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Activity and anion inhibition studies of the α-carbonic anhydrase from *Thiomicrospira crunogena XCL-2* Gammaproteobacterium

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ABSTRACT

Thiomicrospira crunogena XCL-2 expresses an α-carbonic anhydrase (TcruCA). Sequence alignments reveal that TcruCA displays a high sequence identity (>30%) relative to other α-CAs. This includes three conserved histidines that coordinate the active site zinc, a histidine proton shuttling residue, and opposing hydrophilic and hydrophobic sides that line the active site. The catalytic efficiency of TcruCA is considered moderate relative to other α-CAs ($k_{cat}/K_M = 1.1 \times 10^7\ M^{-1}\ s^{-1}$), being a factor of ten less efficient than the most active α-CAs. TcruCA is also inhibited by anions with Cl^-, Br^-, and I^-, all showing K_i values in the millimolar range (53–361 mM). Hydrogen sulfide (HS^-) revealed the highest affinity for TcruCA with a K_i of 1.1 μM. It is predicted that inhibition of TcruCA by HS^- (an anion commonly found in the environment where *Thiomicrospira crunogena* is located) is a way for *Thiomicrospira crunogena* to regulate its carbon-concentrating mechanism (CCM) and thus the organism's metabolic functions. Results from this study provide preliminary insights into the role of TcruCA in the general metabolism of *Thiomicrospira crunogena*.

Carbonic anhydrases (CAs) are mainly zinc metalloenzymes that catalyze the reversible interconversion of CO_2 and water to bicarbonate and a proton.[1–3] CAs are essential enzymes to nearly all organisms as they play significant roles in photosynthesis, CO_2 fixation and transport in plants and bacteria and are involved in physiological processes such as respiration, pH regulation, bone resorption, and the formation of gastric acid and aqueous humor in animals.[3–9] As such, CAs have found importance in terms of drug targeting, clinical medicine, understanding plant physiology, and more recently as bio-catalytic agents for industrial CO_2 sequestration.[4–7] There are five known evolutionarily distinct classes of CA that are classified as α, β, γ, δ, and ζ.[8,9] The α-CAs are found mostly in mammals and are the most catalytically efficient, β-CAs are found in several plants, prokaryotes, and fungi, and γ-CAs have been isolated in several strains of bacteria and archaea that are often found in extreme environments.[9] The δ- and ζ-CAs are typically found in diatoms and types of archaea, and despite having significant sequence disparity between other classes of CAs, they have apparent structural homology.[10,11]

Many bacteria have evolved to express more than one class of CA with the α-, β-, and γ-class typically observed in the same organism.[4] Many of these bacterial CAs have displayed importance in terms of understanding microbial pathogenesis and metabolism, and also as a means to isolate thermo- and pH stable CAs that can be utilized as bio-catalytic agents for industrial CO_2 sequestration.[4,12,13] Such organisms include *Vibrio cholera*,[14] which is responsible for causing cholera in humans, and those found in hot springs or hydrothermal vents such as *Sulfurihydrogenibium yellowstonense YO3AOP1*,[15] *Sulfurihydrogenibium azorense*,[16] and *Thiomicrospira crunogena XCL-2*,[12] all observed to possess thermo- and pH stable CAs that can be utilized for industrial CO_2 sequestration.

Thiomicrospira crunogena XCL-2 is a sulfur-oxidizing gammaproteobacterium that is found in deep-sea hydrothermal vents and expresses four CAs from three evolutionarily distinct classes; a periplasmic α-CA, a cytoplasmic β-CA, a carboxysomal β-CA, and a γ-CA-like protein.[17,18] *Thiomicrospira crunogena* utilizes reduced sulfur compounds as an energy source for carbon fixation and cellular maintainence.[19] The deep-sea hydrothermal vents where *Thiomicrospira crunogena* is found are characterized by a harsh yet dynamic environment that alternates from bottom seawater (2 °C, normoxic, CO_2 concentration = ~0.02 mM, and pH of ~8 to

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dilute hydrothermal fluid (2–35 °C anoxic, highly reduced, CO_2 concentration = >1 mM, and pH of 5–8), temporally and spatially limiting the availability of nutrients accessible to these organisms.[20,21] *Thiomicrospira crunogena* is equipped with a carbon-concentrating mechanism (CCM) that allows it to grow rapidly during bicarbonate and CO_2 scarcity in the rapidly fluctuating environment, as the cell's affinity for these compounds increase during carbon limitation.[19] Previous studies in cell culture have shown that CO_2 and bicarbonate transport plays an active role in the CCM.[17] Therefore, the expression of four CAs suggests a role in bicarbonate and CO_2 transport as part of the CCM.

Recently our group has determined the crystal structure of the α-CA from the gammaproteobacterium, *Thiomicrospira crunogena XCL-2* (TcruCA; PDB ID: 4XZ5[12]). Similar to other bacterial CAs, TcruCA has a high T_M (72 °C) compared to human CA II (hCA II; 56 °C), poising its potential as a bio-catalyst for industrial CO_2 sequestration.[12] The TcruCA monomer displays the signature secondary structure of an α-CA fold with helical and loop structures present toward the surface, and a conical active site cavity comprised of mostly β-strands surrounding a tetrahedrally coordinated (by three histidine residues) active site zinc ion (Fig. 1). The overall characteristics of the active site of TcruCA exhibits defined hydrophobic and hydrophilic pockets similar to most other α-CAs (Fig. 1).[22] This is not surprising as TcruCA shows >30% amino acid sequence identity compared to other human and bacterial α-CAs (Table 1). In addition the TcruCA monomer contains both an intramolecular disulfide bond and a compact overall structure that is postulated to contribute to its increased thermostability compared with hCA II.[12]

The kinetic rates of purified TcruCA[32] were obtained by measurement of the depletion of ^{18}O from species of CO_2 at chemical equilibrium by membrane inlet mass spectrometry as described by Tu, et al.[23,24,33] TcruCA was determined to have a k_{cat}/K_M of 1.1×10^7 M^{-1} s^{-1}, (pH 7.5, 25 °C) which is comparable to the other

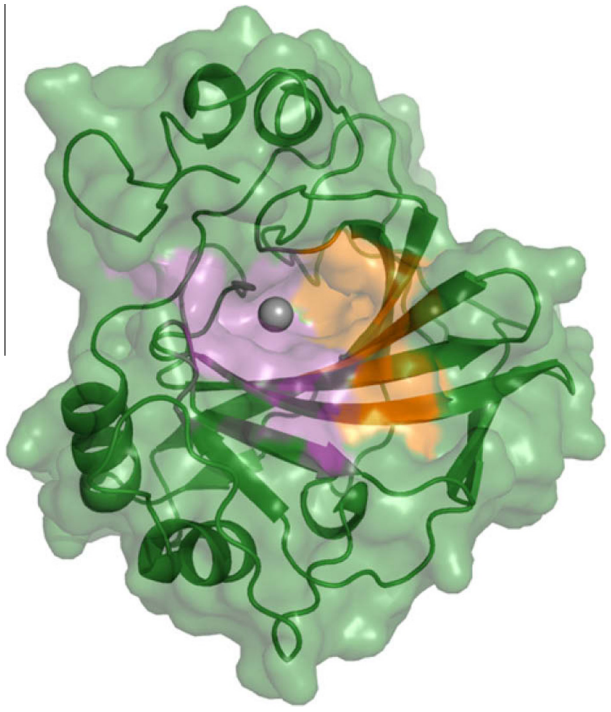

Figure 1. Ribbon and surface (transparent) diagram of the TcCA homodimer. Zinc depicted as a grey sphere. Hydrophobic (orange) and hydrophilic (purple) regions of the TcCA active site are highlighted and show similar arrangement compared to those of hCA II.

Table 1
Comparison of catalytic efficiency and sequence identity of TcCA to other CA isozymes

Isozyme	Activity level	k_{cat}/K_M ($M^{-1} \times s^{-1}$)	Amino acid ID (%)	Ref. #
TcCA	Moderate	1.1×10^7	–	–
SspCA	Very high	1.1×10^8	41.0	21
SazCA	Highest	3.5×10^8	43.4	22
VchCA	Moderate	7.0×10^7	41.1	20
hCA I	Moderate	5.0×10^7	32.4	20
hCA II	Very high	1.2×10^8	32.5	18

CA isozymes (both bacterial and human) and is classified to have moderate catalytic efficiency relative to the CAs with the highest activity (Table 2). Primary sequence alignments (Fig. 2) show that this is expected via conservation of residues that make up the α-CA active site architecture. This includes zinc-coordinating histidines (94, 96, 119; hCA II numbering is used throughout this manuscript), the proton shuttling residue His64, and residues found in the hydrophilic side of the active site that are thought to be responsible for substrate/product entry/exit (positions 7, 62, 67, 92, and 198–202: Fig. 2).[22] The catalytic efficiency of TcruCA is comparable to human CA I (hCA I) and the α-CA found in *Vibrio cholera* (VchCA) (Table 2). In contrast, the catalytic activity of TcruCA is at least an order of magnitude lower compared to the CAs considered to have very high catalytic activity. This includes hCA II, the α-CAs from *Sulfurihydrogenibium yellowstonense YO3AOP1* (SspCA) and *Sulfurihydrogenibium azorense* (SazCA), which has the highest known CA catalytic efficiency (Table 2).[16] Interestingly there appears to be no correlation between catalytic efficiency of TcruCA and primary amino acid sequence identity between the other α-CAs (Table 1). This may be due to significant differences in amino acid positions and compositions in the TcruCA active site compared to the other more active α-CAs, however a more in depth structural analysis is needed to determine this. In addition, we have recently speculated that alterations in the loop region found between residues 230 and 240 might contribute to the altered catalytic efficiency between the TcruCA and the other CAs due to recent work performed by Boone et al. that suggests the importance of this loop region in modulating CA activity.[12,25]

TcruCA activity was assayed in the presence of various anions that are indigenous to the hydrothermal vent environment which include Cl^-, Br^-, HS^-, and I^-.[26] We predict that certain anions in the environment can act as regulators of the *Thiomicrospira crunogena XCL-2* CCM by modulating TcruCA catalytic activity. Inhibition constants of iodide, chloride, and bromide were obtained for TcruCA using the ^{18}O-exchange mass spectroscopy[12,24,33] and compared to the previously reported inhibition constants for both bacterial and human CAs (Table 2). It was shown that Cl^- and Br^- inhibited TcruCA very weakly in the high mM range (361 and 242, respectively). Compared to the other CAs, these anions had the weakest inhibition against TcruCA, except for Cl^- against hCA II, which showed a similar inhibition profile. I^- had a marginally higher affinity for TcruCA relative to Cl^- and Br^- however this anion still showed an inhibition constant in the mM range (K_i = 53; Table 2). Once again hCA II also showed a similar I^- inhibition

Table 2
Comparison of anion inhibition of TcCA to other CA isozymes

Anion	K_i (mM)					
	TcCA	SspCA[21]	SazCA[22]	VchCA[20]	hCA I[20]	hCA II
Cl^-	361	8.3	0.85	0.93	6.0	200
Br^-	242	49	0.94	28	4.0	63
HS^-	0.0011	0.58	0.38	0.074	0.0006	0.0028
I^-	53	0.86	0.87	9.73	0.3	26

Figure 2. Primary amino acid sequence alignments of TcCA, SspCA, SazCA, VchCA, hCA I and hCA II. Conserved zinc-coordinating histidines are highlighted in red. The proton shutting residues, His64 (CA II Numbering) is highlighted in green. Residues that make up regions of the hydrophilic pocket are highlighted in blue and numbered accordingly. The loop region (residues 230–240) that is predicted to be important for both CA catalysis and thermostability is highlighted in purple. All residues that are variable between CA isozymes are underlined.

profile with TcruCA, which is in contrast with SspCA, SazCA and hCA I, all of which having K_i values in the μM range (Table 2). HS^- showed the highest affinity for TcruCA with a K_i value of 1.1 μM. HS^- can be abundant in hydrothermal fluid, and is readily utilized by *Thiomicrospira crunogena* as an electron donor for its general metabolism.[27] Therefore this result suggests that HS^- may act as a regulating agent for the CCM of *Thiomicrospira crunogena* through inhibition of CA activity. As such, in the presence of CO_2-rich hydrothermal fluid there is a reduced need for CA activity (and hence activation of the CCM) since there is an abundant carbon source. Alternatively, in the presence of bottom water, the *Thiomicrospira crunogena* will engage its CCM.[19,26] Comparison of the inhibition profiles of HS^- against the other CAs indicate that it also binds within the μM range. Interestingly, HS^- shows a ~600- and ~400-fold decrease in affinity for SspCA and SazCA, respectively, compared to TcruCA. This is most likely due to the fact that both *Sulfurihydrogenibium yellowstonense YO3AOP1*, *Sulfurihydrogenibium azorense* inhabit environments considered to be more static than deep-sea hydrothermal vents and as a result, continuously maintain high concentrations of hydrogen sulfide.[28,29] Therefore, SspCA and SazCA need to be less sensitive to HS^- inhibition for both *Sulfurihydrogenibium*

yellowstonense YO3AOP1 and *Sulfurihydrogenibium azorense*, and are most likely only function in pH regulation.[13,30] Similar to TcruCA, VchCA, hCA I and hCA II remain sensitive to HS^- inhibition. In this case the sensitivity of these CAs to HS^- inhibition most likely does not infer a similar environmental adaptation similar to the aforementioned hydrothermal vent dwellings organisms. Instead the observation of these CAs being inhibited by HS^- is more useful for drug design, as hCA I, II, and VchCA are all identified drug targets.[22,14]

In conclusion we have shown that TcruCA maintains structural homology and amino acid sequence identity with other human and bacterial α-CAs which translates directly to its comparable catalytic efficiency (Table 1). Furthermore, we have shown that anions typically found in the deep-sea hydrothermal vent where *Thiomicrospira crunogena* resides, readily inhibit TcruCA. Cl^-, Br^-, and I^-, all show relatively weak inhibition of TcruCA (mid to high mM range; Table 2), which is in contrast with the other CAs except for hCA II. HS^- displayed the highest affinity for TcruCA. This result suggests that CA activity might not be essential in CO_2-rich hydrothermal fluid since there is an abundant carbon supply. Therefore the need for *Thiomicrospira crunogena* to activate its CCM is significantly reduced. Comparatively, the weak inhibition of HS^- against SspCA and SazCA, suggests that CA activity is more essential in terms of pH regulation in *Sulfurihydrogenibium yellowstonense YO3AOP1* and *Sulfurihydrogenibium azorense*. Results from this study provide further insight into the role of TcruCA in the metabolism of *Thiomicrospira crunogena* and how it survives in the harsh dynamic environment of the deep-sea hydrothermal vent.

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32. *Protein expression and purification:* TcruCA was expressed in recombinant BL21(DE3) *E. coli* cells. In brief, *E. coli* cells containing the plasmid encoding TcruCA were grown in Luria broth supplemented with 50 μg/mL kanamycin to an OD_{600} of 0.6–1.0, at which point the expression of the TcruCA was induced by addition of isopropyl β-D-1-thiogalactopyranoside (final concentration of 0.1 mg/mL) for ~4 h at 37 °C. Cells containing TcruCA were harvested and enzymatically lysed. TcruCA was purified by affinity chromatography using an agarose resin coupled to the inhibitor *p*-(aminomethyl)benzenesulfonamide (*p*-AMBS; Sigma). The protein was eluted with sodium azide, and buffer-exchanged against 50 mM Tris–HCl pH 7.8, 100 mM NaCl and concentrated using centrifugation. The final protein concentration was determined by UV/Vis spectroscopy at 280 nm, using an extinction coefficient of 42,985 M^{-1} cm^{-1}. Purity was estimated by SDS–PAGE and coomassie-blue staining.
33. *Oxygen-18 exchange kinetic analysis:* The kinetic rates of TcruCA were obtained by measurement of the depletion of ^{18}O from species of CO_2 at chemical equilibrium by membrane inlet mass spectrometry as described by Tu et al.[12,23,24] This occurs via the continuous measure of various isotopic species of CO_2 provided by CO_2 diffusing across a dissolved gas-permeable membrane, the membrane is submerged in the reaction solution and connected by glass tubing to a mass spectrometer (Extrel EXM-200).[24] The catalyzed and uncatalyzed exchange of ^{18}O between CO_2 and water at chemical equilibrium were measured in the absence of buffer at a total substrate concentration of 25 mM. The reaction solution was maintained at 25 °C, and the ionic strength of the solution was normalized at 0.2 M by the addition of Na_2SO_4. CA catalysis of isotope labeled substrate is described by the following two-step process: the first stage of catalysis is the dehydration of ^{18}O-labeled HCO_3^- (Eq. 3). During the second catalytic stage, the zinc-bound ^{18}O-labeled hydroxide is protonated, forming $H_2{}^{18}O$, which is then released into solution (Eq. 4).

$$HCOO^{18}O^- + EZnH_2O \xleftrightarrow{-H_2O} EZnHCOO^{18}O^- \leftrightarrow COO + EZn^{18}OH^- \quad (3)$$

$$H^+His64 - EZn^{18}OH \leftrightarrow His64 - EznH_2{}^{18}O \xleftrightarrow{H_2O} His64 - EZnH_2O + H_2{}^{18}O \quad (4)$$

Data processing is outlined in detail by Diaz-Torres et al.[12] Activity assays were also completed by varying concentrations of anionic inhibitors of TcruCA during data collection. Data processing and calculations of inhibition constants are outlined by Barrese et al.[31] Data were fitted to the data by using non-linear least squares methods in Enzfitter (Biosoft).