Shimadzu Technology) with 180 units of power, accumulating 400 profiles.

Tandem Mass Spectrometry

Two forms of CsoS2 in purified EcS2 were separated via SDS-PAGE (4-20% TGX gel, Bio-Rad) and stained with Coomassie stain. Bands containing CsoS2A and CsoS2B were excised and submitted to the UC Davis Proteomics Core Facility. Analysis was performed on a Q Exactive™ Hybrid Quadrupole-Orbitrap. Acquired spectra were searched in Scaffold against the wild-type CsoS2 sequence and the frameshifted sequence library generated as described in Bioinformatic Methods below. The list of peptides was used to deduce the location of translational frameshifting.

Preparation of samples for negative stain electron microscopy

5 µl of a protein sample was added to a freshly glow-discharged carbon coated copper grid (EMS) and allowed to incubate for two minutes. The grids were washed with deionized water three times, before incubation with 5 µl of 1% uranyl acetate for one minute. Excess liquid was gently wicked off and the grid was allowed to air-dry for two minutes. Negative stain EM was performed with a Tecnai 12 TEM (FEI) operated at 120 kV.

Bioinformatic methods

To construct a library of frameshifted sequence, we wrote a Python script to construct the list of tryptic peptides that would result from digestion of frameshifted CsoS2. The script is available online at <https://github.com/SavageLab/csos2>. In brief, we generated all possible fusions of the primary CsoS2 reading frame with the -1 and -2 reading frames, including only cases in which the fusion event was between 30% and 80% of the way through the CsoS2 coding

sequence. We then simulated a tryptic digest of these ~900 fusion proteins, generating a comprehensive list of possible fusion peptides. This database was then used to match m/z spectra from gel bands corresponding to CsoS2A and CsoS2B. The piling diagram was constructed from peptide counts derived from comparing mass spectra of CsoS2A and CsoS2B gel bands to the primary coding sequence of CsoS2 (i.e. disregarding the potential for frameshifting). We counted the number of times each amino acid was observed in a peptide matched against the primary coding sequence. These counts are plotted in Figure 2 in the main text.

The secondary structure of mRNA was computed using the RNAstructure web server. Nucleotide sequence starting at 50 bases 5' of the slippery sequence and ending at 50 bases 3' of the slippery sequence was used as the input. The temperature parameter was set to 303.15 K. To calculate the stability of the stem-loop alone, the sequence corresponding to the predicted stem-loop was entered without additional nucleotides

To construct a phylogenetic tree mapped with -1 PRF prediction (Fig. S5), species bearing *csoS2* genes were curated from the Joint Genome Institute Integrated Microbial Genomes (JGI IMG) database (<https://img.jgi.doe.gov>). FASTA sequences for the CsoS2-encoding mRNAs were input into the KnotInFrame -1PRF prediction tool (<https://bibiserv.techfak.uni-bielefeld.de>). Significant -1 PRF candidates as predicted by the KnotInFrame tool were further filtered by setting a threshold such that the candidate slip sequence must be found in the last two thirds of the gene and that the structured mRNA element immediately downstream of the 'XXXYYYZ' slip sequence must have minimal free energy value .025 kcal/mol more stable than the nested mRNA structure. Species with candidate -1 PRF sequences meeting these parameters were marked in green on the phylogeny of *csoS2* nucleotide sequences.

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Author contributions

T.C and D.F.S designed the research, analyzed the data, and wrote the manuscript; T.C performed the experiments for Figure 1, 2, and S2; T.C. and R.J.N. performed the experiments for Figure 3; T.C., K.E.K., C.F.N., P.K.T. and L.M.O. performed the experiments for Figure 4 and S4; R.J.N. developed the algorithm for predicting -1 PRF in CsoS2 homologues and produced the phylogenetic tree displayed in the supplementary information

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Figure legends

Fig. 1. CsoS2 is differentially expressed as two proteins without any post-translational modifications. (a) A schematic representation of different regions in CsoS2, including N-terminal Region (NR), Middle Region (MR), C-terminal Region (CR), and a conserved C-terminal Peptide (CTP). NR contains 4 short repeats (yellow). MR has 6 longer repeats (R1-R6). CR has 3 repeats (R7-R9). The repeats are divided by interstitial amino acids (grey), which do not align with any of the repeats. The short isoform, CsoS2A, ends within R6 (asterisk). (b) SDS-PAGE gel of carboxysomes purified from *H. neapolitanus* (HnCB) and from recombinant expression in *E. coli* (EcCB), and CsoS2 recombinantly expressed in *E. coli* (EcS2). The gel is annotated with the identity of each protein band and molecular weight markers. (c) MALDI-TOF spectrum obtained from purified EcS2.

Fig. 2. The *csoS2* gene encodes a programmed ribosomal frameshift event. (a) Western blot of CsoS2 (EcS2) and CsoS2-NonStop (NonStop) expressed in *E. coli*. (b) Piling diagram prepared from the list of peptides discovered by MS/MS. Scale is normalized to maximum count for each sample. CsoS2A is colored translucent green; CsoS2B in blue. (c) Diagram showing *csoS2* codons in the 0 and -1 frames where the -1 PRF occurs. Codons in the -1 frame are underlined. Amino acids in the 0 and -1 frames are written above and below the codons, respectively. The arrow denotes the last amino acid shared between CsoS2A and B. The diagram is mapped back to the corresponding region in the piling diagram (marked by dashed lines)

Fig. 3. Both the slippery sequence and the secondary structure in *csoS2* mRNA are necessary for frameshifting. (a) A predicted stem-loop in the *csoS2* mRNA causes the ribosome to stall during translation. The ribosome may continue translating in the 0 frame, resulting in CsoS2B. Alternatively, the slippery sequence allows the ribosome to slip backward to wobble base-pair and continue translating in the -1 frame to generate CsoS2A. Codons in the original frame are marked by horizontal lines underneath. (b) Predicted secondary structure of the slip sequence and downstream stem-loop (bp 1694-1746). Point mutations were made either in the slippery sequence (orange) or the stem-loop (red). Bases at the end of the arrows outside of the structure are those in the mutated products. Pink and blue bars indicate the slippery sequence and the -1

676 frame stop codon, respectively. (c) Western blot of recombinant wild-type CsoS2 (EcS2),
677 CsoS2A (EcS2A), CsoS2 with an ablated slippery sequence (NoSlip), and CsoS2 with an ablated
678 stem-loop (NoStem). D) GFP fluorescence detected on the SDS-PAGE gel of synthetic EGFP-
679 RFP fluorescent reporters containing a linker (control) or PRF elements (PRF).

680

681 **Fig. 4.** Heterologous expression of carboxysomes without either CsoS2A or CsoS2B. (a) SDS-
682 PAGE of purified carboxysomes containing both CsoS2 isoforms (WT), CsoS2B only (S2B), and
683 CsoS2A only (S2A). Each sample was prepared from fraction 16 of the sucrose gradient (Fig.
684 S4). (b) Electron micrographs of the carboxysome samples used for SDS-PAGE gel. (Scale bar:
685 100 nm). (c) Model of possible differential functions of CsoS2 isoforms, in which CsoS2A
686 organizes luminal RuBisCO while CsoS2B bridges the cargo to the shell. C-terminus of CsoS2B
687 is colored as blue. Areas with unresolved questions are circled with orange dashed lines: 1) the
688 location of CsoS2A; 2) the possibility and the nature of an interaction between CsoS2A and
689 CsoS2B; 3) the exact regions of CsoS2A or B that interact with RuBisCO; and 4) the location of
690 the C-terminus of CsoS2B.

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Figure 1

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