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Carbon Dioxide “Trapped” in a β -Carbonic Anhydrase

Mayank Aggarwal[†], Teck Khiang Chua[‡], Melissa A. Pinard[§], Dolettha M. Szebenyi[‡], and Robert McKenna^{*,§}

[†]Division of Biology and Soft Matter, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, United States

[‡]Cornell High Energy Synchrotron Source (CHESS), Cornell University, Ithaca, New York 14853, United States

[§]Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, Florida 32610, United States

Abstract

Carbonic anhydrases (CAs) are enzymes that catalyze the hydration/dehydration of $\text{CO}_2/\text{HCO}_3^-$ with rates approaching diffusion-controlled limits ($k_{\text{cat}}/K_M \sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$). This family of enzymes has evolved disparate protein folds that all perform the same reaction at near catalytic perfection. Presented here is a structural study of a β -CA (psCA3) expressed in *Pseudomonas aeruginosa*, in complex with CO_2 , using pressurized cryo-cooled crystallography. The structure has been refined to 1.6 Å resolution with R_{cryst} and R_{free} values of 17.3 and 19.9%, respectively, and is compared with the α -CA, human CA isoform II (hCA II), the only other CA to have CO_2 captured in its active site. Despite the lack of structural similarity between psCA3 and hCA II, the CO_2 binding orientation relative to the zinc-bound solvent is identical. In addition, a second CO_2 binding site was located at the dimer interface of psCA3. Interestingly, all β -CAs function as dimers or higher-order oligomeric states, and the CO_2 bound at the interface may contribute to the allosteric nature of this family of enzymes or may be a convenient alternative binding site as this pocket has been previously shown to be a promiscuous site for a variety of ligands, including bicarbonate, sulfate, and phosphate ions.

Abstract

*Corresponding Author: rmckenna@ufl.edu. Phone: (352) 392-5696..

Notes

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Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes that catalyze the reversible interconversion of CO_2 and HCO_3^- . All CAs follow a two-step ping-pong mechanism. In the hydration direction, CO_2 enters the active site where it undergoes nucleophilic attack by a metal-bound hydroxide (the metal is a zinc ion, in most cases), is converted into HCO_3^- , and then is displaced by a water molecule. In the second step of the reaction, the metal-bound water transfers a proton to the bulk solvent to regenerate the metal-bound hydroxide in readiness for the next cycle of catalysis.^{1,2}

CAs are ubiquitous and found in the deepest branches of life. Demonstrating evolutionary convergence, currently five CA classes have been identified, termed α , β , γ , δ , and ζ , with little to no structural homology. To date, all α - and β -CAs have been shown to be zinc metalloenzymes. The α -CAs have been the most extensively studied because of their role in human pathology and drug targeting; however, the other classes play a greater role in the global carbon cycle.¹ In plants, β -CAs are required for transport and maintenance of CO_2 and HCO_3^- concentrations for photosynthesis, and similarly, in prokaryotes they are involved in the maintenance of internal pH and CO_2 and HCO_3^- concentrations required for biosynthetic reactions. Unlike the α -CAs, the β -CAs exhibit much greater phylogenetic diversity.^{3,4} Their importance in prokaryotic biology can be deduced from their widespread presence in metabolically diverse species.⁴ The β -CAs play an essential role in facilitating aerobic growth of microbes at low partial pressures of CO_2 by providing endogenous HCO_3^- .^{5–10} Additionally, β -CAs are involved in multiple roles such as cyanate degradation, host colonization, host survival, and growth in different organisms.^{11–13} Although β -CAs are essential for growth of microbes such as *Escherichia coli*,⁸ *Corynebacterium glutamicum*,⁹ *Saccharomyces cerevisiae*,¹⁴ and *Helicobacter pylori*,¹⁵ their full physiological role in the biosphere is still to be discovered.¹⁶

Pseudomonas aeruginosa is a widely distributed environmental pathogenic bacterium that can cause diseases in animals, including humans. Especially in immune-compromised or otherwise susceptible hosts (patients with HIV, cancer, or burns), it can cause a severe infection of the heart, urinary tract, lungs, and wounds.^{17,18} It is also one of the major pathogenic bacteria that cause nosocomial infections.¹⁹ *P. aeruginosa* is found in a wide variety of habitats, so it has evolved a mechanism for thriving in environments with different concentrations of CO_2 . This adaptation is particularly helpful for the bacteria when they infect lungs, which have ~200-fold higher concentrations of CO_2 as compared to

atmospheric levels (400 ppm);²⁰ the solubility of CO₂ in water at 298 K is 55 mM.²¹ Because *P. aeruginosa* is resistant to most available antibiotics,¹⁹ β -CAs also offer a new target for antibiotic development. Lotlikar et al. have identified three genes encoding three β -CAs (psCA1–3) in *P. aeruginosa* PAO1.²²

Most CAs are extremely fast enzymes that function at a rate that approaches the diffusion-controlled limit ($k_{\text{cat}}/K_M \sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$) and follow Michaelis–Menten kinetics. The overall fold and oligomeric state of the α - and β -CAs are very different. The α -CAs are mainly monomers (there are a few reported cases of dimers, e.g., hCAs IX and XII, but both of these can function as monomers) with an extended 10-strand twisted β -sheet, flanked by six or more α -helices.^{2,23} In contrast, the β -CAs are only functional as dimers or larger oligomeric states and have more compact structures: a β -sheet core composed of four or five strands, and four or more α -helices surrounding this core. Interestingly, the active sites of α - and β -CAs do exhibit certain architectural similarities. In both classes, the active sites are clearly divided into two regions, hydrophobic and hydrophilic (Figure 1A,B). In the α -CAs, the active site zinc is located at the base of a 15 Å deep conical cleft, coordinated by three histidines, and highly solvated (20 ordered waters in hCA II), whereas the β -CAs have a much shallower (<10 Å) active site, constructed by dimer formation; in an active configuration, the zinc is coordinated by two cysteines and a histidine and sparsely solvated (four ordered waters in psCA3) (Figure 1C,D). This implies that the mechanism of proton transfer is most likely different between the two CA classes. In α -CAs, this function is conducted by a well-ordered network of water molecules and a histidine proton shuttle residue (His64 in hCAII) (Figure 1C).²⁴ In contrast, site-directed mutagenesis on β -CAs has led to the widely accepted view that a combination of residues are responsible for proton shuttling, and the identity of the residues differs among β -CAs.^{25–27} However, a unique arrangement of partially conserved amino acid residues in the active site (Asp-Arg dyad, for example) is thought to play an important role in a pH-induced catalytic switch in the opening and closing of the active site, by displacing a zinc-bound solvent molecule (ZBS) (Figure 1D). The ZBS, which can be either OH[−] or H₂O (depending on the pH of the system),¹⁶ is the fourth ligand of the tetrahedrally coordinated zinc for both α - and β -CAs in an active state.

As the CO₂ substrate has no dipole moment, it is expected to bind weakly in the active site of these enzymes.²⁸ The dissociation constant of CO₂ measured for hCA II has been calculated to be 100 mM. This makes capture of the substrate technically difficult; in fact, until this study, only hCA II has been determined in complex with CO₂, using CO₂-pressurized cryo-cooled crystallography.^{28,29}

Here we present the first structure of CO₂ bound to a β -CA, psCA3, which has allowed a comparison of CO₂ binding in two structurally disparate CAs that have undergone convergent evolution to perform the same enzymatic reaction.

EXPERIMENTAL PROCEDURES

Expression, Purification, and Crystallization of psCA3

Cloning, expression, and purification were conducted as described previously.²² The recombinant gene PA4676 was expressed in *E. coli* BL21(DE3) cells with a His tag. The culture was grown at 37 °C in the presence of ampicillin (100 µg/mL) until it reached an OD₆₀₀ of 1.0 and thereafter induced overnight with isopropyl β-D-1-thiogalactopyranoside (IPTG) (100 µg/mL) for protein expression. Cells were resuspended in lysis buffer [20 mM Tris, 5 mM imidazole, and 150 mM NaCl (pH 7.9)] and lysed by sonication. Protein lysate was collected after centrifuging the lysed cells at 16000 rpm for 50 min and purified using His-tag column chromatography using a Sepharose column. Nonspecifically bound proteins were washed off the column using lysis buffer and wash buffer (lysis buffer with 60 mM imidazole); finally, the protein was eluted with elution buffer (lysis buffer with 300 mM imidazole). The eluted protein was then dialyzed [in 20 mM Tris (pH 8.3)] to remove imidazole and thereafter concentrated to 10 mg/mL using a 10 kDa filter. The crystallization condition of psCA3 crystals was optimized using the hanging-drop vapor diffusion technique; 500 µL of a reservoir solution containing 50% polyethylene glycol 200 and 0.1 M Tris-HCl (pH 7.0) was placed in vapor equilibration with a drop size of 5 µL of a reservoir solution and 5 µL of psCA3 protein. The psCA3 crystals were crystallized after 6 days and grew to approximate dimensions of 400 µm × 150 µm × 150 µm.

CO₂ Binding

In order to be trapped as a substrate, CO₂ was used as a pressurizing agent during the cryo-cooling process as described below. Using the capillary shielding method,³⁰ psCA3 crystals were picked and soaked into 20 mM Tris-HCl (pH 7), 10% PEG 200, and a 10% glycerol cryoprotectant solution and inserted into one end of a capillary tube [inside diameter of 991 µm, wall of 25.4 µm (Advanced Polymers, Salem, NH)] to prevent crystal dehydration. The other end of the capillary tube was then filled with 4 µL of cryoprotectant to form a reservoir without touching the crystal. The cryoloop assembly was loaded into the bottom of a high-pressure tube,³¹ where the crystals were pressurized with 9 bar of CO₂ for 10 min. The bottom of the pressure tubes were then immersed into liquid nitrogen and the crystals were cryo-cooled for about 2 min. During this time, the gas pressure was observed to drop below 1 bar as the CO₂ solidified.²⁸ The cooled crystals were then recovered from the high-pressure tubes in liquid nitrogen and transferred into a dewar for storage before data collection.³¹

X-ray Data Collection and Processing

A total of 360 X-ray diffraction image frames were collected at Cornell High Energy Synchrotron Source (CHESS), beamline F1, at a wavelength of 0.978 Å. Data were collected using the oscillation method in intervals of 1° on an ADSC Quantum 270 CCD detector (Area Detector Systems Corp.), with a crystal to detector distance of 195 mm. Indexing, integration, and scaling of X-ray diffraction data were performed using *HKL2000*.³² The data were scaled to a resolution of 1.6 Å, with an overall completeness and *R*_{sym} of 99.3 and 8.0%, respectively (Table 1).

Molecular Replacement and Structure Determination

Starting phases were calculated using molecular replacement from Protein Data Bank (PDB) entry 4RXY³³ with waters removed. The PHENIX package³⁴ was used for refinement. A randomly selected 5% of the unique reflections was excluded from the refinement data set for the purpose of R_{free} calculations,³⁵ and refinements were alternated with manual refitting of the model in *COOT*.³⁶

RESULTS

Protein psCA3 crystallized in space group *I*222, with unit cell dimensions of 71.8, 78.1, and 87.7 Å. The data were phased using coordinates from the recently determined psCA3 structure (PDB entry 4RXY³³). The crystallographic asymmetric unit contains a psCA3 dimer. The structure was refined to a resolution of 1.6 Å, with R_{cryst} and R_{free} values of 0.17 and 0.19, respectively; the first two residues were disordered. The crystallographic data collection and refinement statistics are listed in Table 1, and the coordinates and structure factors have been deposited as Protein Data Bank entry 5BQ1. After the initial refinement of the structure, a difference ($F_o - F_c$) map clearly showed electron density corresponding to three CO₂ binding locations in the psCA3 dimer. As expected, two were positioned in each active site, while the third appeared unexpectedly at the dimeric interface, sandwiched between the two monomers (Figure 2).

Active Site CO₂ Binding Site

CO₂ was observed bound in the active site of each of the psCA3 monomers. As seen in the case of hCA II, the CO₂ displaced a water, termed “deep water” that in the unbound state lies in the hydrophobic pocket and is stabilized by a short strong hydrogen bond (~2.6 Å) with the ZBS28 (Figure 3A). The CO₂ resides within the hydrophobic cleft formed by Val66, Gly102, Gly103, Phe61', Phe83', Val87', and Leu88' (from the second monomer). The CO₂ proximal oxygen is 2.8 Å from the ZBS, with an O-ZBS-Zn angle of 113°, thereby orienting the CO₂ toward the zinc. In addition, the CO₂ is further stabilized by H-bonds with the side chain oxygen of Gln33' from the adjacent monomer (3.4 Å), and the backbone amide N of Gly103 (3.3 Å) (Figure 3B).

Dimer Interface CO₂ Binding Site

Unexpectedly, a third bound CO₂ was observed buried in the dimer interface, sandwiched among symmetry-paired residues Ala49, Val62, and Arg64. The oxygens of CO₂ are stabilized by two paired Hbonds with the side chains of Arg64 (from each monomer), with additional stabilization by the hydrophobic residues of Ala49 and Val62 (Figure 4). On the basis of only structural observations, the dimer interface CO₂ would appear to be more stable than the active site CO₂, as it is completely buried with more stabilizing interactions (in terms of hydrogen bonds and buried surface area) and has a refined B factor of 22.4 Å² compared to a value of 40.6 Å² for the active site-bound CO₂ (Table 1).

Overall Structure

Superposition of the psCA3 structures in the unbound state (PDB entry 4RXY³³) onto the complex with CO₂ (reported here) showed a main chain rmsd of ~0.3 Å, implying very little

structural movement upon substrate binding. However, slight structural perturbations in the side chains of Val66 and Phe81' in the active site were observed. Val66 moved ~ 0.5 Å away from the catalytic zinc, possibly because of repulsion from the electronegative oxygens of CO₂, and Phe81' moved ~ 0.7 Å toward the active site, because of attractive hydrophobic and van der Waals forces on the carbon atom in CO₂ (Figure 5).

DISCUSSION

This work describes the first structure of a β -CA in complex with its substrate CO₂, generated by pressurizing a crystal of psCA3 at room temperature with >5 atm of CO₂, and flash-cooling it in liquid nitrogen, as described previously by our group for the α -CA, hCA II.²⁸ The reason this method is able to capture the substrate without catalysis is that as the crystal is exposed to CO₂, the pH is reduced to <6.0 and the ZBS (which would be OH⁻ for the catalysis to occur in the hydration direction) gains a proton and becomes a water molecule. As a result of this, CA can no longer perform the CO₂ to HCO₃⁻ forward reaction. Thus, the CO₂ is stably bound in the active site (Figures 2 and 3).

At first, this seems like a reasonable and simple explanation for the observed CO₂ binding and is the argument used when CO₂ was first captured in hCA II,²⁸ but the β -CAs class is further divided into two types, based on the structural organization of their active site configuration and catalytic pH range. Type I is active over a broad pH range, while type II is active only for an alkaline pH range. With type I, the zinc is coordinated by three residues and the ZBS [an open active site, over a large pH range (Figure 1D)], whereas in type II, the zinc is in an open state at an alkaline pH, but coordinated by four residues (Asp from the Asp-Arg dyad, in a closed state), at low pH.^{37,38}

The type II β CAs have been shown to “switch” from an active (R) to an inactive (T) state at lower pH.¹⁸ Interestingly, the β -CA psCA3 used in this study has been previously shown to be type II; hence, at the pH of this study, the Asp-Arg dyad should have switched its conformation to the closed state and displaced the ZBS, thereby eliminating the CO₂ binding site.²² However, in unpublished studies, we soaked crystals of psCA3 (initially grown at pH 7) in solutions at pH <6 , and they never underwent the pH-induced structural switch. Therefore, it can be argued that crystal packing constraints of psCA3 may inhibit this conformational switch; in another packing configuration, the Asp-Arg dyad might be induced to transform to the T state. These studies are now ongoing. A direct H-bond between CO₂ and Gln33' is particularly interesting as it supports the previously reported and hypothesized importance of Gln33' (or its equivalent in β -CAs from other organisms) in stabilization of the transition state during the conversion of CO₂ to HCO₃⁻. This finding is a direct corroboration of the proposed role of Gln33' as suggested by site-directed mutagenesis.³⁹

Despite being disparate and evolutionarily distinct structures, the CO₂ active binding sites for psCA3 and hCA II are remarkably similar. In both, the CO₂ is located in a hydrophobic pocket defined by five residues: in psCA3, the residues are Phe61', Val66', Phe83', Val87', and Leu88', while in hCA II, they are Val121, Val135, Leu141, Val143, and Trp209, with some differences in amino acid type and spatial arrangement. Both have an aromatic amino

acid (Phe83' in psCA3 and Trp209 in hCA II) as the assembly core of the hydrophobic cluster cavity. In addition, even though the active site of psCA3 is much shallower and has fewer waters than hCA II, it is noteworthy that the CO₂ buries 129.7 Å² (~87% of its total surface area), comparable to that in hCA II (128.1 Å²). These surface area calculations were performed using PDBePISA⁴⁰ (Figure 6). Also, relative to the zinc, the orientation of the CO₂ is almost identical, with the CO₂ central carbon aligned and distanced 2.8 Å from the ZBS, presumably in readiness for nucleophilic attack. The ZBS is stabilized by a H-bond with Thr199 in HCA II and Asp44 in psCA3 (Figure 6).

Unexpected was the discovery of an additional CO₂ binding site at the dimer interface of psCA3, where the CO₂ is completely surface “inaccessible” and stabilized by four H-bonds. Of course, in the case of hCA II, which functions as a monomer, this interface does not exist. Hence, this dimer binding site begs the question of how the CO₂ binds and whether it has implications for enzyme function, as the active site only forms when psCA3 is a dimer (Figure 1B,D).

It is known that β-CAs, unlike α-CAs, exhibit allostery.¹⁶ Previous studies have shown this interface pocket to be a promiscuous site for a variety of ligands, including bicarbonate, sulfate, and phosphate ions. Also, the existence of a noncatalytic bicarbonate ion binding site in *Haemophilus influenzae* β-CA (HICA, PDB entry 2A8D³⁸) and *E. coli* β-CA (ECCA, PDB entry 1I6P⁴¹) that is characterized by a Trp39-Arg64-Tyr181 (HICA numbering) triad has been identified. In these type II β-CAs, binding of a bicarbonate ion in this allosteric site relocates the side chain of Val47 (HICA numbering) and causes the reorganization of the 44–48 loop; the Asp44-Arg46 dyad is also disrupted. Interestingly, psCA3 also contains this Trp39-Arg64-Tyr182 triad (psCA3 numbering). Though bicarbonate was not found bound in this potential allosteric binding site in psCA3, the CO₂ molecule at the dimer interface was only ~6 Å from this site. The CO₂ at this site is completely buried, and the only way this molecule could have entered this site seems to be due to the protein dynamics and molecular “breathing”. Possibly, under alkaline pH conditions, CO₂ would be converted into HCO₃[−] and bind in the allosteric site; it might then adopt the closed active site configuration as its 44–48 loop underwent reorganization. In addition to HCO₃[−], other ions such as sulfate,³⁸ phosphate,³⁹ and chloride⁴² have also been reported to be bound at this site. Hence, we propose that this site may have a biochemical, structural, and physiological role to play and is not just an experimental artifact caused by exposure to pressurized CO₂, but it could also just be a highly promiscuous site that can potentially bind many different small molecules. Further experimental studies would be needed to answer this question.

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ABBREVIATIONS

CA	carbonic anhydrase
ZBS	zinc-bound solvent
PDB	Protein Data Bank
rmsd	root-mean-square deviation

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Figure 1.

Surface representation of (A) hCA II and (B) psCA3. Beige and green regions represent hydrophilic and hydrophobic residues of the active site, respectively. Stick representations of the active site for (C) hCA II and (D) psCA3. The active site zinc is depicted as a magenta sphere (only one active site of the psCA3 dimer is visible in this view). For all subsequent figures, residues will be as labeled, ordered waters depicted as red spheres, zinc depicted as a magenta sphere, H-bonds represented by red dashes, and distances given in angstroms.

Figure 2. Cartoon representation of the functional dimeric form of psCA3. The green mesh is the difference ($F_o - F_c$) electron density for the two CO₂ binding sites, contoured at 2.0σ . The right panels show details of the CO₂ binding sites. The blue mesh is the refined ($2F_o - F_c$) electron density after modeling building and refinement, contoured at 1.6σ .

Figure 3. Hydrogen bond network in the psCA3 active site: (A) open (without CO₂) and (B) with CO₂. Residues are as labeled. Beige and green regions represent hydrophilic and hydrophobic residues, respectively. Note the displacement of deep water upon CO₂ binding.

Figure 4. Secondary binding site for another molecule of CO₂ shown at the dimeric interface as (A) a cartoon and (B) sticks (close-up). The two monomers are colored yellow and cyan.

Figure 5. Superposition of unbound psCA3 (gray) and CO₂-bound (green) represented as a (A) cartoon and (B) close-up sticks, showing the slight perturbation in two active site residues.

Figure 6. Cross-eyed stereoview of CO₂ in the active site of (A) hCA II and (B) psCA3. Residues are as labeled.

Table 1**Crystallographic Data Collection and Refinement Statistics (PDB entry 5BQ1)**

Data Collection	
temperature (K)	100
wavelength (Å)	0.978
space group	<i>I</i> 222
unit cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	71.8, 78.1, 87.7
no. of reflections (theoretical, measured)	32819, 32530
resolution (Å)	29.1–1.6 (1.65–1.60) ^a
R_{sym}^b	0.08 (0.50)
<i>I</i> /σ(<i>I</i>)	72.8 (6.1)
completeness (%)	99.3 (98.3)
redundancy	14.1 (10.9)
Final Model	
R_{cryst}^c (%)	17.3 (21.4)
R_{free}^d (%)	19.9 (22.3)
no. of atoms ^e (protein, ligand, ^f water)	1675, 3 (2), 103
rmsd [bond lengths (Å), angles (deg)]	0.006, 0.99
Ramachandran statistics (%) (favored, allowed, outliers)	98.5, 1.5, 0.0
average B factor (Å ²) [main chain, side chain, CO ₂ (active site), CO ₂ (interface), solvent]	29.4, 34.8, 40.6, 22.4, 35.5

^aValues in parentheses represent data for the highest-resolution bin.

$$^b R_{\text{sym}} = \Sigma(|I - \langle I \rangle|) / \Sigma \langle I \rangle.$$

$$^c R_{\text{cryst}} = (\Sigma |F_o| - |F_c| / \Sigma |F_o|) \times 100.$$

^d R_{free} is calculated in same manner as R_{cryst} , except that it uses 5% of the reflection data omitted from refinement.

^eIncludes alternate conformations.

^fThe value in parentheses represents the total number of ligands bound in the whole structure.