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RESEARCH ARTICLE

Interaction of anions with a newly characterized alpha carbonic anhydrase from *Halomonas* sp

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Abstract

The inhibition and characterization of the α-class carbonic anhydrase (CA, EC 4.2.1.1) from the Halomonas sp. are reported for the first time. The enzyme was purified 91-fold with a yield of 39%, and a specific activity of 600 U/mg proteins was obtained. It has an optimum pH at 7.5, an optimum ionic strength at 20 mM and an optimum temperature at 20 °C. The following anions, SCN^-, Br^-, Cl^-, I^-, N_3^-, NO_2^-, NO_3^-, CO_3^{2-}, SO_3^{2-} and SO_4^{2-} showed inhibitory effects on the hydratase activity of the enzyme. Sulfate, sulfide, azide, nitrate, nitrite and iodide exhibited the strongest inhibitory activity, in the micromolar range (K_I-s of 5.5–15.5 μM). SCN^-, Br^-, Cl^-, CO_3^{2-} were moderate inhibitors, whereas other anions showed only weak activities. Our findings indicate that these anions inhibit the Halomonas sp. CA (HmCA) enzyme in a similar manner to other α-CAs from mammals investigated earlier, but the susceptibility to various anions differs significantly between the Halomonas sp. and other organism CAs.

Keywords

Anions, carbonic anhydrase, enzyme interaction, Halomonas sp

History

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Introduction

Carbon dioxide and bicarbonate are essential components in microorganisms as well as other living organism. The required CO_2 by the cell is transported into the cell by hydration reaction depending upon the bicarbonate, carbon dioxide concentration inside and outside the cell. This transportation is performed depending upon the amount of carbon dioxide conversion into bicarbonate and occurs very frequently in the cell. The reaction of this conversion is very slow and should be speed up somehow. Carbonic anhydrase (CA, EC 4.2.1.1), one of the fastest known enzyme, catalyzes the reaction with typical catalytic rates of the different forms of this enzyme ranging between 10^4 and 10^6 reactions per second[1–3]. CAs are mainly zinc metalloenzymes that catalyze the reversible interconversion of carbon dioxide and water to bicarbonate and a proton[1–3]. These isoenzymes are thus involved in many significant processes such as photosynthesis, respiration, pH regulation, CO_2 fixation and CO_2 transport in plants and bacteria[4–7].

CAs have attracted attention in terms of drug design, clinical medicine, highlighting plant physiology and more recently as biocatalytic agents for industrial CO_2 sequestration[4–7]. There are six known evolutionarily distinct classes of CA that are classified as α, β, γ, δ, ζ and η[7]. 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[8]. 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[8].

In mammals, 16 α-CA isozymes have been described in terms of their subcellular localization, catalytic activity and susceptibility to different classes of inhibitors. These isozymes are found in various locations as membrane bound (CA IV, CA IX, CA XII and CA XIV), cytosolic (CA I, CA II, CA III, CA VII and CA XIII), mitochondrial (CAVA and CA VB) and saliva (CA VI)[1–4]. Some microorganisms (*Neisseria gonorrhoeae, Helicobacter pylori, Vibrio cholerae, Sulfurihydrogenibium yellowstonense, Sulfurihydrogenibium azorense* and *Ralstonia eutropha*) expressed CAs belonging to the α-class[9].

Halomonas is a member of halophilic proteobacteria. The member of this genus grows well in wide range of sodium chloride (5–25%). The member of this genus are Gram-negative rods and described as halotolerant or halophilic. The species of *Halomonas* have been isolated from different saline habitats, including salt lakes, saline soils and marine environments[10,11]. Recently, *Halomonas* species have been largely investigated for their potential use in biotechnology[12,13]. The production of compatible solutes, exopolysaccharides and extracellular enzymes are among their potential use in biotechnology[14,15].

Although there are studies regarding purification of CA from various tissues, no reports have been found on purification and characterization of the enzyme from *Halomonas sp*. In the present study, we purified and characterized an α-CA from *Halomonas sp*. for the first time, and investigated its kinetic properties and

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inhibitory effects of anions including SCN^-, Br^-, Cl^-, I^-, N_3^-, NO_2^-, NO_3^-, CO_3^{2-}, SO_3^{2-} and SO_4^{2-} on enzyme activity.

Experiment section

Chemicals

NaSCN, KBr, KCl, KI, NaN_3, $NaNO_2$, $NaNO_3$, Na_2CO_3, Na_2SO_3, Na_2SO_4, Sepharose-4B, protein assay reagents and 4-nitrophenyl acetate (NPA) were obtained from Sigma-Aldrich Co. (St. Louis, MO). All other chemicals were of analytical grade and obtained from Merck (Kenilworth, NJ).

Isolation and characterization data of strain owing CA activity

The microorganism was isolated from salt-affected soils in the south east Anatolian region, Turkey (Şanlıurfa). The bacterial isolate was incubated on moderate halophilic medium with salt consisting of (g/l): NaCl 81, $MgSO_4.7H_2O$ 9.6, $MgCl_3.6H_2O$ 7.0, $CaCl_2.2H_2O$ 3.6, KCl 2.0, $NaHCO_3$ 0.06, NaHBr 0.026, agar 20[16]. The isolate was screened for its Gram reaction and spore formation as described previously[17]. This was followed by the determination of the optimal growth pH (5, 7, 9 and 11) in the 10% MH agar medium and salt concentrations (5, 10, 15 and 20) in nutrient agar. The motility of the isolates was determined with wet mounts. Other test that were used for the characterization of the isolate was the determination of catalase, oxidase, hydrolysis of casein, gelatin, esculin, Tween-80 and starch, nitrate and nitrite reduction and acid from carbohydrates (glucose, fructose, lactose, mannitol, maltose, mannose, inositol, sorbitol, sucrose and galactose)[18]. Genomic DNA extraction of the isolate was performed with DNA extraction kit (Qiagen, Venlo, Netherlands). The 16S rRNA isolates were amplified by PCR using universal primers 27f (CATCTCAGTGCAACTAAA) and 1492r (CAGGAAACAGCTATGAC)[19]. According to the conventional tests, the strain is Gram-negative, short rods, non-motile, catalase and oxidase-negative, nitrate reduction positive. The strain grew optimally in media containing 7.5–10% NaCl at pH 7.0–11.0. The strain was negative for hydrolysis of starch, casein, gelatin, and aesculin but positive for the hydrolysis Tween-80. The strain produces acid from D-fructose, α-D-glucose, myo-inositol, maltose, D-mannose, sucrose, lactose and D-galactose. The strain has an antibiotic susceptibility to kanamycin, tetracycline and neomycin. Based on the conventional and molecular techniques, the bacterial isolate was identified and characterized as *Halomonas sp*. (Accession number: KR706322).

Purification of CA from *Halomonas sp.* by affinity chromatography

Halomonas sp. CA (*Hm*CA) was purified from bacteria grown in the liquid nutrient medium. Bacteria culture cells mixed lysis buffer and later samples were centrifuged at 10 000 rpm for 30 min, and plasma and precipitate were removed. The pH of the homogenate was adjusted to 7.5 with solid Tris. The homogenate was applied to the prepared sepharose-4B-L-tyrosine-sulfanilamide affinity column which had been equilibrated with 20 mM Tris-HCl/100 mM Na_2SO_4 (pH 7.5)[20]. The affinity gel was washed with 20 mM Tris-HCl/25 mM Na_2SO_4 (pH 7.5), and the enzyme was eluted by 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3). All procedures were performed at 4 °C.

Esterase activity assay

CA esterase activity was assayed by following the change in absorbance at 348 nm of 4-NPA to 4-nitrophenylate ion over a period of 3 min at 25 °C using a spectrophotometer according to the method described by Verpoorte et al.[21] The enzymatic reaction, in a total volume of 3.0 ml, contained 1.4 ml 0.05 M Tris-SO_4 buffer (pH 8.0), 1 ml 3 mM 4-NPA, 0.5 ml H_2O and 0.1 ml enzyme solution.

Protein determination

Protein quantity was determined spectrophotometrically at 595 nm during the purification steps according to the Bradford method, using bovine serum albumin as the standard[22].

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzyme. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure[23]. The electrophoretic pattern was photographed (see Figure 1).

In vitro effects of anions

SCN^-, Br^-, Cl^-, I^-, N_3^-, NO_2^-, NO_3^-, CO_3^{2-}, SO_3^{2-} and SO_4^{2-} anions were tested. Five different volumes (0.1, 0.2, 0.3, 0.4 and 0.5 ml) of anion solutions at a constant concentration were added to *Hm*CA activity determination medium (total volume: 4.2 ml). CA activities with anions were assayed by following the hydration of CO_2[24]. Activity % values of *Hm*CA for five different concentrations of each anion were drawn by using regression analysis graphs on a computer. *Hm*CA activity without an anion was accepted as 100% activity. For the anions having an inhibition effect, the inhibitor concentrations causing up to 50% inhibition (IC_{50} values) were determined from the graphs[25]. The curve-fitting algorithm allowed us to obtain the IC_{50} values, working at the lowest concentration of substrate of 0.15 mM, from which K_I values were calculated by using the Chenge–Prusoff equation[26].

Results and discussion

Purification and characterization of CA from *Halomonas sp.*

Bacteria encode enzymes belonging to the α-, β- and γ-CA classes[9]. They contain zinc ion (Zn^{2+}) in their active site, coordinated by three histidine residues and a water molecule/hydroxide ion (in the α- and γ-CAs) or by two Cys and one His residues (in the β class), with the fourth ligand being a water molecule/hydroxide ion. Some of the catalytically active α-CAs can also catalyze the hydrolysis of esters, for example, 4-NPA (and other hydrolytic reactions as well)[9,27].

In this study, we have purified and characterized the *Hm*CA enzyme. And then, anion inhibition of the *Hm*CA enzyme investigated. The purification procedure was carried out by affinity chromatography on sepharose-4B-tyrosine-sulfanilamide, as this zinc enzyme has a high affinity for sulfonamides, which bind in deprotonated to the metal ion from the enzyme active site[2].

The enzyme was purified up to 91-fold with a recovery ratio of 39% compared to homogenate (Table 1). After the sample had completely passed through, the column was washed with 20 mM Tris-HCl/100 mM Na_2SO_4 buffer whose pH was 7.5. During washing, absorbance of fractions were measured at 280 and 348 nm by means of the spectrometer.

These values showed that some proteins, bound to the affinity material, have been removed from the column by the washing solutions. Then, the enzyme was eluted with 1 M NaCl/25 mM Na_2HPO_4 pH 7.0. At the end of the last step, a highly purified enzyme was obtained exhibiting a single band on SDS-PAGE (Figure 1).

Figure 1. SDS-PAGE photograph. Lane 1 and 2, standard proteins: bovine albumin (66 kDa), chicken ovalbumin (45 kDa) and bovine carbonic anhydrase (29 kDa). Lane 3, affinity chromatography purified *Hm*CA enzyme.

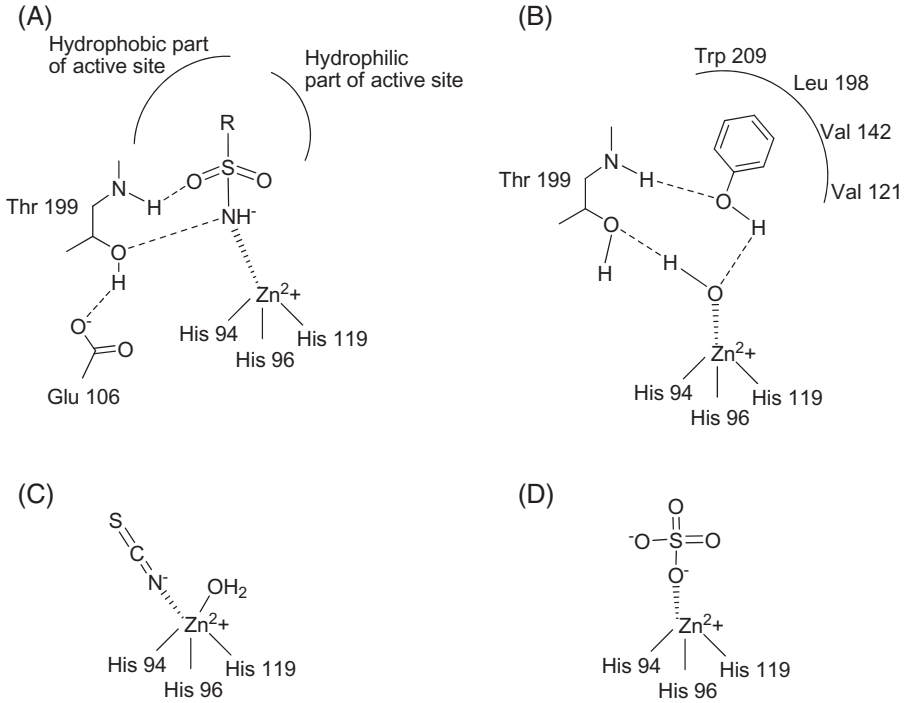

Table 1. Summary of the carbonic anhydrase enzyme purification steps in *Halomonas sp*.

Purification steps	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Hemolysate	87	15	13.2	198	1305	6.59	100	1
Affinity column chromatography	102	5	0.17	0.85	510	600	39	91

We used a single step chromatographic technique; employing sepharose-4B-tyrosine-sulfanilamide affinity chromatography which strongly binds α-CAs[28–31]. The optimum pH for the purification of the enzyme was determined to be 7.5; the optimum temperature of 20 °C; optimum ionic strength 20 mM. The stable pH profile of the enzyme was determined at four different pHs in 50 mM Tris-HCl and five different pHs in 50 mM K-phosphate buffer. The enzyme maintained 83% of the maximum catalytic activity at the end of 14 d in 20 mM Tris-HCl buffer (pH 7.5), proving it to be stable, similar to many other α-CAs[28–31]. To determine the native molecular weight of the enzyme, SDS-PAGE was carried out.

The molecular weight was determined to be 32 kDa. Similar results have been observed for the enzyme from different sources. For example, human erythrocyte CA is 29 kDa,[28–31] teleost fish *Dicentrarchus labrax* CA is 29 kDa[5] and rainbow trout gill CA is 29 kDa[28–31]. The molecular weight was proved to be 32 kDa by SDS-PAGE (Figure 1).

α-CAs have some catalytic versatility, acting also as esterases, phosphatases[19,32,33]. Thus, we have investigated the esterase activity of the *Hm*CA enzyme with 4-NPA as a substrate. This enzyme has thus comparable esterase activity with the human isoforms hCA I and II, investigated earlier[19,32,33].

CA inhibition

It is well-known that metal-complexing anions bind to CAs, representing a rather well investigated class of inhibitors[34]. Most anions coordinate directly to the metal ion from the enzyme active site, replacing the coordinated water molecule/hydroxide ion, or add to the coordination sphere, leading to trigonal bipyramidal geometries of the Zn (II) ion[1–3,34]. In our study, we examined the inhibitory activities of anions on the alpha class CA from *Halomonas sp.*, by assaying the inhibition of the CO_2 hydratase activity mentioned above.

Inhibition data of the current paper and previous studies were discussed in Table 2. As seen from data of Table 2 and Figure 2, all investigated anions show inhibitory activity, but sulfate was the strongest one, with K_I value of 5.5 μM, which is similar to that of the (coral *Stylophora pistillata* CA) STPCA[35], but quite different from α-isozyme hCA I, II, β-isozyme (*Methanobacterium Thermoautotrophicum*) Cab CA and γ-isozyme (*Methanosarcina thermophila*) Zn-Cam CA reported earlier[36]. Iodide and sulfite ions were also an excellent *Hm*CA inhibitor, with a potency intermediate between that of sulfate on one part, and azide and nitrate on the other one. Azide and nitrate exhibited quite effective inhibition that is similar for STPCA, but rather different than hCA II, Cab CA and Cam CA[35–37]. Interestingly, nitrate showed much more effective inhibition on *Hm*CA compared to human isoforms CA I, II, IV, Cab CA and Cam CA[19,29–31,32,33]. Carbonate was found to be rather weak inhibitor of the *Hm*CA with inhibition value of 121 μM, which are very close to that of hCA IV, whereas they are much lower than those of hCA I, II, Cab CA and Cam CA[35–37].

Thiocyanate, bromide and chloride were found to be moderate inhibitors of the *Hm*CA with inhibition values of 27.5–39.0 μM, which were close to that of *Pseudomonas gingivalis* (PgiCA), whereas they are much lower than those of hCA I, II, VI, STPCA, Zn-Cam and Cab CA[35–37].

It should be noted the important difference of affinity of these anions for *Hm*CA and human CA enzymes. All tested anions here

Table 2. Inhibition constants of anionic inhibitors against *Hm*CA, for the CO_2 hydration reaction, at 20 °C.

	K_I (μM)							
Anion	*Hm*CA	hCA I*	hCA II*	hCA VI*	STPCA*	PgiCA*	Zn-Cam*	Cab*
SCN^-	28.5	200	1600	890	680	93	7000	520
Br^-	27.5	4000	63 000	730	9.7	920	160 000	42 100
Cl^-	39.0	6000	200 000	720	500	940	>200 000	152 000
I^-	8.50	300	26 000	810	9.0	8700	160 000	13 200
N_3^-	10.0	12	1500	70	520	73	5800	55 700
NO_2^-	15.5	8400	63 000	820	770	3100	6800	44 800
NO_3^-	10.0	7000	35 000	760	560	8500	36 500	7800
CO_3^{2-}	121	15 000	73 000	690	10.0	890	6700	9600
SO_3^{2-}	8.0	–	–	–	–	–	–	–
SO_4^{2-}	5.5	63 000	>200 000	9900	910	8700	>200 000	950 000

Errors were in the range of 3–5% of the reported values from three different assays.
*From[35–37].

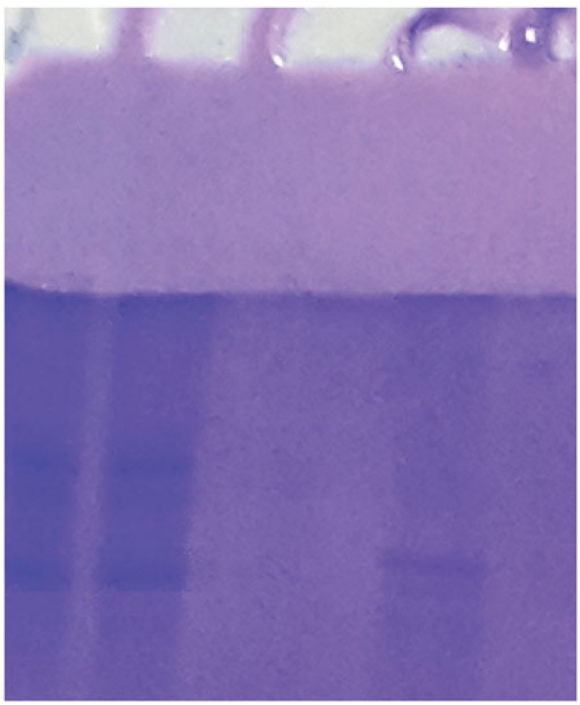

Figure 2. CA inhibition with: (A) zinc binders such as sulfonamide compounds (B) anchoring to the zinc-bound water/hydroxide ion, such as phenol, (C) inorganic anion SCN^- and (D) inorganic anion SO_4^{2-}. Figures represent distances (in Å), as determined by X-ray crystallographic techniques[35–37]. Hydrogen bonds are represented as dashed lines. All these binding modes have been proven by means of X-ray crystallography on enzyme-inhibitor adducts[36].

exhibited competitive inhibition which might indicate that these anions are in competition with the CO_2 binding site.

Conclusion

We purified CA from the *Halomonas sp.* which has not been reported earlier, and analyzed the features of this enzyme. As a strong esterase and hydratase, enzyme activity was determined from raw homogenate obtained from a liquid medium, the colonization to a microorganism (e.g. *Escherichia coli*) were not tested. The kinetic data for the hydratase activity of this enzyme with CO_2 as substrate are in good agreement with others reported in the literature. The inhibitory effects of several anions on the enzyme activity were reported. Our findings indicate that these anions inhibit the *Hm*CA enzyme in a similar manner to other α-CAs from mammals investigated earlier, but the susceptibility to various anions differs between the *Halomonas sp.* and mammalian CAs. All tested anions exhibited competitive inhibition with the CO_2 substrate, which indicates that these anions are in competition with CO_2 for binding to the active site.

Soil salinization severely affects the health of the soil (socio-economic wellbeing) and thus the production of food[38]. Throughout the world, salinity is an increasing problem in arid and semi-arid lands. Saline soils are estimated to increase at a rate of 7% in the world[39]. The increasing salinity threatens the sustainable agriculture and food production of the world. In this respect, the sustainable use of natural sources (arable lands) for the human beings is of utmost importance. As *Halomonas* is a genus inhabit in saline soil, the investigation of the presence of CA and anions interfering CA physiological function are of vital importance in the maintenance of the food security.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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