Deciphering molecular details in the assembly of alpha-type carboxysome

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Bacterial microcompartments (BMCs) are promising natural protein structures for applications that require the segregation of certain metabolic functions or molecular species in a defined microenvironment. To understand how endogenous cargos are packaged inside the protein shell is key for using BMCs as nano-scale reactors or delivery vesicles. In this report, we studied the encapsulation of RuBisCO into the α-type carboxysome from *Halothiobacillus neapolitanus*. Our experimental data revealed that the CsoS2 scaffold proteins engage RuBisCO enzyme through an interaction with the small subunit (CbbS). In addition, the N domain of the large subunit (CbbL) of RuBisCO interacts with all shell proteins that can form the hexamers. The binding affinity between the N domain of CbbL and one of the major shell proteins, CsoS1C, is within the submicromolar range. The absence of the N domain also prevented the encapsulation of the rest of the RuBisCO subunits. Our findings complete the picture of how RuBisCOS are encapsulated into the α-type carboxysome and provide insights for future studies and engineering of carboxysome as a protein shell.
sequence analysis of gene clusters of the cluster. Modified carboxysomes were purified from tag to the C terminus of the CbbL protein encoded by the cso gene cluster. This permitted an investigation of how RuBisCO is encapsulated inside the shell of the carboxysome. In this study, we present experimental evidences to demonstrate that the N domain of CbbL is essential for encapsulation function of N-terminal sequences of metabolosome enzymes, and to investigate whether the α-domain may play a key role in the RuBisCO encapsulation into the α-type carboxysome.

Results and Discussion

Encapsulation of wild-type CbbL into the heterologously expressed carboxysome. We first established a technical platform for the studies, including the expression, isolation, and detection of carboxysome, shell proteins, and/or cargo proteins. The carboxysome from Halothiobacillus neapolitan (ATCC 23641) was used as a model system in this study. The Halothiobacillus neapolitan encodes the α1/α-carboxysome, which consists of eight large (CbbL, 52.6 kDa) and eight small (CbbS, 15.8 kDa) subunits (L4S8, PDB: 1SVD). The core of the enzyme is made up of four functional dimers of CbbL arranged around a 4-fold axis, and capped at each end by four noncatalytic CbbS proteins. The CbbL is built up of an N domain and a C domain, which harbors the major structure of the active site. A few residues of the N domain from the adjacent subunit of the dimer complete the substrate binding cavity. Intrigued by the encapsulation function of N-terminal sequences of metabolosome enzymes, we seek to investigate whether the N-terminal domain of the large subunit participates in the encapsulation of RuBisCO into the α-type carboxysome. In this study, we present experimental evidences to demonstrate that the N-domain is essential for the encapsulation of the enzyme. Its interaction with the major shell protein has an apparent Kd in the submicrometer range. In silico docking experiments reveal that a short α-helix structural motif embedded in the middle of the N-domain interacts with the major shell protein and plays a key role in the interaction. Our data also show that the previously identified interaction between scaffold protein CsoS2 and RuBisCO is engaged through the RuBisCO small subunit, CbbS. Overall, this study reveals new details of protein-protein interaction in the process of RuBisCO encapsulation into the α-type carboxysome and provides insights for future studies and applications of BMCs.

Figure 1. Expression and purification of H. neapolitan carboxysome. (A) The cso gene cluster in plasmid pZS-23641; (B) Electron microscopy image of wild-type carboxysome purified from E. coli cells transformed with pZS-23641.
E. coli cells and subjected to immunoprecipitation experiments. Samples of intact and broken carboxysome were incubated with anti-6xHis antibody, which was subsequently precipitated by protein A agarose at low centrifugation speed. Obtained supernatant fractions of the two samples contained either the intact carboxysome...
or proteins of broken carboxysome. Released proteins from the agarose beads were analyzed as the precipitate fractions. Western blots using the anti-6xHis antibody detected the CbbL-6xHis protein in supernatant fractions from both intact and broken carboxysome samples, which confirmed that the protein was expressed from the modified cso gene cluster (Fig. 2B). Furthermore, the CbbL-6xHis protein was only detected in the precipitate fraction of the broken carboxysome, but not in the sample of the intact carboxysome (Fig. 2B). These observations support the conclusion that the CbbL-6xHis protein was inaccessible to anti-6xHis antibody when it was encapsulated into the intact protein shell of carboxysome.

N domain of CbbL (CbbL(N)) is essential for encapsulation. We hypothesized that the N domain of CbbL is necessary for its encapsulation into functional carboxysome. To this end, we initially sought to apply the same immunoprecipitation method to examining the encapsulation of CbbL protein that lacks the N domain (a.a. 1–136). However, protein expression analysis showed that the C domain of CbbL (CbbL(C), a.a. 137–473) was mainly expressed as inclusion bodies (Fig. S1A). We were unable to separate the inclusion bodies from the carboxysome fraction using sucrose gradient ultracentrifugation method. As a result, the C domain of CbbL was detected in both the broken and the intact fraction of the purified carboxysome in the immunoprecipitation experiment, which complicated the interpretation of the results (Fig. S1B). To solve the problem, we improved the solubility of the C domain by fusing it to the C-terminus of the maltose binding protein (MBP) (Fig. S1C). Plasmid was then constructed by replacing CbbL6xHis with MBP-CbbL(C)6xHis gene. Carboxysome was purified from E. coli strain transformed with plasmid pZS-23641-MBP-CbbL(C)6xHis. In comparison to the SDS-PAGE of wild-type carboxysome derived from pZS-23641, protein bands of major shell proteins and encapsulated proteins (CsoS2A and CsoS2B) derived from pZS-23641-MBP-CbbL(C)6xHis were still visible (Fig. 2A, lane 1). However, a band with the size of MBP-CbbL(C)6xHis could not be unambiguously identified. Immunoprecipitation experiment to detect the MBP-CbbL(C)6xHis protein in the purified carboxysome also showed negative result (Fig. 2B). These data indicated that the C domain of CbbL was not encapsulated in the carboxysome. Close inspection of SDS-PAGE of the wild-type (Fig. 2A, lane 2) and 23641-MBP-CbbL(C)6xHis carboxysomes (Fig. 2A, lane 1) also revealed a significantly reduced presence of the small subunit of RuBisCO, CbbS, when the N domain of CbbL was not expressed.

As it is possible that the MBP itself and not the lack of the N domain prevented the encapsulation of the MBP-CbbL(C)6xHis fusion protein, we designed and conducted another set of experiments. In these experiments, a red fluorescent protein (mCherry) was fused either to the intact or to the C domain of CbbL. The fluorescence of E. coli strains transformed with pZS-23641-CbbL-mCherry or pZS-23641-CbbL(C)-mCherry was studied under confocal microscope (Fig. 2C). Fluorescence foci formation was clearly observed in cells expressing CbbL-mCherry (Fig. 2C, top panel), which is a sign of localization in the cytosol due to the encapsulation by the carboxysome32. On the other hand, evenly dispersed fluorescent signals were observed in cells expressing the CbbL(C)-mCherry fusion protein (Fig. 2C, middle panel). Above data suggest that the N domain of CbbL is essential for its packaging into the carboxysome.

To further investigate whether the N domain of CbbL is capable of localizing foreign proteins to carboxysome, we fused either MBP6xHis or mCherry6xHis to the C-terminus of the domain. Carboxysome was purified from E. coli cells transformed with pZS-23641-CbbL(N)-MBP6xHis (Fig. 3A, lane 2). In comparison with purified wild-type carboxysome (Fig. 3A, lane 1), a new protein band corresponding to the size of CbbL(N)-MBP6xHis

Figure 3. N domain of CbbL directs foreign proteins to carboxysome. (A) SDS-PAGE analysis of purified carboxysomes (original image, Fig. S7). M, protein size markers; lane 1, from cells transformed with plasmid pZS-23641-CbbL6xHis; lane 2, from cells transformed with pZS-23641-CbbL(N)-MBP6xHis. (B) Immunoprecipitation of purified carboxysome (original image, Fig. S8). I, samples from intact carboxysome; B, samples from broken carboxysome.
A fusion protein was observed. Western blot experiment confirmed the protein band contained His tag (Fig. 2B). The intensity of this band is significantly lower than the band of CbbL in the wild-type carboxysome, although the two samples of purified carboxysome contained similar amounts of shell proteins (CsoS1A/1B/1C) and scaffold proteins (CsoS2A/2B) (Fig. 3). The purified carboxysome was then subjected to immunoprecipitation experiment (Fig. 3B), which showed that the CbbL(N)-MBP<sub>6xHis</sub> fusion protein was located to the purified carboxysome (Fig. 3B). We further studied the distribution of mCherry protein in <i>E. coli</i> cells expressing pZS-23641-CbbL(N)-mCherry (Fig. 2C, bottom panel). Significant heterogeneity in fluorescence distribution was observed within the cell population. Analysis showed that around 35% of the fluorescence signal formed foci in 23641-CbbL(N)-mCherry sample, while 100% of the signals formed foci in 23641-CbbL-mCherry sample and less than 5% in 23641-CbbL(C)-mCherry sample. Above data support the conclusion that the N domain of CbbL is capable of directing foreign protein to carboxysome, although the efficiency is low due to apparent stochastic effect. The expression of the N domain of CbbL also largely recovered the encapsulation of small subunit of RuBisCO, CbbS (Fig. 3A).

Scaffold protein CsoS2 interacts with CbbS. To understand how the N domain of CbbL participates in the encapsulation process, we first examined its interaction with CsoS2 protein. It is known that CsoS2 is absolutely conserved in organisms that express α-carboxysomes. Recent studies demonstrated that this structurally flexible protein interacts with both major shell proteins and RuBisCO<sup>29</sup>. It was further hypothesized that CsoS2 serves as a scaffold to recruit shell proteins and RuBisCO prior to the assembly of carboxysome<sup>29</sup>. However details on the interaction between CsoS2 and RuBisCO have not been studied. To probe whether the N domain of CbbL plays any role in this process, we conducted pull-down assays using cells that co-expressed different combinations of CsoS2 (with N-6xHis tag) and CbbL/CbbS proteins (Fig. 4). When CsoS2 and CbbS were co-expressed in <i>E. coli</i> cells, the fraction purified using immobilized Ni resin contained both CsoS2 and CbbS (Fig. 4, lane 3). On the other hand, the co-purification was not observed for CbbL when it was expressed together with CsoS2 (Fig. 4, lane 2). Furthermore, when the RuBisCO complex, including both CbbL and CbbS, was co-expressed with CsoS2, CbbL was also observed in the pull-down fraction (Fig. 4, lane 1). The results support the notion that scaffold CsoS2 recruits RuBisCO through its interaction with the small subunit (CbbS) of the RuBisCO, which eliminates the possibility that the role of CbbL(N) in RuBisCO encapsulation is through its interaction with CsoS2 proteins.

The N domain of CbbL (CbbL(N)) interacts with major shell proteins. The lack of direct interaction between CsoS2 proteins and CbbL(N) prompted us to investigate whether the N domain of CbbL interacts with any shell proteins of carboxysome. To co-express CbbL(N) and shell proteins in <i>E. coli</i> for pull-down assays, two plasmids were constructed. The first plasmid encodes a fusion protein of CbbL(N) and mCherry with a C-terminal 6xHis tag. The fluorescence protein was fused to the C-terminus of CbbL(N) and used as a
visual signal to facilitate the protein purification. All genes that encode the *H. neapolitan* shell proteins, including CsoS1A/1B/1C/1D and CsoS4A/4B, were cloned into a second plasmid, pZS-23641-CsoS41. Above two plasmids were co-transformed into *E. coli* for protein expression and the cell extract was subjected to the pull-down assay. By comparing to a control sample of the CbbL(N)-mCherry-6xHis protein that was purified separately in parallel, an additional band was unambiguously identified in the SDS-PAGE of the pull-down sample (Fig. S3). Proteomics analysis confirmed that the identified band consisted of CsoS1A and CsoS1C with 91% sequence coverage for each protein. Since the two shell proteins are of the same length (98 a.a.) and differ only in two amino acid residues, they could not be resolved in our SDS-PAGE experiments.

To verify our above observations and to exclude the possibility that interactions between CsoS1A/1C and mCherry led to the pull-down effect, we generated a control construct in which the 6xHis tag was fused directly to the C terminus of CbbL(N). Plasmids encoding individual shell protein were also constructed. Another round of pull-down assays were conducted between Cbb(N)-6xHis and each shell protein separately. The results confirmed that the N domain of CbbL interacts with shell proteins CsoS1A and CsoS1C (Fig. 5). In addition, interaction between CbbL(N) and CsoS1B was also detected (Fig. 5). On the other hand, no pull-down effect was observed for CsoS1D, CsoS4A, and CsoS4B (Fig. 5). It was estimated in previous reports that CsoS1A/1B/1C (Pfam00936, BMC-H)33,34 exist in 3510 copies per carboxysome, while the copy numbers of CsoS1D (BMC-T) and CsoS4A/4B (Pfam 03319, BMC-P) were too low to be determined in wild-type carboxysomes purified from *H. Neapolitan*35. Interactions between the N domain of CbbL and the three major shell proteins indicate possible role that is played by CbbL(N) during the encapsulation of RuBisCO.

Structural motifs of CbbL(N) that is responsible for the interaction. To investigate whether the entire N domain (a.a. 1–136) of CbbL contribute to the interaction with major shell proteins, we expressed five truncated segments of CbbL(N) based on its secondary structural units (Fig. 6A). Segment 1–20 represented the flexible N terminus (red, Fig. 6A) that was mainly ‘invisible’ in the X-ray structure. Segment 1–73 contained one of the central β strand followed by one large and one short α helices (yellow, Fig. 6A). Segment 1–100 covered two additional central β strands (green, Fig. 6A), while segment 1–122 covered the last α helix (blue, Fig. 6A) and 1–136 included an additional β strand (cyan, Fig. 6A). Each segment was fused to the N terminus of MBP that contained a C-terminal 6xHis tag. The interaction between truncated CbbL(N) with major shell proteins CsoS1A and CsoS1C was studied using pull-down assays (Fig. S4). The ligand binding ratio between the CbbL(N) segment and the shell protein was calculated using signals from densitometry measurement in the assays. The relative binding strength of a truncated segment was expressed as the percentage of its ligand-binding ratio to that of the entire N domain (Fig. 6B). Data indicated that the unstructured N-terminal peptide that contains the first 20 amino acids played insignificant role in the binding of CbbL(N) with the shell proteins, which is different from the observations of signal peptides in functional proteins of the Pdu and Eut metabolosomes. For CsoS1A and CsoS1C, the relative binding strength with truncated N domain continuously increased and reached maxima when CbbL(1–122) was used. Only a small difference in binding strength was observed between CbbL(1–122) and CbbL(N).

Due to the high sequence homology between CsoS1A and CsoS1C and their similar behaviors in the pull-down assay against truncated CbbL(N) protein, further studies on the interaction between the N domain of CbbL and major shell proteins focused on CsoS1C. The binding affinity between CbbL(N) and CsoS1C was examined by semi-quantitative ELISA experiments36. In brief, wells of microtiter plates were coated with MBP tagged CbbL(1–136), into which different concentrations of the CsoS1C-6xHis protein were applied. Following removal of the unbound proteins by washing, the amount of CsoS1C that bound to CbbL(1–136) was quantified by immunoassays. The interaction between CbbL(1–136) and CsoS1C showed a strong binding affinity with an apparent $K_d$ of 120.5 ± 4.6 nM.
In silico docking experiment was subsequently conducted to simulate the possible mode of interaction between CbbL(N) and CsoS1C. The hexameric structure of CsoS1C (PDB: 3H8Y) and the CbbL (PDB: 1SVD) were used in rigid body docking in the ZDOCK module of Discovery Studio suite. The top three conformations with the lowest energy all showed possible interaction between a short α helix formed by residues 63 to 67 of CbbL(N) and the convex side of the CsoS1C hexamer (Fig. 6D). The simulation result supported observations in CbbL(N) truncation experiments where significant interactions were first observed when fragment 1–73 was examined. The identified short α helix is held between α helix (a.a. 42–54) and β sheet (a.a. 76–82), which indicates that additional structural units may further constraint the short α helix for optimal interaction with the shell proteins.

Conclusion

BMC is a promising type of protein scaffold for applications in synthetic biology, drug delivery and material science. Understanding the assembly mechanism of BMCs is critical for further engineering efforts. This research focused on studying the interactions between subunits of RuBisCO and shell/scaffold proteins of the α-type carboxysome from H. neapolitan. Our data showed that the small subunit (CbbS) of RuBisCO interacts with the scaffold proteins (CsoS2), while the N domain of the large subunit (CbbL(N)) of RuBisCO interacts with all major shell proteins CsoS1A, 1B and 1C with submicromolar binding affinity. Computer simulation revealed a possible mode of interaction between the CbbL(N) domain and shell proteins. The findings reveal molecular details of protein-protein interactions in the process of α-type carboxysome assembly and further support previous hypothesis of simultaneous assembly of the shell and encapsulation of RuBisCO enzymes. In addition to completing the picture of α-type carboxysome assembly, findings from this work also provide insights for future engineering of carboxysome for biotechnological applications.

Methods

Material and general methods. Primers (Table S1) were ordered from Sigma. Plasmids (Table S2) were constructed using standard molecular biology techniques. DNA sequencing services were provided by Eurofins MWG Operon. Restriction enzymes, Antarctic phosphatase (AP) and T4 DNA ligase were purchased from New England Biolabs. KOD hot start DNA polymerase was purchased from EMD Millipore. All solutions were prepared in deionized water that was further treated by Barnstead Nanopure ultrapure water purification system. LB medium (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl.
Glycine-HCl buffer (150 mM, pH 2.94) and incubated for 5 minutes. Following centrifugation at 2,500 g, the lysate was harvested at 5,000 g for 10 min. Collected cells were suspended in TEMB with 200 mM NaCl, then lysed by sonication. Following removal of cell debris by centrifugation at 16,000 g for 20 min, the supernatant was applied to a Ni Sepharose 6 Fast Flow (GE Healthcare) column. The column was eluted stepwise with five column volumes of buffers containing 5 mM, 50 mM, then 250 mM of imidazole. All elution fractions were analyzed by SDS-PAGE to test for co-elution of proteins with and without the 6xHis tag.

Pull-down assays. Two plasmids, each encoding one protein of interest, were co-transformed into E. coli BL21(DE3). Transformed cells were cultured in LB medium with appropriate antibiotics at 37 °C until OD₆₀₀ reached 0.6. Protein expression was induced with IPTG at 0.4 mM and carried out at 30 °C for 12 h. The cells were harvested by centrifugation at 5,000 g for 10 min. Collected cells were suspended in TEMB with 200 mM NaCl, then lysed by sonication. Following removal of cell debris by centrifugation at 16,000 g for 20 min, the supernatant was treated with Ni Sepharose 6 Fast Flow (GE Healthcare) column. The column was eluted stepwise with five column volumes of buffers containing 5 mM, 50 mM, then 250 mM of imidazole. All elution fractions were analyzed by SDS-PAGE to test for co-elution of proteins with and without the 6xHis tag.

Immunoprecipitation. Protein A agarose beads ( Pierce™, ThermoFisher Scientific) were first washed with PBS buffer, then diluted tenfold in PBS prior to mixing with 6xHis tag monomolecular antibody (Invitrogen™, ThermoFisher Scientific). The bead suspension was incubated at 4 °C for 1 h with gentle shaking. Purified carboxysome was first treated with Ni Sepharose 6 Fast Flow resin to remove His-tagged protein that was leaked out of the carboxysome during the sample preparation process. To obtain broken carboxysome, purified sample was subjected to sonication using Model 120 sonicator (Fisherbrand™) at 50% amplitude for 2 min. Samples of broken or intact carboxysome were mixed with antibody-treated protein A beads. Following incubation at 4 °C for 1 h with gentle shaking, the beads were collected by centrifugation at 2,500 g for 2 min. The supernatant was saved as the supernatant fractions for further analysis. The beads were washed three times with 0.5 mL of IP buffer (25 mM Tris–HCl, pH 7.2, 150 mM NaCl). To elute the protein, the beads were suspended in 50 μL of glycine–HCl buffer (150 mM, pH 2.94) and incubated for 5 minutes. Following centrifugation at 2,500 g for 2 min, the supernatant was collected as the sediment fraction from the immunoprecipitation step. The supernatant and sediment fractions were analyzed by Western blots for the presence of 6xHis tag using the 6xHis tag monomolecular antibody together with the goat anti-mouse IgG-HRP conjugate (BioRad) and Opti-4CN detection kit (BioRad).

Enzyme-linked immunosorbent assay (ELISA). The semi-quantitative ELISA experiments followed reported protocols with minor modifications. In brief, MaxiSorp 96 well ELISA plates were coated with 100 μL of MBP-tagged CbbL(1–136) proteins (30 nM in 0.1 M Na₂CO₃ (pH 9.6)) at 4 °C for 12 h. After removal of the coating solution, blocking buffer (1 mM CaCl₂ and 1% BSA in TBS buffer) was added and the plates were incubated at room temperature for 1 h. Following three times of washing with washing buffer (1 mM CaCl₂ and 0.05% Tween 20 in TBS buffer), solutions of CsoS1C-6xHis protein at varied concentrations (5 nM to 5 μM) in blocking buffer were applied to each well. The plates were incubated for 1 h at room temperature, then washed three times to remove unbound protein. Detection of bound protein used the 6xHis tag monomolecular antibody together with the goat anti-mouse IgG-HRP conjugate and the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. Following the reaction, the absorbance at 450 nm was measured to quantify the activities of HRP. Dissociation constants were calculated by curve fitting with Hill Equation using Prism 7 (Graphpad).

Proteomics analysis. Gel band sample was washed, then digested with trypsin overnight at 37 °C. Tryptic peptides were extracted from the gel pieces, dried down, and re-dissolved in 25 μL of 2.5% acetonitrile, 0.1% formic acid. The digests were run on a Q-Exactive-HF mass spectrometer (ThermoFisher Scientific) equipped with a U3000 RSLc nano LC system. All MS/MS data were analyzed using Mascot (Matrix Science, v 2.5.1). Mascot was set up to search the databases with a fragment ion mass tolerance of 0.060 Da and a parent ion tolerance of 10 ppm. Modifications of residues, including deamination of asparagine and glutamine, oxidation of methionine and carbamidomethyl of cysteine, were specified as variable modifications. Scaffold (Proteome Software Inc., v 4.7.5) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability and protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides, with a false discovery rate less than 1%.

Microscopy methods. For transmission electron microscopy analysis, purified carboxysomes were first fixed with glutaraldehyde (4% in PBS buffer) for 30 min at room temperature, washed three times with PBS, then adsorbed for 1 min to a carbon-coated grid. The samples were stained with 1.0% uranyl acetate in deionized water. The grids were imaged using 80 kV accelerating voltage using a Hitachi H7500 transmission electron microscope. For scanning imaging using fluorescent microscope, E. coli cells transformed with either pZS-23641-CbbL-mCherry or pZS-23641-CbbL-C-mCherry were cultured, induced for carboxysome expression, then collected by centrifugation at 5,000 g for 10 min. Cells were washed three times with PBS buffer, then fixed with paraformaldehyde (4% in PBS buffer) for 30 min at room temperature. Cells were then washed, resuspended in PBS, loaded on a glass slide, then imaged using a Nikon A1R-Ti confocal microscope. Texas red channel was excited at 595 nm and imaged at 620 nm. The percentage of localization of fluorescence signal was analyzed using ImageJ.
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Author Contributions
W.N. and Y.L. designed the study. Y.L., X.H., W.L., J.M., L.K. and W.N. constructed the plasmids, performed protein purifications and conducted pull-down assays. Y.L., J.L. and W.N. purified carboxysomes and conducted immunoprecipitation assays. Y.L. and X.H. conducted ELISA assays. W.N., J.G. and Y.L. analyzed the data and wrote the manuscript.

Additional Information
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