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CO₂ Fixation Kinetics of *Halothiobacillus neapolitanus* Mutant Carboxysomes Lacking Carbonic Anhydrase Suggest the Shell Acts as a Diffusional Barrier for CO₂*

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The widely accepted models for the role of carboxysomes in the carbon-concentrating mechanism of autotrophic bacteria predict the carboxysomal carbonic anhydrase to be a crucial component. The enzyme is thought to dehydrate abundant cytosolic bicarbonate and provide ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) sequestered within the carboxysome with sufficiently high concentrations of its substrate, CO₂, to permit its efficient fixation onto ribulose 1,5-bisphosphate. In this study, structure and function of carboxysomes purified from wild type *Halothiobacillus neapolitanus* and from a high CO₂-requiring mutant that is devoid of carboxysomal carbonic anhydrase were compared. The kinetic constants for the carbon fixation reaction confirmed the importance of a functional carboxysomal carbonic anhydrase for efficient catalysis by RubisCO. Furthermore, comparisons of the reaction in intact and broken microcompartments and by purified carboxysomal RubisCO implicated the protein shell of the microcompartment as impeding diffusion of CO₂ into and out of the carboxysome interior.

Many bacteria form intracellular polyhedral microcompartments that act as microbial organelles. They sequester metabolically important enzymes and enhance or regulate their activity. Several molecular mechanisms have been postulated for the way in which microcompartments function (1–4); all of these assume that the bounding proteinaceous shell of the microcompartment acts as a selective diffusion barrier, effectively separating the enclosed enzymes and the reactions they catalyze from the cell cytoplasm (5). Two families of small shell proteins appear to be the only common genetic and structural elements among the bacterial microcompartments formed by such metabolically diverse prokaryotes as heterotrophs and autotrophs (1, 6). By far the best studied microcompartments are the carboxysomes of cyanobacteria and chemolithoautotrophic bacteria, which contain ribulose 1,5-bisphosphate carbox-

ylase/oxygenase (RubisCO),² the CO₂-fixing enzyme of the Calvin-Benson-Bassham cycle, and are thought to act as the terminal element of the carbon-concentrating mechanism in these bacteria (reviewed in Refs. 3, 5). Genetic and physiological studies suggest that in β -cyanobacteria (7) inorganic carbon is actively transported into the cell interior and concentrated in the cytoplasm as bicarbonate, which must first be converted to CO₂ by a carboxysome-associated carbonic anhydrase before it can be fixed by RubisCO (8). Direct biochemical studies of β -carboxysomes from cyanobacteria have been hampered by difficulties with the purification of intact particles (1, 9). Carboxysomes of the α -type found in chemolithoautotrophs and α -cyanobacteria (7) and exemplified by those of the sulfur bacterium *Halothiobacillus neapolitanus*, have been purified to homogeneity and shown to be composed of eight major proteins (10). The CbbL and CbbS polypeptides, which account for 60–70% of the total carboxysome protein (3, 10), represent the large and small subunit, respectively, of RubisCO. The balance of the carboxysome mass is accounted for by polypeptides that are associated with the thin protein boundary membrane, also known as shell. The major structural shell elements are CsoS1A, CsoS1B, and CsoS1C, highly conserved small proteins that are the products of duplicated genes. Recent crystallographic studies of CsoS1A and its cyanobacterial homologs, CcmK2 and CcmK4, have shown that the proteins form hexamers. These pack into sheets and likely form the facets of the icosahedral carboxysome (11, 12). Each of the hexamers contains a pore that might be involved in the controlled passage of substrates and products into and out of the carboxysome. The other conserved microcompartment constituents, OrfA, OrfB, and CcmL, crystallize as pentamers and may be located on the vertices of the icosahedral particle (13). The CsoS2A and CsoS2B polypeptides, which together account for nearly 12% of the carboxysomal protein by weight (3), are products of a single gene in the *cs* operon (14). Their high calculated pI value has led to the speculation that they may act as rudimentary transporters for the phosphorylated RubisCO substrates and products (15). The remaining shell protein CsoSCA, which is encoded by the *cs* gene and only a minor component by weight (15), is the carbonic anhydrase of α -carboxysomes (16). Its lack of sequence homology with known carbonic anhydrases

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² The abbreviations used are: RubisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; RubP, ribulose 1,5-bisphosphate; DIC, total dissolved inorganic carbon; rCsoSCA, recombinant CsoSCA protein; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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led to its original categorization into a novel class (ϵ) (16). Subsequent crystallographic analysis, however, revealed that CsoSCA constitutes a subclass of β -carbonic anhydrases that appears to be populated exclusively by α -carboxysomal enzymes (17). The CsoSCA protein is very tightly associated with the shell and is released only under conditions that result in disassembly of the shell. The likely functional form of the carboxysomal carbonic anhydrase is a dimer that is present in only 40 copies/carboxysome. Despite this low number, kinetic characterization of recombinant CsoSCA (rCsoSCA) revealed that the enzyme could supply the ~ 270 holoenzyme molecules of carboxysomal RubisCO with a sufficient amount of CO₂ to maintain saturating substrate conditions (15).

In an effort to further assess the biological role of the carboxysomal CA, we have taken advantage of the homogeneous carboxysome preparations that can be obtained from *H. neapolitanus* to directly compare the biochemical properties of wild type microcompartments with those isolated from a *csoS3* insertion mutant that is devoid of carboxysomal carbonic anhydrase activity and requires elevated CO₂ levels to grow at normal rates.

EXPERIMENTAL PROCEDURES

Cultures—Wild type *H. neapolitanus* (ATCC 23641) and mutant *csoS3::Km* cultures were maintained in a bioreactor (INFORS-HT, Switzerland) at a dilution rate of 0.08 h⁻¹ and a pH of 6.4, as previously described (16). The *csoS3::Km* mutant was grown in air supplemented with 5% CO₂. Growth of wild type and mutant culture over time was monitored by determining the optical density of batch cultures at 600 nm.

Construction of *H. neapolitanus* *csoS3::Km*—Plasmid pTnEP2.2 (18), which contains a 2.2-kb EcoRI/PstI fragment encompassing nucleotides 4192–6364 of the *cso* operon (GenBankTM accession number AF038430) in the vector pT7T3 α 13, was digested with ClaI. The kanamycin resistance cassette was excised from the plasmid pUC4K (19, 20) by digestion with BamHI. The overhangs generated by the restriction enzymes were filled in with *Escherichia coli* PolI Klenow fragment, and the cassette was blunt end-ligated into the linearized pTnEP2.2 to generate pHncsoS3::Km. The wild type *csoS3* gene of *H. neapolitanus* was replaced by the mutant version via homologous recombination following electroporation with pHncsoS3::Km (21). Transformants were allowed to recover in medium without antibiotic for 24 h before being transferred to selective medium containing 50 μ g·ml⁻¹ kanamycin. All of the cultures were maintained in a 5% CO₂-enriched atmosphere. Gene replacement in *H. neapolitanus* *csoS3::Km* was verified by sequence determination of genomic DNA and by Southern hybridization.

Complementation of *H. neapolitanus* *csoS3::Km*—A single colony of the *H. neapolitanus* *csoS3::Km* mutant was used to inoculate a liquid culture that was grown at 30 °C in medium containing 50 μ g/ml kanamycin. Cells from this culture were transformed with p*csoS3*ProEx plasmid DNA (17) by electroporation (21). This expression construct contains a mutated *csoS3* gene that yields enzymatically active rCsoSCA with a His residue instead of Tyr⁹². Following a 24-h recovery period at elevated CO₂ levels, cells that had regained the ability to grow at

ambient CO₂ levels were selected in medium without antibiotics.

Genomic DNA was isolated as described previously (22) and digested with EcoRI or ClaI. Following gel electrophoresis the DNA fragments were blotted onto a nylon support via alkaline transfer (23). The blot was probed with ³²P-labeled *csoS3* DNA at 68 °C overnight. Replacement of *csoS3::Km* by an uninterrupted copy of the gene in *H. neapolitanus* was confirmed by sequencing of genomic DNA.

Isolation of Carboxysomes from Wild Type and Mutant *H. neapolitanus*—Wild type and *csoS3::Km* mutant cells from 6–8-liter cultures were used to purify carboxysomes as described before (16). Because elevated CO₂ levels are known to suppress carboxysome formation (24), it was necessary to switch *csoS3::Km* cells to ambient CO₂ levels once the culture had reached the desired density during growth in CO₂-supplemented air. All of the protein samples were desalted using Micro-Spin 6 columns (Bio-Rad) equilibrated in 50 mM Bicine, pH 8.0.

Electron Microscopy—Formvar/carbon-coated copper grids (EMS, Fort Washington, PA) were floated on a solution containing purified carboxysomes for 3 min. The samples were allowed to dry in air for 10 s before being stained with 1% (w/v) ammonium molybdate in 10 mM Tris-HCl, pH 8.0, for 40 s. The grids were air-dried for 10 min prior to being observed under a Zeiss EM-109 transmission electron microscope. Photographs of carboxysomes were taken by exposing Kodak EM film 4489 for 2 s.

SDS-PAGE and Immunoblot Analysis—Carboxysome proteins were separated by electrophoresis in pre-cast Criterion SDS, 4–20% polyacrylamide gradient gels (Bio-Rad) and stained with Gelcode Blue (Pierce). Protein blots were probed with polyclonal antiserum raised against *H. neapolitanus* rCsoSCA (16) as primary antibody and with goat anti-rabbit, alkaline phosphatase-conjugated IgG as secondary antibody. The blots were developed with One-Step NBT-BCIP reagent (Pierce). Densitometric analysis was performed using the Quantity One program supplied with the VersaDoc imaging system (model 4000 MP; Bio-Rad).

Radiometric RubisCO Assays—Desalted protein samples were activated in 50 mM Bicine-NaOH, pH 8.0, 10 mM MgCl₂, and 10 mM Na¹⁴HCO₃ (0.1 μ Ci· μ mol⁻¹) for 10 min. Each 0.5-ml assay contained 50 mM Bicine-NaOH, pH 8.0, 20 mM MgCl₂, 0.5 mM ribulose 1,5-bisphosphate (RubP) (Fluka brand; Sigma), and the appropriate amount of NaHCO₃ to give a final concentration range from 1.45 to 80.2 mM (this includes the contribution of the bicarbonate used to activate RubisCO). In parallel assays, the NaHCO₃ concentration was kept at 60 mM, and the RubP concentration was varied from 12.5 to 800 μ M. CO₂ fixation was initiated by adding 10 μ l of activated protein sample. To determine the relative carbon assimilation rates in intact wild type and mutant cells, 25-ml aliquots of bioreactor-grown wild type and mutant cultures were centrifuged at 12,000 \times g for 10 min, and the pelleted cells were resuspended in 1 ml of sterile medium. To start the reaction, 20- μ l aliquots of the cell suspension were added to growth medium that contained radioactive bicarbonate at various concentrations (1.0 μ Ci· μ mol⁻¹). The reactions were stopped at 1-min intervals by

adding 100- μ l assay aliquots to 300 μ l of glacial acetic acid. The vials containing the acidified samples were heated to remove excess ¹⁴C-¹⁴CO₂, cooled, and mixed with 4 ml of ScintiVerse II

mixture (Fisher). Radioactivity was counted in a LS 6000SC scintillation counter (Beckman Coulter, Fullerton, CA). Quench and counting efficiency were determined from a standard curve of ¹⁴C-labeled *n*-hexadecane in 0.3 ml of glacial acetic acid.

The initial velocities were calculated from linear regression lines plotted using Prism 4. The V_{max} and K_m values for CO₂ (K_C) and RubP were obtained by fitting the results to the Michaelis-Menten equation. Kinetic assays were performed in triplicate and repeated with a minimum of three independent preparations.

Protein Determination—Protein concentrations in carboxysome and RubisCO preparations were determined with the BCA assay (Pierce); those in intact cell samples were estimated with a modified Lowry assay (Pierce). Bovine serum albumin served as standard.

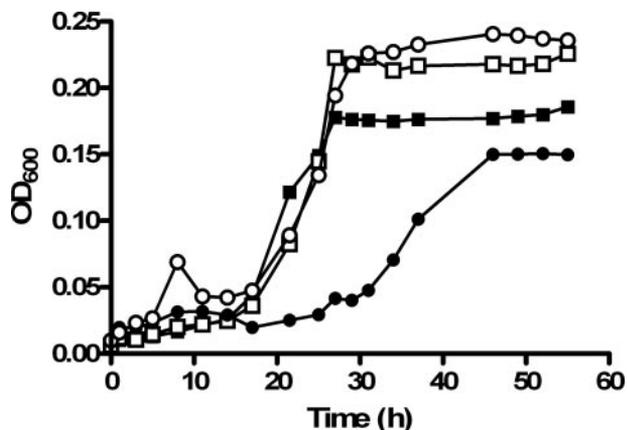


FIGURE 1. Growth curves of *H. neapolitanus* wild type and *csoS3::Km* mutant cultures. Cell density was assessed by measuring optical density at 600 nm. ■, wild type in air; □, wild type in CO₂-enriched air; ●, mutant in air; ○, mutant in CO₂-enriched air.

RESULTS

Generation of the *csoS3::Km* Mutant—The wild type *csoS3* gene of *H. neapolitanus*, which encodes CsoSCA, was interrupted by insertion of a kanamycin resistance cassette between the ClaI sites in the *csoS3* coding sequence to produce plasmid pHnc*csoS3::Km*. Following electroporation of mid-exponential phase, *H. neapolitanus* cells with this construct and selection of kanamycin-resistant transformants in 5% CO₂-enriched air colonies with a high CO₂-requiring phenotype were recovered that grew at close-to-wild type rates only at elevated CO₂ levels. Gene replacement was confirmed by Southern blotting and sequence analysis of genomic DNA (data not shown). A single high CO₂-requiring colony was used to establish the working culture of *csoS3::Km*. Comparison of mutant and wild type growth rates demonstrated extremely slow growth of *csoS3::Km* at ambient CO₂ levels but nearly wild type rates when the culture was aerated with 5% CO₂ in air (Fig. 1). Immunoblot analysis of crude cell extracts with anti-CsoSCA antibodies revealed a strong signal for the wild type but undetectable CsoSCA levels in the mutant (Fig. 2C).

Rescue of Wild Type Phenotype by Homologous Recombination—The sizes of DNA restriction fragments that hybridize to a *csoS3* probe on genomic Southern blots were different between mutant and wild type *H. neapolitanus*. Insertion of the kanamycin resistance cassette

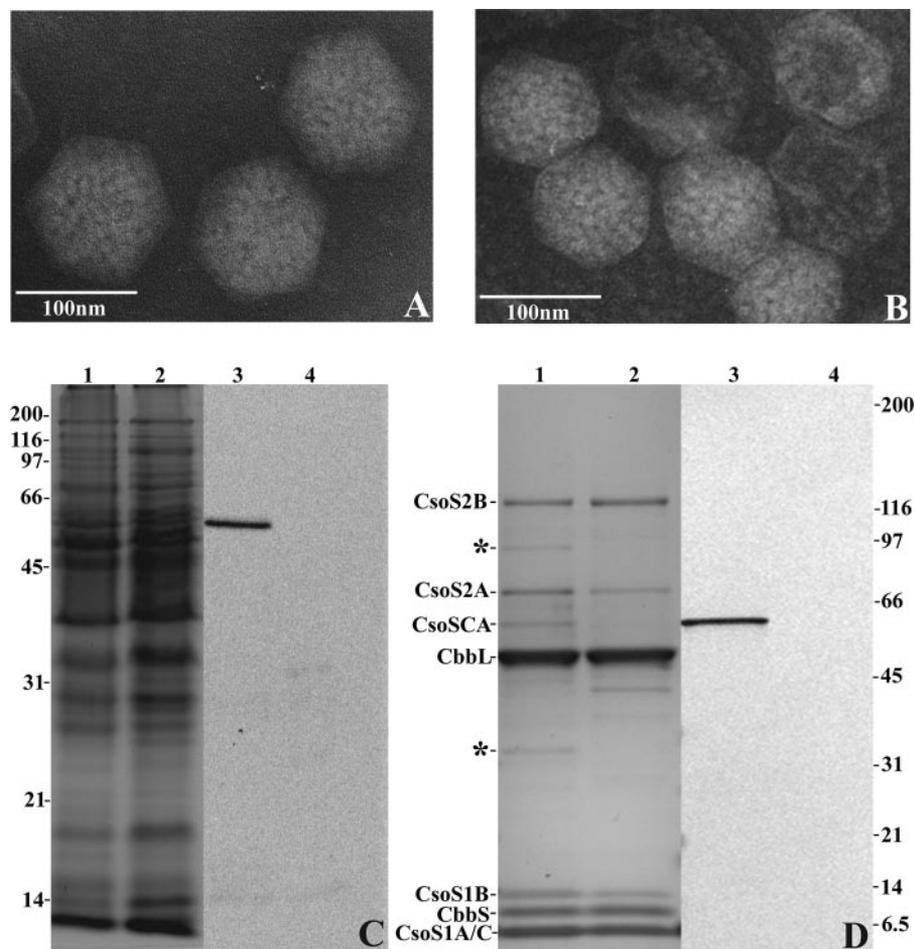


FIGURE 2. Morphology and composition of wild type and *csoS3::Km* mutant carboxysomes. *A* and *B* show transmission electron micrographs of purified, negatively stained wild type (*A*) and mutant (*B*) carboxysomes. *C* and *D* show the polypeptide composition of cell extracts (*C*; 20 μ g of protein) and purified carboxysomes (*D*; 7.5 μ g of protein) from wild type (lanes 1 and 3) and mutant (lanes 2 and 4). Lanes 1 and 2, stained polypeptides separated by SDS-PAGE; lanes 3 and 4, immunoblots probed with anti-CsoSCA antiserum. As determined by mass spectrometry, the polypeptide bands marked with asterisks are carboxysome protein aggregates that were not disrupted during electrophoresis.

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between the two ClaI sites in the *csoS3* gene of the mutant *csoS3::Km* yielded two diagnostic ClaI restriction fragments of ~8.3 and 1.1 kilobase pairs, respectively. ClaI-digested genomic DNA from wild type and from clones complemented with plasmid *pcsoS3ProEx* produced the two wild type size fragments of ~0.9 and 7.3 kilobase pairs, respectively, indicative of restored *csoS3* ClaI sites in the transformants. Furthermore, the restored *csoS3* gene, as expected, encoded a His instead of the wild type Tyr residue at position 92 of CsoSCA. This fortuitous mutation, which was introduced during subcloning of the *H. neapolitanus* *csoS3* gene (17), has no effect on the carbonic anhydrase activity of rCsoSCA (15). This mutation was therefore instrumental in documenting that the wild type phenotype in the complemented *H. neapolitanus* clones resulted from replacement of the interrupted genomic *csoS3* copy with a fully functional plasmid-borne gene, presumably by homologous recombination.

Isolation of Carboxysomes Lacking CsoSCA—*H. neapolitanus* *csoS3::Km* cells were grown in a chemostat under conditions identical to those used to cultivate wild type cells, except that the mutant culture was aerated with air supplemented with 5% CO₂. Under these conditions, a dilution rate of 0.08 h⁻¹ was maintained. Removal of the supplemental CO₂ resulted in rapid wash-out of the mutant cells and confirmed that a wild type growth rate was supported only at elevated CO₂ concentrations. Chemostat efflux was collected and aerated with 5% CO₂ in air until ~6 liters of culture had accumulated. Because the expression of high cellular carboxysome levels is known to be suppressed by high CO₂ concentrations (24), the mutant culture in the collection tank was switched to ambient CO₂ for 18–24 h prior to cell harvest and subsequent carboxysome purification. Yields of mutant carboxysomes purified by the standard procedure (16) were comparable with those from wild type cells (~10 mg of carboxysome protein/8 g of wet weight of cells). Furthermore, isolated *csoS3::Km* carboxysomes did not appear to be morphologically different from wild type microcompartments, as judged by electron microscopic analysis (Fig. 2, A and B). The mutant microcompartments were, however, consistently found to be ~15–20% larger in diameter than their wild type counterparts when analyzed by dynamic light scattering (data not shown).

The stopped flow changing indicator assays (25) detected no carbonic anhydrase activity in purified mutant carboxysomes at protein concentrations that yielded clear evidence of enzyme activity in wild type microcompartments (Fig. 3). Comparison of the polypeptide composition of both types of carboxysomes by denaturing SDS-PAGE revealed similar but not identical patterns. The most obvious difference was the absence of the CsoSCA band at ~60 kDa in mutant carboxysomes (Fig. 2D). Immunoblot analysis using polyclonal anti-rCsoSCA antibodies (16) confirmed the absence of detectable amounts of CsoSCA from the mutant microcompartments. Somewhat surprisingly, the molar ratio of CsoS2A to CsoS2B, which is close to 1:1 in wild type carboxysomes (3, 10), was shifted to favor the larger form 2:1 in mutant carboxysomes (Fig. 2D).

Carbon Fixation by Mutant Cells—Rates of carbon fixation by bioreactor-grown wild type and mutant cells were deter-

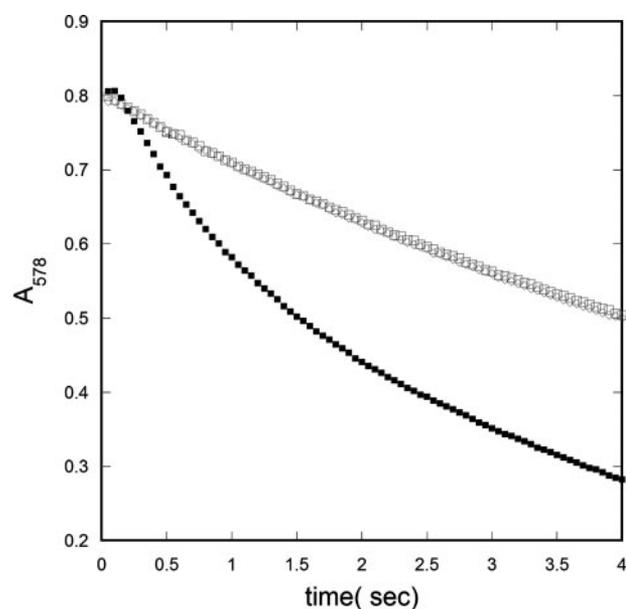


FIGURE 3. Carbonic anhydrase activity of purified carboxysomes from wild type and *csoS3::Km* mutant *H. neapolitanus*. The stopped flow changing indicator assays (23) were performed as described previously (13). ○, background; ■, wild type; □, mutant.

mined at various DIC concentrations. Although maximal fixation rates were comparable (0.12 ± 0.01 and 0.13 ± 0.01 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$ for wild type and mutant, respectively), the concentration of dissolved inorganic carbon (bicarbonate, carbonate, and CO₂) required to produce the half-maximal rate (K_{DIC}) was more than three times higher for mutant ($K_{\text{DIC}} = 3.9 \pm 1.0$ mM) than for wild type cells ($K_{\text{DIC}} = 1.1 \pm 0.2$ mM) (Fig. 4). Moreover, the ratio of wild type to mutant CO₂ fixation rate is 3.3 ± 1.0 at ambient CO₂ ([DIC] = 26 μM) and 1.5 ± 0.3 at 5% CO₂ ([DIC] = 3.3 mM) (26), consistent with the measured differences in growth rates (Fig. 1).

CO₂ Fixation Kinetics in Isolated Carboxysomes—To judge the effect of the shell-bound carbonic anhydrase on carboxysomal CO₂ fixation rates, the RubisCO activity of mutant and wild type carboxysomes was determined radiometrically (27) (Fig. 5). For these experiments we report K_C as $\mu\text{M CO}_2$, calculated from the input HCO₃⁻ concentration at pH 8.0, to allow comparison with the true K_m of free RubisCO and of the enzyme in broken carboxysomes. We refer to the concentration of HCO₃⁻ required to produce half-maximal velocity as K_C to distinguish it from the true K_{MCO_2} , which can be measured for carboxysome-bound RubisCO only if the flux of CO₂ across the shell does not affect the accessibility of RubisCO to its substrate.

CO₂ fixation rates at standard assay conditions (60 mM bicarbonate) and varying RubP concentrations yielded a V_{max} for mutant carboxysomes of 1.1 ± 0.1 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein, which is ~60% of the maximal rate observed for wild type particles (1.6 ± 0.1 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein). The apparent K_m for RubP, on the other hand, was essentially the same for mutant and wild type carboxysomes (174.7 ± 16.1 and 146.2 ± 5.6 μM , respectively). Likewise, V_{max} obtained at a fixed, saturating RubP concentration of 0.5 mM and increasing bicarbonate concentrations from 1.45 to 80.2 mM was consistently slightly lower for the mutant carboxysomes (1.3 ± 0.1

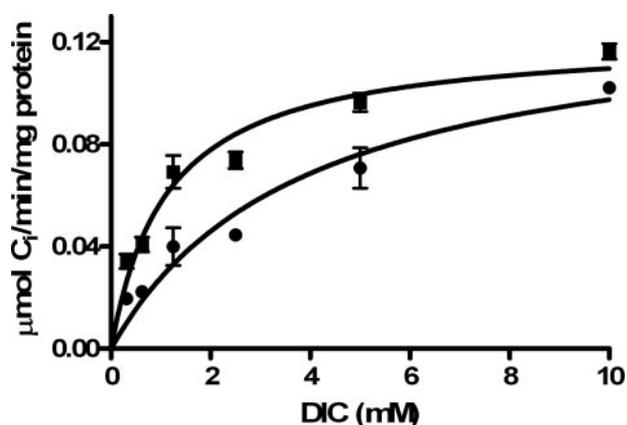


FIGURE 4. Assimilation of inorganic carbon by *H. neapolitanus* wild type and *csoS3::Km* mutant cells. Bioreactor-grown cultures were kept in air (ambient CO₂) for 24 h before being concentrated by centrifugation. The cell pellets were resuspended in fresh medium and assayed as described under "Experimental Procedures." ■, wild type; ●, mutant. The error bars signify standard error.

$\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein) than for wild type particles ($1.7 \pm 0.1 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein) (Table 1). The K_C for mutant carboxysomes ($499.3 \pm 47.1 \mu\text{M}$), however, was nearly 3-fold higher than that measured for wild type microcompartments ($177.1 \pm 16.3 \mu\text{M}$) (Table 1).

To assess whether exogenously added carbonic anhydrase could functionally complement the missing carboxysome component, purified rCsoSCA was mixed with purified *csoS3::Km* carboxysomes at an activity level that was 5-fold higher than that measured for wild type carboxysomes (15). No significant effect on V_{max} and K_C values was observed (Table 1).

To further evaluate how the presence and location of CsoSCA within the carboxysome affect carboxysome function, the carboxysome shell was disrupted by a freeze/thaw treatment (15, 16, 28). Electron microscopic examination of the remaining structures revealed the presence of shell ghosts that retained their icosahedral shape to some degree but contained breaks and tears (3, 28). As reported previously, some RubisCO was retained within the ruptured structures but greater than 70% was released into the supernatant, as estimated by differential centrifugation and SDS-PAGE (15). Disruption of the *csoS3::Km* carboxysomes resulted in an increase in V_{max} ($2.0 \pm 0.1 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein) compared with the value obtained for intact mutant particles ($1.3 \pm 0.1 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein). The K_C of broken mutant carboxysomes ($167.3 \pm 13.2 \mu\text{M}$) was only slightly higher than that of broken wild type particles ($126.7 \pm 10.8 \mu\text{M}$) and within the range of that calculated for intact wild type carboxysomes ($177.1 \pm 16.3 \mu\text{M}$) (Fig. 5, A and B). Control assays with disrupted wild type carboxysomes revealed a small increase in V_{max} ($2.0 \pm 0.1 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein) and a slight decrease in K_C ($126.7 \pm 10.8 \mu\text{M}$). RubisCO preparations derived from the supernatants of disrupted wild type and mutant carboxysomes, as expected, displayed no significant differences in their kinetic constants (Table 1 and Fig. 5C).

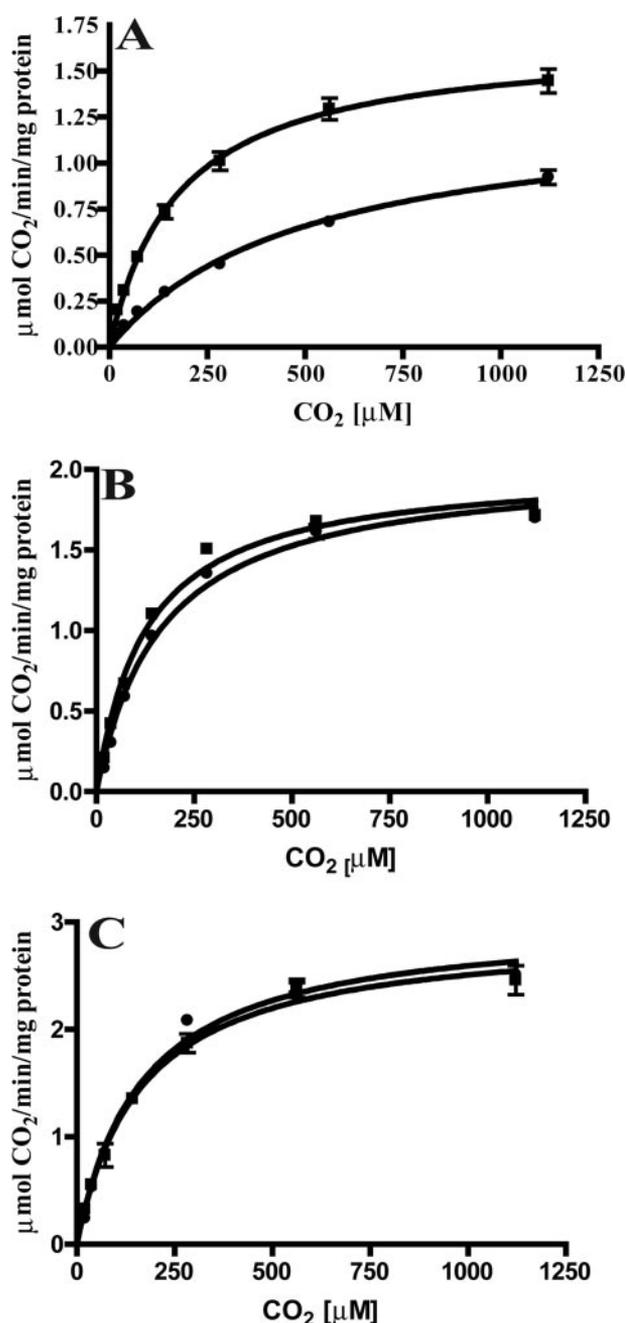


FIGURE 5. CO₂ fixation by *H. neapolitanus* wild type and *csoS3::Km* mutant carboxysomes. The figure shows carbon fixation by purified intact carboxysomes (A), broken carboxysomes (B), and freed RubisCO (C). ■, wild type; ●, mutant. The error bars signify standard error.

DISCUSSION

Numerous biochemical and genetic studies support the widely accepted notion that the carboxysome functions as the terminal component of a CO₂-concentrating mechanism (1, 2). Its carbonic anhydrase is thought to rapidly convert the abundant cytosolic bicarbonate to CO₂, the sole form of inorganic carbon that can be utilized by RubisCO. In the work reported here, knocking out CsoSCA, the carboxysomal carbonic anhydrase of *H. neapolitanus*, by insertion mutagenesis resulted in the *csoS3::Km* mutant that required elevated CO₂ for growth at wild type rates. The high CO₂-requiring phenotype is common

TABLE 1

Kinetic constants for the RubisCO reaction

Radiometric RubisCO assays were performed as described under "Experimental Procedures." The values for K_C are shown with standard error.

	K_C	V_{\max}
	$\mu\text{M CO}_2$	$\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$
Intact carboxysomes		
Wild type	177.1 ± 16.3	1.7 ± 0.1
Wild type + rCsoSCA	163.1 ± 10.7	1.6 ± 0.1
Mutant	499.3 ± 47.1	1.3 ± 0.1
Mutant + rCsoSCA	430.6 ± 49.6	1.2 ± 0.1
Broken carboxysomes		
Wild type	126.7 ± 10.8	2.0 ± 0.1
Mutant	167.3 ± 13.2	2.0 ± 0.1
Freed RubisCO		
Wild type	163.7 ± 17.1	2.9 ± 0.1
Mutant	165.8 ± 14.2	3.0 ± 0.1

for mutants of cyanobacteria and chemoautotrophs that are deficient in carboxysome components. Some of these mutants produce carboxysomes of normal appearance that are, however, obviously functionally compromised (8, 21, 29–33). Maybe cells can assemble microcompartments from a subset of gene products, but to yield a functional carboxysome all proteins found in wild type particles are required. In agreement with this possible scenario is our recent report that in *H. neapolitanus* all genes for carboxysome proteins, including those that exist in multiple copies in the *cso* operon, are transcribed (34).

Although negatively stained mutant and wild type carboxysomes were indistinguishable by transmission electron microscopy, dynamic light scattering measurements consistently revealed *csoS3::Km* carboxysomes to be 15–20% larger than their wild type counterparts. This difference, although barely exceeding the error margins of dynamic light scattering, was reproducible for all carboxysome preparations; a smaller-than-wild type diameter of mutant carboxysomes was never observed. Because CsoSCA accounts for only 2–3% of carboxysomal protein, this difference in size may be attributed to changes in the carboxysome assembly pattern. Alternatively, a different polydispersity of the mutant carboxysome population caused by the absence of CsoSCA may have rendered the apparent diameter artificially high. The larger size of mutant carboxysomes may also be related to their CsoS2B to CsoS2A ratio, which was twice that of wild type particles, in which both CsoS2 variants are represented in approximately equimolar amounts (3, 10).

Clearly, the carboxysomes of the *csoS3::Km* mutant were functional in that they supported CO₂ fixation by the sequestered RubisCO even in the absence of CsoSCA. However, the requirement of *csoS3::Km* cells for higher CO₂ levels to support efficient growth suggests that mutant carboxysomes were not able to provide the catalytic advantage RubisCO derives from wild type microcompartments. These results support the idea that CsoSCA is instrumental in providing sufficient levels of CO₂ to the sequestered RubisCO and are consistent with the existence of a carbon-concentrating mechanism in *H. neapolitanus* similar to that shown to operate in *Thiomicrospira crunogena* and in several cyanobacteria (5, 35, 36). The path by which the bicarbonate that is concentrated in the cytosol enters the carboxysome and the way in which the resulting CO₂ is distrib-

uted to the RubisCO in the microcompartment core are not well understood. Previous quantitative models predict that to maintain effectively higher concentrations of CO₂ at the active sites of RubisCO, either the shell of the carboxysome prevents CO₂ escape, or the carbonic anhydrase is localized in the center of the microcompartment, and the surrounding densely packed RubisCO molecules slow CO₂ leakage (37, 38). In *H. neapolitanus* and other α -carboxysomes, the carbonic anhydrase (CsoSCA) is tightly associated with the shell (15, 16, 39). This makes the model that posits a central location for the enzyme (37) unlikely, at least for α -carboxysomes. The results obtained in this study show that to reach half-maximal CO₂ fixation rates intact mutant carboxysomes lacking CsoSCA required nearly 3-fold higher concentrations of bicarbonate than wild type carboxysomes. Furthermore, exogenously added excess rCsoSCA did not affect this requirement for higher bicarbonate concentrations. To be effective in enhancing the carbon fixation rate of RubisCO, the carbonic anhydrase activity must reside on the inside of the microcompartment boundary. If the shell were freely permeable to CO₂, exogenously added carbonic anhydrase should catalyze the formation of CO₂ rapidly enough to allow diffusion into the particle. This should at least partially overcome the requirement for high inorganic carbon levels to support wild type carbon fixation rates. Our results are consistent with the idea that the protein shell limits the diffusion of CO₂ into and out of the carboxysome.

If the carboxysomal shell limits access of CO₂ to the carboxysome interior, its disruption should relieve any diffusional limits. Indeed, the observed kinetic constants of RubisCO were restored to near wild type values in mutant carboxysomes whose shell integrity had been compromised. These results are consistent with the previously reported increase in *in situ* carbonic anhydrase activity upon disruption of the carboxysome shell (15), which was also interpreted as relieving the apparent limitation on diffusion of HCO₃⁻ and/or CO₂ across the carboxysome boundary.

Comparison of K_C and V_{\max} values for intact and disrupted wild type carboxysomes revealed a slight increase in maximal CO₂ fixation rate upon disruption of the shell and no significant difference in K_C . This result is in stark contrast to the significant improvement in RubisCO kinetic performance measured for disrupted mutant carboxysomes and affirms the important role CsoSCA plays in facilitating the diffusion of cytosolic bicarbonate across the microcompartment shell. Furthermore, according to one of the quantitative models, the shell should also act as a barrier that reduces leakage of CO₂ from the carboxysome interior before it can be fixed by RubisCO (38).

Although considerable strides have recently been made toward establishing a model of carboxysome architecture that is based on protein structure information (11, 12, 13, 17), our current understanding of shell structure is incomplete, and a satisfactory model that could form the basis for carboxysome function has yet to be formulated. Existing crystallographic data have established nearly seamless interactions between neighboring hexamers of CsoS1A. Because the three CsoS1 proteins make up the bulk of the shell (12), it is difficult to envision how CsoSCA dimers or the filaments they form in crystals (11) can be embedded in or transverse the shell within

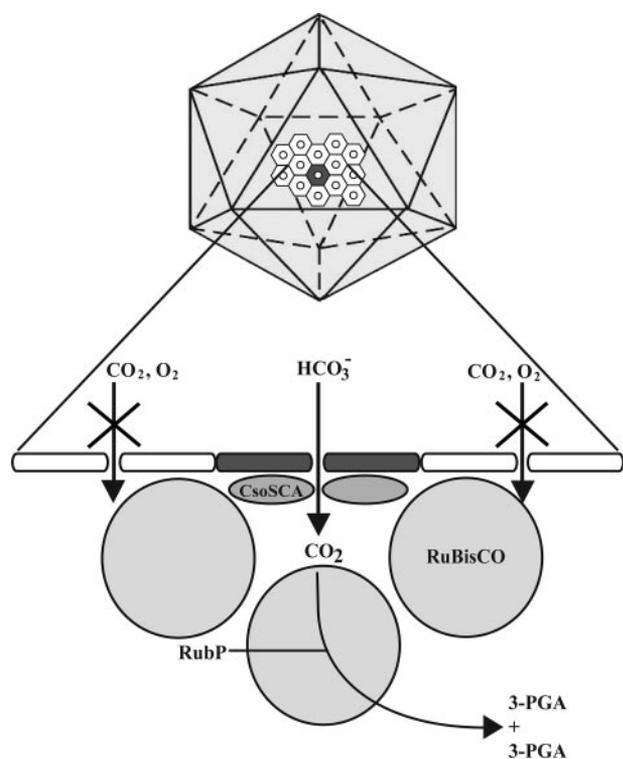


FIGURE 6. Model for the location and function of CsoSCA in the carboxysome shell. This model depicts an interaction of a CsoSCA dimer with one CsoS1 hexamer, facilitating transfer of CO₂ through the pore of the interacting hexamer into the carboxysome interior via dehydration of bicarbonate.

the context of the CsoS1 arrays. Perhaps the ~40 CsoSCA dimers predicted to exist in each carboxysome are instead associated with some of the estimated 600 central pores in the CsoS1 hexamers (Ref. 12 and Fig. 6). All three CsoS1 paralogs of *H. neapolitanus* are almost identical in amino acid sequence and are thought to form hexamers of similar, if not identical structure. It is possible that the C-terminal extension of 12 amino acids in CsoS1B, which distinguishes this shell protein from CsoS1A and CsoS1C, permits interaction with CsoSCA.

The pores of *H. neapolitanus* CsoS1A hexamers bear a positive charge and have been hypothesized to facilitate flux of small, negatively charged molecules such as HCO₃⁻ across the carboxysome shell (12). If CsoSCA binds tightly to such a pore on the side that faces the inside of the carboxysome, its catalytic activity might create a sink that “pulls” bicarbonate through the pore (Fig. 6). Conversion of HCO₃⁻ to CO₂ would create an inorganic carbon species that is slow to diffuse out of the carboxysome interior. Furthermore, because CO₂ is the substrate for RuBisCO, the product of the bicarbonate dehydration reaction would be consumed in the carboxysome interior and would drive continued directed “transport” of cytosolic bicarbonate into the microcompartment in a manner that is analogous to a chemical diode.

Such an arrangement would be consistent with the data presented here in that CO₂ fixation in mutant carboxysomes lacking CsoSCA would be dependent on high concentrations of CO₂ to overcome the diffusional resistance offered by the shell. Breaching shell integrity to allow free flux of bicarbonate and CO₂ would produce kinetic parameters similar to those

observed for intact wild type carboxysomes, where the 40 dimers of CsoSCA are capable of producing sufficient CO₂ to saturate the carboxysomal RuBisCO (15).

The data reported here support this model but at the same time raise a more fundamental question: because ruptured and intact wild type carboxysomes catalyze CO₂ fixation *in vitro* with similar efficiencies, the advantage these microcompartments offer the cell *in vivo* is not immediately obvious. Indeed, RuBisCO freed from either wild type or *csoS3::Km* mutant carboxysomes catalyzes the fixation of CO₂ with efficiencies very similar to those measured for intact wild type carboxysomes (10, 40). Perhaps this suggests that the substrate concentrations used to measure CO₂ fixation *in vitro* do not adequately reflect the *in vivo* conditions. One can envision that carboxysomes may provide an advantage for the cells under conditions of rapidly changing environmental pH and/or bicarbonate concentration. Perhaps, as previously suggested (1, 15), in addition to its role in the carbon-concentrating mechanism, the carboxysome fulfills the important function of protecting carboxysomal RuBisCO from the detrimental effects of oxygen (Fig. 6).

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CO₂ Fixation Kinetics of *Halothiobacillus neapolitanus* Mutant Carboxysomes Lacking Carbonic Anhydrase Suggest the Shell Acts as a Diffusional Barrier for CO₂

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