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Sulfonamide inhibition studies of the γ-carbonic anhydrase from the Antarctic bacterium *Colwellia psychrerythraea*

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ABSTRACT

The Antarctic bacterium *Colwellia psychrerythraea* encodes for a γ-class carbonic anhydrase (CA, EC 4.2.1.1), which was cloned, purified and characterized. The enzyme (CpsCAγ) has a moderate catalytic activity for the physiologic reaction of CO_2 hydration to bicarbonate and protons, with a k_{cat} 6.0×10^5 s^{-1} and a k_{cat}/K_m of 4.7×10^6 M^{-1} s^{-1}. A series of sulfonamides and a sulfamate were investigated as inhibitors of the new enzyme. The best inhibitor was metanilamide (K_I of 83.5 nM) followed by indisulam, valdecoxib, celecoxib, sulthiame and hydrochlorothiazide (K_Is ranging between 343 and 491 nM). Acetazolamide, methazolamide as well as other aromatic/heterocyclic derivatives showed inhibition constants between 502 and 7660 nM. The present study may shed some more light regarding the role that γ-CAs play in the life cycle of psychrophilic bacteria as the Antarctic one investigated here, by allowing the identification of inhibitors which may be useful as pharmacologic tools.

Organisms capable of surviving under non-standard conditions in non-conventional environments are classified as 'extremophiles'. They include thermophiles (living at temperatures >80 °C), psychrophiles (living at temperatures <15 °C), halophiles (high salt), alkaliphiles (pH >9), acidophiles (pH <2–3), and piezophiles (pressure-loving organisms).[1–4] These organisms produce enzymes functional under such extreme conditions. Recently, enzymes from psychrophiles have become interesting for industrial application, partly because of ongoing efforts to decrease energy consumption.[5–7] For example, the polymer-degrading industry, like the pulp and paper ones, are demanding catalysts that are active at lower temperatures than enzymes used until now, which work optimally at temperatures of 30–37 °C. Moreover, food-processing applications would also benefit from the availability of lower temperature acting enzymes.[5–7] Many scientists share a great interest to the Antarctic continent especially in relation to the molecular evolutionary mechanisms developed by the Antarctic organisms and microorganisms in order to adapt to such an extreme habitat.[8–14] Low temperatures, in fact, influence the rates of the chemical reaction by dropping enzyme activity, which result in a decrease of their catalytic constant (k_{cat}).[15,16] Enzymes from psychrophiles have a higher activity at lower temperatures with respect to those from mesophilic or thermophilic counterparts, as they evolved an activity, stability and flexibility in order to compensate the 'freezing effect' of the frosty habitats.[8,13] Generally, psychrophilic enzymes enhance their flexibility through many structural modifications that lead to attenuation of the strength and number of stabilizing factors, such as the reduction of the ion pairs number, hydrogen bonds and hydrophobic interactions; decreased inter-subunit interactions; increased interaction with the solvent; a reduced apolar fraction in the core; higher accessibility to the active site; increased exposure of apolar residues to the solvent; decreased cofactor binding; clustering of glycine residues; and a lower

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proline and arginine content.[8–11,13,17] This increased flexibility may possibly concerns the entire molecule or could be restricted only to a specific region, such as the amino acids involved in the enzyme catalytic cycles. For many psychrophilic enzymes, a more labile binding of the substrate, which is observed through the high K_M values, represents the main effect of the enhanced flexibility.[9–11,17] This strategy increases the k_{cat} constant at the expense of K_M, whereas in some intracellular enzymes this adaptive drift of K_M is counteracted by the retention of rigid structural domains.[9–11,17] Therefore, enzyme adaptation to cold seems to rely on a higher flexibility of the molecular structure determining a decreased stability, which compensates the freezing effect of low temperatures on the three-dimensional structure.[9–11,17]

In this context, we started to investigate in detail (e.g., kinetic properties, inhibition profile with various classes of compounds) of carbonic anhydrases (CAs, EC 4.2.1.1) identified in the genome of psychrophiles.[18–22] CAs are ubiquitous metalloenzymes that catalyze the reversible hydration of carbon dioxide with the production of bicarbonate and protons.[23–27] CAs have been classified into several classes including the α-, β-, γ-, δ-, ζ- and η-CAs. α-, β-, δ- and, probably η-CAs use Zn(II) ions at the active site, γ-CAs are Fe(II) enzymes but they are active also with bound Zn(II) or Co(II) ions, whereas ζ-class uses Cd(II) or Zn(II) to perform the physiologic reaction catalysis.[23–32] The α-, β-, γ- and ζ-CAs have been crystallized, but not δ- and η-CAs. The metal ion from the enzyme active site is coordinated by three His residues in the α-, γ- and δ-classes, by one His, and two Cys residues in β- and ζ-CAs or by two His and one Gln residues in η-class with the fourth ligand being a water molecule/hydroxide ion acting as nucleophile in the catalytic cycle of the enzyme.[29,33,34]

CAs have been thoroughly investigated in mammalian and other mesophilic species, but a limited number of studies are available on psychrophiles species. Bacterial genome encodes for three CA classes that in agreement with the CA family nomenclature are designed as α, β and γ.[25,27] Here, we explored the susceptibility of the recombinant γ-CA from the Antarctic bacterium *Colwellia psychrerythraea* (CpsCAγ) to inhibition with the main class of CA inhibitors (CAIs), the sulfonamides and their isosteres (sulfamates). *C. psychrerythraea* is an obligate psychrophile and Gram-negative bacterium isolated from the from cold ice sediments of Antarctica. The recombinant CpsCAγ was isolated and purified to homogeneity using the nickel affinity resin. We noted the oligomeric state of the enzyme detecting the CpsCAγ activity on the SDS–PAGE gel using the protonography technique[35–37] (Fig. 1). The protonogram showed two bands: one corresponding to a monomer with an apparent molecular weight of 22 kDa; while the second band showed an apparent molecular weight of 55 kDa, which corresponded to the trimeric form of the γ-CA, PgiCA (Fig. 1).

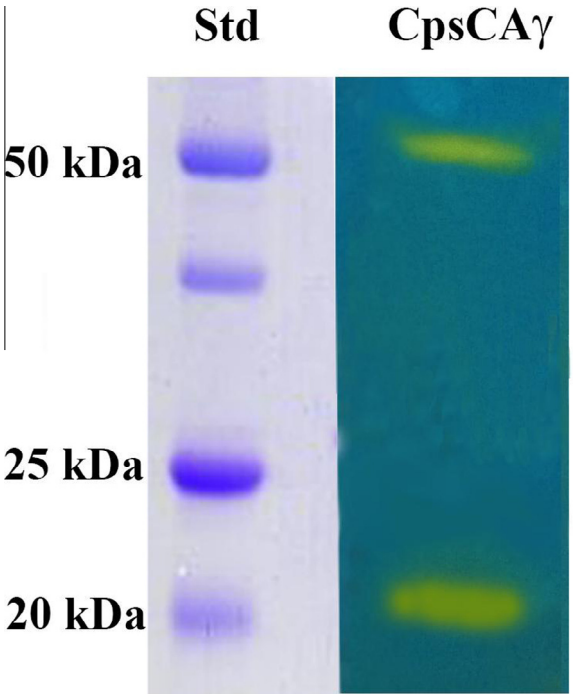

Figure 1. CpsCAγ protonography. The yellow bands correspond to the PgiCA position on the gel responsible for the drop of pH from 8.2 to the transition point of the dye in the control buffer. Incubation time was of 20 s. The Antarctic enzyme is present in two oligomeric states, the monomeric (20 kDa) and the trimeric (55 kDa) forms.

We compared the kinetic constants of CpsCAγ identified in the genome of the Antarctic *Colwellia psychrerythraea* with those of other γ-CAs from different species, such as two Antarctic γ-CAs (PhaCA from *Pseudoalteromonas haloplanktis* and NcoCA from *Nostoc commune*), CAM from *Methanosarcina thermophila* (thermophilic bacterium), PgiCA from *Porphyromonas gingivalis* (mesophilic bacterium). In the comparison we also included two α-CAs (hCA I and hCA II) and a cyanobacterial β-CA (CahB1). As shown in Table 1 CpsCAγ showed a significant catalytic activity for the CO_2 hydration reaction, similar to other γ-CAs investigated earlier such as CAM, PgiCA, NcoCA and PhaCAγ. The kinetic parameters for the CpsCAγ-catalyzed CO_2 hydration to bicarbonate and protons, were: k_{cat} of 6.0×10^5 s^{-1} and a k_{cat}/K_m of 4.7×10^6 $M^{-1} \cdot s^{-1}$. Attracting, the k_{cat} of CpsCAγ is in the same order of those determined for the hCA I (α-CA) and CahB1 (β-CA). CpsCAγ activity is inhibited by the sulfonamide CA inhibitor (CAI) acetazolamide, with a K_I of 502 nM, which is in the same range of the PgiCA from *P. gingivalis*, the Antarctic PhaCAγ and the human slow isoform hCA I (see Table 1).

A phylogenetic tree was build for understanding the evolutionary relationship of the CpsCAγ with other γ-CAs present in the genomes of other organisms such as Archaea and bacteria, but also with other CA classes like the α- and β-CAs present in vertebrates and bacteria. The tree shown in Figure 2 clearly showed that the bacterial β- and γ-CAs clustered close forming two main branches. Among the γ-CAs, the Archaea enzymes CAMH and CAM are very distantly related to each other and to all other γ-CAs from bacteria. This is expected, as the separation of Archaea and Bacteria is probably a very ancient event in the history of life on earth. It is interesting to note that all the bacterial γ-CAs clustered together on nearby branches, proving their similarity but CpsCAγ and NoCA, the two Antarctic enzymes, seems to posses an amino acid sequence which is a transition point from the Archaea and bacteria γ-CAs (Fig. 2).

We investigated the susceptibility of CpsCAγ to inhibition with the main class of CA inhibitors (CAIs), the sulfonamides and their isosteres (sulfamates).[23,29,38–42] A panel of 40 such derivatives was included in this study. Derivatives **1**–**24** and **AAZ**–**HCT** are either simple aromatic/heterocyclic sulfonamides widely used as building blocks for obtaining new families of such pharmacological agents, or they are clinically used agents, among which acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA** and dichlorophenamide **DCP**, are the classical, systemically acting antiglaucoma CA inhibitors (CAIs). Dorzolamide **DZA** and brinzolamide **BRZ** are topically-acting antiglaucoma agents, benzolamide **BZA** is an orphan drug belonging to this class of pharmacological agents, whereas topiramate **TPM**, zonisamide **ZNS** and sulthiame **SLT** are widely used antiepileptic drugs. Sulpiride **SLP** and indisulam **IND** were also shown by our group to belong to this class of pharmacological agents, together with the COX2 'selective' inhibitors celecoxib **CLX** and valdecoxib **VLX**. Saccharin and the diuretic