Characterization of the Carboxysomal Carbonic Anhydrase CsoSCA from Halothiobacillus neapolitanus

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In cyanobacteria and many chemolithotrophic bacteria, the CO₂-fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) is sequestered into polyhedral protein bodies called carboxysomes. The carboxysome is believed to function as a microcompartment that enhances the catalytic efficacy of RubisCO by providing the enzyme with its substrate, CO₂, through the action of the shell protein CsoSCA, which is a novel carbonic anhydrase. In the work reported here, the biochemical properties of purified, recombinant CsoSCA were studied, and the catalytic characteristics of the carbonic anhydrase for the CO₂ hydration and bicarbonate-dehydration reactions were compared with those of intact and ruptured carboxysomes. The low apparent catalytic rates measured for CsoSCA in intact carboxysomes suggest that the protein shell acts as a barrier for the CO₂ that has been produced by CsoSCA through directional dehydration of cytoplasmic bicarbonate. This CO₂ trap provides the sequestered RubisCO with ample substrate for efficient fixation and constitutes a means by which microcompartmentalization enhances the catalytic efficiency of this enzyme.

A wide variety of bacterial species package some metabolically important enzymes into polyhedral microcompartments that are bounded by shells comprised of three to seven highly conserved proteins (reviewed in reference 20). Microcompartmentalization is thought to enhance the catalytic efficacy of the enzyme(s) sequestered inside by providing metabolic channeling through enzyme colocalization and by reducing the effects of product/substrate diffusion (47). The structures may also protect the enzyme(s) from inhibitors (13) or prevent escape of products that are toxic to other portions of cellular metabolism (19). The best-studied example of these microbial microcompartments is the carboxysome, found in many chemolithotrophic bacteria and in all cyanobacteria thus far studied (Fig. 1). Carboxysomes are organelles that derive their name from their content, the CO₂-fixing enzyme of the Calvin-Benson-Bassham cycle, ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) (40). Genetic and metabolic studies have revealed that carboxysomes are required for growth at normal atmospheric CO₂ levels (reviewed in reference 13). In some species, the number of carboxysomes per cell and the proportion of cellular RubisCO packaged into the microcompartments are regulated in a manner dependent on the level of inorganic carbon available to the cells (9, 15, 29, 32, 52). However, in some cyanobacteria, such as Synechocystis 6803, the number of carboxysomes seems to remain constant regardless of available inorganic carbon (Ci) levels (48).

The carboxysome shell polypeptides of Halothiobacillus neapolitanus are encoded by a cluster of genes that are arranged in an apparent operon (cso), which includes cbbL and cbbS, the genes for the large subunit and the small subunit, respectively, of RubisCO (12–14). A similar organization of carboxysome genes has been observed in all carboxysome-bearing chemolithotrophs thus far examined and in α-cyanobacteria (5, 14), exemplified by the genus Prochlorococcus, which dominates the oligotrophic oceans (47). In contrast, the carboxysome genes of β-cyanobacteria are arranged in a less-defined cluster termed ccmKLMN, which is typically located upstream from the genes encoding the large and small subunits of RubisCO (6). It has been proposed that carboxysomes play a role in an inducible CO₂-concentrating mechanism (CCM) in some cyanobacteria (reviewed in references 4, 6, and 24). In the β-cyanobacteria Synechocystis and Synechococcus, as many as five energy-dependent Ci accumulation modes have been described, which result in cytoplasmic concentrations of HCO₃⁻ that are 1,000 times higher than the concentrations of Ci on the outside of the cell (reviewed in references 4, 17, and 24). Since the substrate for RubisCO is CO₂ and the uncatalyzed rate of HCO₃⁻ dehydration to CO₂ is much lower than needed to sustain the observed photosynthetic rates, it is generally accepted that a carbonic anhydrase (CA) is colocalized with RubisCO inside the carboxysome. Bicarbonate is thought to be able to diffuse into the carboxysome, where its conversion to CO₂ is catalyzed by the resident CA. Based on calculations from two quantitative models, it has been proposed that some feature of the carboxysome structure limits the outward diffusion of CO₂, although no direct experimental results supporting a molecular mechanism that could give rise to this purported characteristic have been put forth so far (11, 24, 37, 38).

The existence of a CCM in chemolithotrophic bacteria is supported by the fact that H. neapolitanus accumulates Ci as HCO₃⁻ in its cytoplasm, although at levels below those observed in cyanobacteria with a functional CCM (22). An energy-dependent CCM is also present in the deep sea hydrothermal vent sulfur bacterium Thiomicrospira crunogena and probably...
in other autotrophs (16). The role—if any—of carboxysomes in the CCM of chemoautotrophs has not been established to date. An alternative hypothesis suggests that the carboxysomal shell plays a role as a selective permeability barrier favoring the influx of HCO$_3$\(^{-}\) into the carboxysome and limiting the diffusion of O$_2$, the competitive inhibitor of CO$_2$ fixation by RubisCO.

The recent report (25) describing the crystal structure of the major carboxysomal shell protein from Synechocystis suggests a role for the carboxysomal shell in the regulation of metabolite passage into and out of the microcompartments. This role is consistent with the alternative hypothesis but to date cannot explain how this control can be achieved through structures and interactions of the five putative α-carboxysomal shell proteins.

We have identified one of the carboxysomal shell proteins, CsoS3 (referred to here as CsoSCA for carboxysomal shell CA), as a CA that appears to be limited to organisms containing α-carboxysomes (47). The novel primary structure of CsoSCA led us to propose a new class of CA that we termed ε in keeping with the nomenclature for the four other known classes of CA (α, β, γ, and δ) (50). Previous structural studies using immunogold electron microscopy suggested that CsoS3 is a carboxysomal shell protein (8), and CA assays of purified carboxysome shells confirmed a tight association of the enzyme with the shell (47). It is not clear at this point if CsoSCA is embedded in the shell and perhaps plays a role in facilitating the diffusion of HCO$_3$\(^{-}\) into the carboxysome or if it is tightly bound to the inside surface of the structure, converting bicarbonate within the interior of the particle to CO$_2$ for use by the carboxysomal RubisCO.

Recent solution of the crystal structure of CsoSCA has revealed a remarkable similarity to the secondary and tertiary structure of β-CAs and a near-identical three-dimensional arrangement of active-site residues, suggesting that the enzyme is best described as a subclass of β-CAs (39). In this study, we have utilized stopped-flow spectrophotometry to assess the activity of CsoSCA in carboxysomes and as purified recombinant protein.

**MATERIALS AND METHODS**

**Metals analysis.** Semiquantitative and quantitative metals analyses were performed by inductively coupled plasma atomic emission spectroscopy at the Center for Trace Analysis, Department of Marine Science, The University of Southern Mississippi.

**Carboxysome purification.** A chemostat culture of *H. neapolitanus* was grown as previously described (15) with a dilution rate of 0.08 h$^{-1}$ and at a pH of 6.4. Cells (5 to 8 g wet weight) were harvested by centrifugation and resuspended into 20 ml of TEMB buffer (10 mM Tris-HCl, pH 8.0, 10 mM MgCl$_2$, 20 mM NaHCO$_3$, 1 mM EDTA) before disruption by sonication as previously reported. The resulting cell break was mixed with 1 volume of BPER II (Pierce Biotechnology, Inc., Rockford, IL), incubated with shaking at room temperature for 30 min, and clarified by centrifugation at 12,000 × g for 10 min. The supernatant was then centrifuged at 48,000 × g for 30 min to sediment the carboxysomes, which were resuspended and purified by sucrose density gradient centrifugation as previously described (47).

**Carboxysome shell disruption.** Homogeneous preparations of carboxysomes (50 to 100 μl) at a protein concentration of 6 to 8 mg ml$^{-1}$ in TEMB were collected as a pellet by centrifugation at 14,000 × g for 30 min. The resulting supernatant was carefully removed and the remaining semidry pellet frozen at −20°C for 10 to 15 min. The pellet was then rapidly resuspended in 50 to 100 μl of the desired buffer (typically TEMB). Transmission electron microscopy of the preparation revealed that more than 95% of the carboxysome shells were disrupted, allowing most of the RubisCO to be released from the carboxysome interior into the surrounding buffer. The shells retained their polyhedral shape, with some fraction of RubisCO always evident lining the inside.

**Expression and purification of CsoSCA.** Recombinant histidine-tagged CsoSCA (rCsoSCA) was expressed from plasmid pProExCsoSCA in *Escherichia coli* and purified by affinity chromatography on a Ni column as previously described (39).

**Measurement of CA activity.** Steady-state CA activity was measured by stopped-flow spectrophotometry using the changing pH indicator method of Khalilah (26). Assays were performed at 25°C with an Applied Photophysics SX.18M stopped-flow spectrophotometer/fluorometer. The buffer/indicator pairs and wavelengths used were MES (morpholineethanesulfonic acid)/chlorophenol red ($A_{620}$) at pH 5.5 to 6.8; HEPES/phenol red ($A_{620}$) at pH 7.5 to 8.0; and TAPS [N-tris(hydroxymethyl)methyl-3-ammonopropanesulfonic acid]/m-cresol purple ($A_{550}$) at pH 8.0 to 9.0. For measurements of CO$_2$ hydration reactions, the appropriate amount of water saturated with CO$_2$ (32.9 mM) was diluted with N$_2$-saturated water to yield, when mixed in the stopped-flow cell, final dissolved CO$_2$ concentrations from 5 to 25 mM. For measurements of bикарбонат dehydron reactions, KHCO$_3$ solutions in final assay concentrations in the range of 10 to 100 mM were used. In both instances, sufficient sodium sulfate was added to maintain an ionic strength of 0.5 M. Initial reaction rates were obtained from progress curves, corrected by subtracting the uncatalyzed rate, and fitted to the Michaelis-Menten equation using KaleidaGraph (Synergy Software).

The ability of rCsoSCA to hydrolyze p-nitrophenylacetate was tested at 25°C following the method of Armstrong et al. (3). The enzyme was incubated with 1.5 mM p-nitrophenylacetate in 100 mM potassium phosphate buffer, pH 7.2, and the change in A$_{400}$ was followed over time.

**RESULTS**

The carboxysomal CA CsoSCA is tightly associated with and therefore believed to be an integral component of the carboxysome shell (8, 47). Upon disruption of carboxysomes by freezing and thawing, the CA activity copurifies with the shell fraction through differential and density gradient centrifugation steps (47). To further characterize the carboxysomal enzyme, attempts were made to purify CsoSCA from the carboxysomes under conditions that would preserve its enzymatic activity. Ion-exchange and immunofinity chromatography of disrupted carboxysomes did not yield preparations of active CsoSCA protein (data not shown), presumably because of the protein’s tight interaction with other carboxysome shell com-
For this study, the csoS3 tag to facilitate purification, was soluble and typically yielded protein rCsoSCA, which carries an N-terminal hexahistidine vector was used exclusively, because the resulting recombinant protein contained a mutation that resulted in the replacement of a phenylalanine residue at position 92 with a histidine. This change created a second Zn$^{2+}$-binding site at position 92 with a histidine. This change created a second Zn$^{2+}$-binding site, which likely represents a domain. When these interactions were disrupted by treatment of shell-enriched carboxysomes with strong denaturants such as urea or guanidinium hydrochloride, CsoSCA could be purified by denaturing size-exclusion chromatography, but it was not possible to recover enzymatic activity after removal of the denaturants by dialysis. Although the CA activity of CsoSCA is easily measurable in purified carboxysomes or in isolated shells, the protein represents only a small portion of the total shell protein (approximately 8%) (15, 20, 41). To be able to characterize a homogeneous preparation of the carboxysomal carbonic anhydrase, it was therefore necessary to produce the recombinant enzyme in E. coli and purify the recombinant protein by affinity chromatography. The basic catalytic parameters determined for CsoSCA were compared with those measured with purified carboxysomes and purified shells and were corrected for the total protein amounts.

Expression and purification of recombinant CsoSCA. The carboxysomal CA CsoSCA from H. neapolitanus was produced in E. coli by use of several different expression systems (47). For this study, the csoS3 expression construct in the pProEx vector was used exclusively, because the resulting recombinant protein rCsoSCA, which carries an N-terminal hexahistidine tag to facilitate purification, was soluble and typically yielded 10 mg of homogeneously pure protein from 1 liter of E. coli culture (Fig. 2). The purified protein had an enzymatic activity similar to that of recombinant enzyme produced in the IMPACT system, which was devoid of any extraneous amino acids but could be obtained only in considerably lower yields (47). One of the clones obtained was subsequently found to contain a mutation that resulted in the replacement of a phenylalanine residue at position 92 with a histidine. This change created a second Zn$^{2+}$-binding site, which likely represents a domain. The purified recombinant enzyme in E. coli by use of buffers without any added metal ions, but no special provisions were made to eliminate existing trace metals. Addition of Zn$^{2+}$, Co$^{2+}$, or Fe$^{2+}$ to a final concentration of 1 mM did not significantly alter the CA activity (Table 1). However, extended dialysis of the purified recombinant enzyme against buffer that had been treated with Chelex-100 to remove traces of metal ions resulted in the loss of nearly 75% of its activity compared to what was seen for samples that were dialyzed against buffers containing 1 mM ZnCl$_2$. The addition of 1 mM ZnCl$_2$ to the metal-depleted samples restored the CA activity to nearly the level observed for nondialyzed samples, while neither CoCl$_2$ nor MgCl$_2$ was able to reverse the inactivation of the enzyme (Table 1).

Qualitative analysis of the purified enzyme by inductively coupled plasma atomic emission spectroscopy clearly revealed the presence of Zn$^{2+}$ in the purified enzyme but not in buffer blanks. In addition, low levels of both Co$^{2+}$ and Cr$^{2+}$ were observed in the enzyme preparation. Quantitative inductively coupled plasma atomic emission spectroscopy analysis yielded Zn$^{2+}$ contents of between 0.8 and 1.1 mol per mole of the rCsoSCA 57-kDa monomer, with an average value of 0.9 mol of Zn$^{2+}$ per mol of monomer. This is consistent with the existence of a single Zn$^{2+}$-binding site per protein monomer, as predicted from the protein’s crystal structure (39).

Inhibitor effects. The effects of some common CA inhibitors on rCsoSCA activity were assessed by stopped-flow spectrometry at 25°C to confirm results from earlier electrometric and mass spectrometric assays (Table 2). The recombinant carboxysomal enzyme was inhibited by the dithiol dithiothreitol (DTT) (50% inhibitory concentration [IC$_{50}$] = 2.6 × 10$^{-4}$ M) and, to a lesser extent, by the monothiol β-mercaptoethanol (IC$_{50}$ = 3.4 × 10$^{-3}$ M), suggesting two different mechanisms of inhibition for these two reducing agents. As previously reported, ethoxyzolamide inhibits rCsoSCA but to an extent that is significantly lower than that observed for other bacterial enzymes (35, 44, 47), while the effect of cyanide on rCsoSCA was similar to that on the chloroplast CA Cah6 from Chlamydomonas (30). Recombinant CsoSCA was remarkably resistant to azide, with an IC$_{50}$ value of 4.1 mM, which is in the range of those reported for the β-CA and the γ-CA of the archaea Methanobacterium thermautotrophicum and Methanosarcina thermophila, respectively (1, 43). Sodium chloride, Na$_2$SO$_4$,

![FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel of heterologously expressed CsoSCA. Lanes: 1, 20 μg of cell extract from uninduced E. coli cells containing pProExCsoSCA; 2, 20 μg of cell extract of induced cells; 3, 2 μg of rCsoSCA protein eluted from a Ni$^{2+}$ affinity column; 4, purified carboxysomes (from a separate gel).](http://jb.asm.org/)

**TABLE 1. Metal dependence of recombinantly expressed carboxosomal carbonic anhydrase rCsoSCA from H. neapolitanus**

<table>
<thead>
<tr>
<th>Metal added (1 mM)</th>
<th>Relative activity (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>92.8</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>101.8</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>98.1</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>98.4</td>
</tr>
<tr>
<td>Chelex-treated buffer</td>
<td>28.4</td>
</tr>
<tr>
<td>Chelex-treated buffer + Zn$^{2+}$</td>
<td>84.6</td>
</tr>
<tr>
<td>Chelex-treated buffer + Co$^{2+}$</td>
<td>25.9</td>
</tr>
<tr>
<td>Chelex-treated buffer + Fe$^{2+}$</td>
<td>33.4</td>
</tr>
<tr>
<td>Chelex-treated buffer + Mg$^{2+}$</td>
<td>26.7</td>
</tr>
</tbody>
</table>

$^a$ CO$_2$ hydration by rCsoSCA (0.5 μM) was measured by stopped-flow spectrophotometry at pH 8 (buffer/indicator pair, TAPS-m-cresol purple). Values are averages of three determinations with maximum variations of less than 12%.
and KCl at concentrations up to 100 mM did not affect the activity of rCsoSCA. Unlike many α-CAs from animal sources, rCsoSCA exhibited no detectable esterase activity when assayed spectrophotometrically with p-nitrophenylacetate as a substrate.

**Enzymatic activity of rCsoSCA.** The CsoSCA protein from *H. neapolitanus* and its homologues in other α-carboxysome-producing autotrophs represent a novel CA class (ε) based on their unique primary structure. Their secondary and tertiary structures, however, strongly suggest that they constitute a variant of the β-class of these enzymes. The CA activity of the carboxysomal enzyme was demonstrated electrophoretically with the classical Wilber-Andersen assay and with the mass spectrometry-based 18O exchange assay (47). However, under the conditions employed neither of these methods yields data that are suitable for rate parameters. The kinetic constants for rCsoSCA were determined with the colorimetric stopped-flow method of Khalifah (26), which allows one to follow the progress of the hydration of CO2 and, under some conditions, the dehydration of bicarbonate.

The rate of CO2 hydration by rCsoSCA was dependent on pH, and *k*<sub>cat</sub> increased over a pH range from 6.5 to 8.5. The marked inflection between pH 7.0 and 6.5 implicated a histidine residue participating in the catalytic chemistry, which is consistent with the previously proposed mechanism for this enzyme (39) (Fig. 3). The dehydration rate was also pH dependent but showed a decrease in *k*<sub>cat</sub> with increasing pH over the pH range of 6.0 to 7.5. At pH values outside of this range, measurement of the dehydration progress curves was unreliable.

The efficiency (*k*<sub>cat</sub>/*K*<sub>m</sub>) of CsoSCA for the hydration of CO2 at pH 8.0 was calculated to be 2.8 × 10<sup>7</sup> M<sup>-1</sup> s<sup>-1</sup> (Table 3), while a *k*<sub>cat</sub>/*K*<sub>m</sub> of 4.9 × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup> was determined for the dehydration of bicarbonate at pH 7.0. The cytoplasmic pH of *H. neapolitanus* and related thiobacilli has been estimated to be between 7 and 8 (16, 22), a pH range for which the efficiency of HCO<sub>3</sub><sup>-</sup> dehydration is predicted to be approximately threefold lower than that of CO2 hydration. To date, no estimate of the pH of the carboxysome interior, which might be different from that of the cytoplasm, has been reported. However, recent studies of Penrod and Roth (33) examining the effects of CO2 and acetaldehyde exchange on wild-type *Salmonella enterica* and on various *S. enterica*<sub>eut</sub> mutants suggest that the microcompartments in that organism may provide a

**TABLE 2. Susceptibility of the *H. neapolitanus* carboxysomal carbonic anhydrase rCsoSCA to inhibitors**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (M) *&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dithiothreitol</td>
<td>2.4 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>3.4 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethoxylazomide</td>
<td>1.9 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>KCl (&lt;100 mM)</td>
<td>No effect</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt; (&lt;100 mM)</td>
<td>No effect</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>4.1 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sodium cyanide</td>
<td>2 × 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> IC<sub>50</sub> is the molar inhibitor concentration that reduced the CO<sub>2</sub> hydration activity of rCsoSCA by 50% at pH 8. Initial rates of CO<sub>2</sub> hydration activity were determined by stopped-flow spectrophotometry in the presence of the indicated conditions, the dehydration of bicarbonate. At pH 8.0, a *k*<sub>s</sub> was determined for the hydration of CO2 and, under some conditions, the dehydration of bicarbonate.

**TABLE 3. Kinetic constants of the *H. neapolitanus* carboxysomal carbonic anhydrase CsoSCA**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Hydration reaction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Dehydration reaction&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>k</em>&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td><em>K</em>&lt;sub&gt;m&lt;/sub&gt; (mM)</td>
</tr>
<tr>
<td>rCsoSCA</td>
<td>(8.9 ± 0.5) × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.2 (± 0.4)</td>
</tr>
<tr>
<td>Broken carboxysomes</td>
<td>(6.5 ± 0.9) × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.6 (± 0.7)</td>
</tr>
<tr>
<td>Intact carboxysomes</td>
<td>(1.8 ± 0.2) × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>12.2 (± 2.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The hydration reaction was performed at pH 8.0.
<sup>b</sup> The dehydration reaction was performed at pH 7.0. ND, not determined.
related in some manner to the structural integrity of the carboxysomal shell. The general trend of HCO$_3^-$ dehydratation was also much lower in assays with intact carboxysomes than in those with broken carboxysomes or with recombinant enzyme. However, the poor signal obtained for such low levels of activity made it impossible to reliably fit the dehydratation kinetics to Michaelis-Menten equations and prevented comparisons to results obtained with disrupted carboxysomes and rCsoS3 (Table 3). Since the rate of the CA-catalyzed reaction is influenced by the pH of its environment, as indicated by the dependence of $k_{cat}$ on pH (Fig. 3), the $k_{cat}$ for the hydration reaction was measured over the pH range from 6.5 to 8.5 for intact and broken carboxysomes (Fig. 3). An internal carboxysome pH that is significantly lower than that of the surrounding buffer could explain the difference in CA activity observed between disrupted and intact carboxysomes; however, the pH profiles of CA activity plotted in Fig. 3 for all three forms of CsoS3 assayed did not display significant differences in shape.

**DISCUSSION**

We have previously shown that the carboxysomal shell protein encoded by csoS3 from a number of α-carboxysome-containing prokaryotes is a carbonic anhydrase (47). The protein is a minor component of the carboxysome, comprising approximately 2.3% of total carboxysomal protein by weight. This value corresponds to 80 copies of the monomer per carboxysome, compared to the estimated 270 molecules of RubisCO holoenzyme per particle (15, 20, 41). The lack of significant primary sequence homology of CsoS3 with representatives of the other CA classes (α, β, γ, and δ) led us to propose a new class (ε) for the carboxysomal enzyme (47). However, subsequent elucidation of the protein’s crystal structure established unequivocally that CsoS3 and its homologues are unique variants of β-CAs (39). To signify the connection of these CAs with the carboxysomal shell, the term CsoS3 has been replaced by CsoS3A. The association of CsoS3A with the carboxysomal shell is so tight that it is impossible to remove the protein from shell-enriched fractions of broken carboxysomes without the use of denaturants. Since the treatment needed to completely disassemble the shell irreversibly inactivates the CA activity, CsoS3A from *H. neapolitanus* was expressed heterologously in *E. coli*, purified, and characterized for this study. Consistent with its crystal structure (39), CsoS3A was found to bind one Zn$^{2+}$ per monomer. Removal of the metal by dialysis or by treatment with chelators resulted in a loss of enzymatic activity that could not be restored by other divalent metal ions. Interestingly, CsoS3A was potently inhibited by the dithiol reducing agent DTT (reference 47 and this study) and to a lesser extent by the monothiol β-mercaptoethanol. The inhibition by DTT is similar to that reported for the cyanobacterial β-CA CcaA, which shares some sequence homology with higher plant chloroplast carbonic anhydrases and may be the carboxysome-associated enzyme in *Synechocystis PCC 6803* and other β-cyanobacteria (49). The mechanism of inhibition by DTT is generally assumed to be the destabilization of tertiary or quaternary protein structure through the disruption of disulfide bonds (23, 28). However, the low concentration of DTT, compared to that of β-mercaptoethanol, required to bring about a
50% inhibition of CsoSCA suggests that DTT might interact directly with an active-site residue, such as one of the Zn$^{2+}$-coordinating cysteines (39), that is not reactive with β-mercaptoethanol. The sulfamidamide ethoxyzanilamide inhibited rCsoSCA as it does all known carbonic anhydrases, presumably through binding the active-site Zn$^{2+}$. The ethoxyzanilamide concentration necessary to effect a 50% reduction of enzymatic activity, however, is orders of magnitude higher than that expected for most bacterial carbonic anhydrases (1, 2, 35, 44, 51), suggesting that the Zn$^{2+}$ in CsoSCA may be blocked from interaction with the inhibitor.

The molecular mass of CsoSCA calculated from its primary structure is 57.3 kDa, which agrees well with denaturing polyacrylamide gel electrophoresis-based molecular weight estimates for the carboxysome-associated enzyme from *H. neapolitanus* and for rCsoSCA without the hexahistidine tag. Other CAs from autotrophic microorganisms are usually considerably smaller and fall into the range of 30 kDa. The notable exception is the β-CA from *Porphyridium purpureum*, which has a molecular weight of approximately 60,000 and is comprised of two very similar (70% sequence similarity) domains that probably arose through a gene duplication event (31, 51). CsoSCA also has two domains that are likely to have arisen from gene duplication but have diverged considerably more than those of the *P. purpureum* enzyme, such that only one of the domains contains a functional Zn$^{2+}$-binding site. This is consistent with the single Zn$^{2+}$ ion per monomer that was predicted from the protein’s crystal structure (39) and was detected in this study.

The catalytic ability of rCsoSCA, as indicated by its $k_{cat}$ and efficiency of CO$_2$ hydration, is well within the range of values reported for other β-CAs (1.7 x 10$^{-4}$ to 2.3 x 10$^{-3}$ s$^{-1}$) (21, 27, 44, 45). If the *H. neapolitanus* carboxy enzyme functions as the terminal component of a CCM, as is hypothesized for the carboxysomes of β-cyanobacteria (6, 36), the presumed role of CsoSCA would be to convert the abundant cytoplasmic HCO$_3^-$ to CO$_2$ within the carboxysome. RubisCO inside the microcompartments would fix the CO$_2$ onto ribulose 1,5-bisphosphate (RubP) and produce 3-phosphoglycerate (3-PG), which would be returned to the cytoplasm for completion of the Calvin-Benson-Bassham cycle. This would account for the significantly lower apparent catalytic rate observed with intact carboxysomes than that observed with ruptured carboxysomes, where CO$_2$ and HCO$_3^-$ equilibrate rapidly and the inside and the outside of the microcompartment. The measured reduction in the hydration rate could be explained in the same manner if it is assumed that CO$_2$ must first diffuse into the carboxysome before it reaches the side of the shell-bound CsoSCA that is capable of binding and converting it to HCO$_3^-$.

In vivo under physiological conditions, the fixation of CO$_2$ onto RubP and the subsequent conversion to 3-PG within the carboxysome would lower the CO$_2$ concentration, allowing more cytoplasmic HCO$_3^-$ to be converted to CO$_2$ by CsoSCA and be “transported” into the microcompartment. As long as bicarbonate remained plentiful in the cytoplasm, this hypothetical scenario would effectively constitute an “on-demand” CO$_2$ supply system for RubisCO within the carboxysome. This model of carboxysome function requires that there be a means of rapidly “transport” of RubP into the carboxysome and of 3-PG out of the microcompartment. While it may seem difficult to envision a protein barrier to CO$_2$ that permits facile passage of negatively charged organic molecules such as RubP and 3-PG, it must be noted that another carboxysomal shell protein, CsoS2, might be a candidate for interaction with the negatively charged phosphates of RubP and 3-PG. This protein
is the only shell component with a basic isoelectric point and a positive net charge at the presumed cytosolic pH of 7 to 8 (16, 22). Little is known about the structure of CsoS2 other than the fact that in *H. neapolitanus* the polyhedral organelles formed in microcompartments has been performed (18, 19), the shell of other case in which limited biochemical analysis of isolated microcompartment’s primary diffusion barrier. It is interesting to note in this context the widespread occurrence of homologues of carboxysome shell protein genes in aquatic photosynthetic microorganisms. Can. J. Bot. 83:695–697.


affinity for inorganic carbon in Anabaena strain PCC 7119 (Cyanophyta) in response to modification of CO₂ and Na⁺ supply. Plant Cell Physiol. 42: 46–53.


