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Anion inhibition studies of an α-carbonic anhydrase from the thermophilic bacterium *Sulfurihydrogenibium yellowstonense* YO3AOP1

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ABSTRACT

The newly discovered thermophilic bacterium *Sulfurihydrogenibium yellowstonense* YO3AOP1 encodes an α-carbonic anhydrases (CAs, EC 4.2.1.1) which is highly catalytically active and thermostable. Here we report the inhibition of this enzyme, denominated SspCA, with inorganic and complex anions and other molecules interacting with zinc proteins. SspCA was inhibited in the micromolar range by diethyldithiocarbamate, sulfamide, sulfamic acid, phenylboronic and phenylarsonic acid, trithiocarbonate and selenocyanide (K_Is of 4–70 μM) and in the submillimolar one by iodide, cyanide, (thio)cyanate, hydrogen sulfide, azide, nitrate, nitrite, many complex anions incorporating heavy metal ions and iminodisulfonate (K_Is of 0.48–0.86 mM). SspCA was not substantially inhibited by bicarbonate and carbonate, hydrogensulfite and peroxidisulfate (K_Is in the range of 21.1–84.6 mM). The exceptional thermostability and lack of strong affinity for hydrogensulfide, bicarbonate, and carbonate make this enzyme an interesting candidate for biotechnological applications of enzymatic CO_2 fixation.

Carbonic anhydrases (CAs, EC 4.2.1.1) are a class of enzymes which catalyze a simple but physiologically relevant process in all life kingdoms, carbon dioxide hydration to bicarbonate and protons.[1–4] Five different genetically distinct CA families are known to date, the α-, β-, γ-, δ- and ζ-CAs, and all of them are metalloenzymes, using Zn(II), Cd(II) or Fe(II) at their active sites.[3–6] Bacteria, the most successful organisms on earth, encode CAs belonging to the α-, β-, and/or γ-CA families.[1,2,5,6a] Many such enzymes started to be investigated in detail ultimately in pathogenic bacteria, in the search of antibiotics with a novel mechanism of action, since it has been demonstrated that in many such bacteria CAs are essential for the life cycle of the organism.[6a] Indeed, the α-CAs from *Neisseria* spp. and *Helicobacter pylori* as well as the β-class enzymes from *Escherichia coli, H. pylori, Mycobacterium tuberculosis, Brucella* spp., *Streptococcus pneumoniae*, *Salmonella enterica* and *Haemophilus influenzae* have been cloned and characterized in detail in the last years.[6a] For some of them, X-ray crystal structures of the encoded CAs were also determined, and in vitro and in vivo inhibition studies with various classes of inhibitors, such as anions, sulfonamides and sulfamates reported.[2,6a] Although efficient inhibitors have been reported for many such enzymes, only for *Nessseria spp., H. pylori, Brucella suis* and *S. pneumoniae* enzymes has it been possible to evidence inhibition of bacterial growth in vivo.[6–12] Although the bacterial CA inhibition studies are in their infancy at this moment, the cloning of more bacterial genomes may lead to the discovery of genes and proteins which may have interesting applications both in the biomedical and biotechnological fields.

Recently, our group reported[13] the cloning and purification of a bacterial α-CA from a newly discovered thermophilic bacterium, *Sulfurihydrogenibium yellowstonense* YO3AOP1,[14] denominated SspCA. The genus *Sulfurihydrogenibium* belongs to the chemosynthetic bacterial communities living in hot springs, at temperatures up to 110 °C, and in the presence of rather high hydrogensulfide concentrations (between 1 and 100 μM).[14] There are several chemolithotrophic, sulfide-oxidizing species belonging to the genus *Sulfurihydrogenibium* (Aquificales), and they were discovered starting with 2003, and found in hot springs all over the world, from the Yellowstone National park, to the Azores Islands and Japan.[14,15] The CA (or maybe CAs, as very probably, several distinct such enzymes may be present) of these bacteria are probably involved in the CO_2 fixation and biosynthetic processes, as for other bacteria or as for algae and plants, in which this role of the various classes of CAs is well established.[14–16]

In the preliminary communication from this group[13] it has been discovered that SspCA has an exceptional thermal stability,

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retaining its high catalytic activity for the CO_2 hydration reaction even after being heated at 70 °C for several hours. As a consequence, this enzyme may show important biotechnological applications in the field of 'artificial' CO_2 fixation, considering the green gas (among which CO_2 is highly relevant) emission problems. However, no inhibition studies of this enzyme were reported to date. In this Letter we measured the catalytic activity for the CO_2 hydration reaction of this enzyme, comparing it to that of other α-CAs from bacterial or mammalian sources. We also investigate its inhibition with a large number of simple and more complex inorganic anions and small organic molecules known to interact with zinc enzymes and more precisely CAs.[17–19]

Data of Table 1 show that SspCA has a high catalytic activity for the physiologic reaction, CO_2 hydration to bicarbonate and protons, with the following kinetic parameters: k_{cat} of 9.35×10^5 s^{-1}, Km of 8.4 mM and k_{cat}/K_m of 1.1×10^8 $M^{-1} \times s^{-1}$ (at 20 °C and pH of 7.5) being thus 73.3% as active as the human (h) isoform hCA II, one of the best catalysts known in nature.[1,2] Furthermore, SspCA is 7.3 times more effective as a catalyst for the physiologic reaction compared to a similar bacterial enzyme, the α-CA from *H. pylori*, hpαCA investigated earlier by some of us,[11] and 2.2-times more effective compared to the slow human isoform hCA I[1,2] (Table 1). All these enzymes (except hCA I) were effectively inhibited by the sulfonamidein clinical use acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), with inhibition constants in the range of 4.5–21 nM (the K_I of this sulfonamide against hCA I is of 250 nM). It may be observed that acetazolamide is an excellent inhibitor of SspCA, being much more effective against this isoform than against hpαCA or hCA II (Table 1).

These activity data can be easily rationalized taking into consideration the amino acid sequence of this enzyme, which has been aligned with that of other α-CAs, such as the human isoforms hCA I and II, and the bacterial ones hpαCA and NgCA (from *Neisseria gonorrhoeae,* Accession number: CAA72038.1), as well as SsCA (from *Streptococcus salivarius* PS4) (Fig. 1). It may be observed that similar to the other investigated members, SspCA has the conserved three His ligands which coordinate the Zn(II) ion crucial for catalysis (His94, 96 and 119, hCA I numbering system). The proton shuttle residue (His64) which assists the rate-determining step of the catalytic cycle, that is, the transfer of a proton from the water coordinated to the Zn(II) ion as the fourth ligand to the environment, with formation of the zinc hydroxide, nucleophilic species of the enzyme is also conserved. SspCA has the gate-keeping residues (Glu106 and Thr199) which orientate the substrate for catalysis, and are also involved in the binding of inhibitors, similar to the other α-CAs discussed here.[1–4] All these residues are in fact conserved in all these α-CAs of mammalian or bacterial origin.

In Figure 2, a phylogenetic analysis of SspCA is shown, comparing it to the bacterial/mammalian α-CAs mentioned above. Unexpectedly, it may be observed that SspCA is phylogenetically more related to the two mammalian isoforms hCA I and II than to the other bacterial α-CAs considered here, such as the ones from *Neisseria* spp., *Helicobacter pylori*, and *Streptococcus salivarius*.

As inorganic anions represent a well-known[17] class of CA inhibitors (CAIs) due to their affinity for metal ions in solution or when bound within metalloenzymes active sites, we investigated a rather large number of such species for their interaction with SspCA (Table 2). The interest in this class of CAIs is due to the fact that *Sulfurihydrogenibium* spp. live both in marine and terrestrial water environments which may contain various concentrations of salts and rather high amounts of sulfur-containing compounds such as sulfate, sulfite and HS^-.[14,15] Data of Table 2, in which the inhibition of the human enzymes hCA I and II,[20] as well as that of hpαCA[19c] with the same anions is reported for comparison reasons, show the following:

(i) The two anions with the smallest propensity to coordinate metal ions,[17] perchlorate and tetrafluroborate, were not inhibitors of SspCA up to concentrations of 200 mM. The same is true for the human isoforms hCA I and II as well as the bacterial one hpαCA. However for this last enzyme, perchlorate did show some inhibition (K_I of 10.1 mM).[19c]

(ii) Another group of anions, among which fluoride, bromide, bicarbonate, carbonate, hydrogensulfite, and peroxydisulfate, behaved as very weak SspCA inhibitors, with inhibition constants in the range of 21.1–84.6 mM. The fact that bicarbonate and carbonate do not significantly inhibit SspCA is a very interesting situation, also considering the fact that these anions are submillimolar inhibitors of hpαCA,[19c] inhibit 2–3 times better hCA I than SspCA, but also do not significantly inhibit hCA II (Table 1). This may represent an evolutionary adaptation of SspCA to be less sensitive to these two anions probably because the enzyme must function well as a catalyst for CO_2 hydration, in an environment which is rich in these two chemical species. The same may be true regarding sulfite, one of the reaction products of sulfide oxidation. However, just regarding the difference between the inhibition constant of hydrogensulfite, hydrogensulfide, sulfate and peroxydisulfate, it can be noted that only hydrogensulfite is a weak, millimolar inhibitor, whereas the remaining species are submillimolar SspCA inhibitors. Indeed, HS^-, and SO_4^{2-} show K_Is in the range of 0.58–0.82 mM (Table 1).

(iii) A rather large number among the investigated anions were submillimolar SspCA inhibitors, with K_Is in the range of 0.48–0.86 mM. They include iodide, (thio)cyanate, cyanide, azide, nitrate, nitrite, hydrogensulfide, stannate, selenate, tellurate, diphosphate, divanadate, tetraborate, perrhenate, perruthenate, sulfate, fluorosulfate, and iminodisulfonate (Table 2). Only chloride was a weaker inhibitor compared to these anions, with a K_I of 8.3 mM (Table 2). It is rather probable that most of these anions can inhibit SspCA similar to the human isoforms hCA I and II, by coordinating to the

Table 1
Kinetic parameters for CO_2 hydration reaction catalyzed by some human α-CA isozymes (hCA I and II) and the bacterial enzymes hpαCA (*Helicobacter pylori*) and SspCA (*Sulfurihydrogenibium yellowstonense* YO3AOP1) at 20 °C and pH 7.5, and their inhibition data with acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a clinically used drug.

Enzyme	Activity level	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \times s^{-1}$)	K_I(acetazolamide) (nM)
hCA I[a]	Medium	2.00×10^5	4.0	5.0×10^7	250
hCA II[a]	Very high	1.40×10^6	9.3	1.5×10^8	12
hpαCA[b]	Low	2.5×10^5	16.6	1.5×10^7	21
SspCA[c]	High	9.35×10^5	8.4	1.1×10^8	4.5

[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method, from Refs. 2a,11a.
[b] From Ref. 11a.
[c] Recombinant enzyme, stopped flow CO_2 hydrase assay method, this work.

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hCAII    ----------------------------MSHH------WGYGK-HNGPEHW---HKDFP
hCAI     ----------------------------MASPD------WGYDD-KNGPEQW---SKLYP
hpαCA    ---MKKTF---------LIALALTASLIGAENTK-----WDYKNKENGPHRWDKLHKDFE
SsCA     MTKKCLTLTGVLSLMASTILLVACQSKTDTETSKSKDVPWGYTG-ETGPEHWGDLSKDYE
NgCA     MPRFPRTL-----PRLTAVLLLACTAFSAAAHGNHTH--WGYTG-HDSPESWGNLSEEFR
SspCA    ---MRKIL-------ISAVLVLSSISISFAEHE------WSYEG-EKGPEHWAQLKPEFF
                                                  *.*     .*  *      :

                                                        64
hCAII    IAK-GERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQDKAVLK
hCAI     IAN-GNNQSPVDIKTSETKHDTSLKPISVSYNPATAKEIINVGHSFHVNFEDNDNRSVLK
hpαCA    VCKSGKSQSPINI-EH-YYHTQDKADLQFKYAASKPKAVFFTHHTLKASF-EPTNHINYR
SsCA     LSKNGKEQSPINI-TG--AEDVDLPELNLHNQESK-GQVENNGHTIEVSFKNPKNSLTIG
NgCA     LCSTGKNQSPVNI-TE--TVSGKLPAIKVNYKPSM-VDVENNGHTIQVNYPEGGNTLTVN
SspCA    WCK-LKNQSPINI-DKKYKVKANLPKLNLYYKTAKESEVVNNGHTIQINIKE-DNTLNYL
          ..  : ***::*          .   :..    :    :    *::  .  :  :

                     94 96       106          119
hCAII    GGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNT-KYGDFGKAVQQPDG
hCAI     GGPFSDSYRLFQFHFHWGSTNEHGSEHTVDGVKYSAELHVAHWNSAKYSSLAEAASKADG
hpαCA    G----HDYVLDNVHFH------APMEFLINNKTRPLSAHFVHKDA------------KGR
SsCA     D----DVYKLQQFHFH------APSENEIDGKTYPLEGHFVYKTD------------TGK
NgCA     G----RTYTLKQFHFH------VPSENQIKGRTFPMEAHFVHLDE------------NKQ
SspCA    G----EKYQLKQFHFH------TPSEHTIEKKSYPLEIHFVHKTE------------DGK
         .      * * :.***          *  :.  . . . *..:

                                                                  199
hCAII    LAVLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADFTNFAARGLLPESLDYWTYPGSLTT
hCAI     LAVIGVLMKVGEANPKLQKVLDALQAIKTKGKRAPFTNFDPSTLLPSSLDFWTYPGSLTH
hpαCA    LLVLAIGFEEGKENPNLDPILEGIQ------KKQNLKEVALDAFLPKSINYYHFNGSLTA
SsCA     ITVVSVLYNYGDENQALKQIWDKMPQ-AANTETELSQPISLDDFYPEDKGYYNFEGSLTT
NgCA     PLVLAVLYEAGKTNGRLSSIWNVMPM--TAGKVKLNQPFDASTLLPKRLKYYRFAGSLTT
SspCA    ILVVGVMAKLGKTNKELDKILNVAPA--EEGEKILDKNLNLNNLIPKDKRYMTYSGSLTT
           *:.:  : *. :  *. : :         :      .    : *.   :  : ****


hCAII    PPLLECVTWIVLKEPISVSSEQVLKFRKLNFNGEGEPEELMVDNWRPAQPLKNRQIKASF
hCAI     PPLYESVTWIICKESISVSSEQLAQFRSLLSNVEGDNAVPMQHNNRPTQPLKGRTVRASF
hpαCA    PPCTEGVAWFVIEEPLEVSAKQLAEIKKRMKNSP---------NQRPVQPDYNTVIIKSS
SsCA     PPCTEGVNWIVFKNQETVSKEQVEKFTQTLGFE----------NNRPIQETNGRKIKE--
NgCA     PPCTEGVSWLVLKTYDHIDQAQAEKFTRAVGSE----------NNRPVQPLNARVVIE--
SspCA    PPCTEGVRWIVLKKPISISKQQLEKLKSVMVNP----------NNRPVQEINSRWIIEGF
         **  * * *:: :    :.  *  ::                 *.** *      :


hCAII    K---
hCAI     ----
hpαCA    AETR
SsCA     ----
NgCA     ----
SspCA    ----
```

Figure 1. Alignment of the amino acid sequences of α-CAs from human and bacterial organisms. The proton shuttle residue (His64 in blue), the zinc ligands (His94, 96 and 119, in red) and the gate keeper residues (Glu106 and Thr199, in cyan) are conserved in all these enzymes. The asterisk (*) indicates identity at all aligned positions; the symbol (:) relates to conserved substitutions, while (.) means that semi-conserved substitutions are observed. Multialignment was performed with the program Clustal W, version 2.1. Legend: hCA I, *Homo sapiens*, isoform I (Accession number: NP_001158302.1); hCA II, *H. sapiens*, isoform II (Accession number: AAH11949.1); hpαCA, *Helicobacter pylori* J99 (Accession number: NP_223829.1) CA; SspCA, *Sulfurihydrogenibium sp.* YO3AOP1 (Accession number: ACD66216.1); NgCA, *Neisseria gonorrhoeae* enzyme (Accession number: CAA72038.1); SsCA, *Streptococcus salivarius* PS4 CA, (Accession number: EIC81445.1). The hCA I numbering system was used.

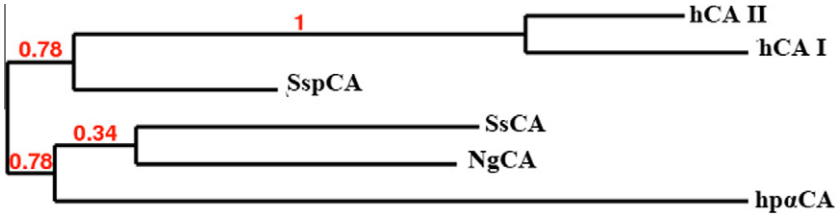

Figure 2. Phylogenetic trees of the sequences of the α-CAs shown in Figure 1. The tree was constructed using the program PhyML 3.0. Branch support values are reported at branch points.

Zn(II) ion from the enzyme active site.[17] Indeed, there are many such X-ray crystal structures of human CA isoforms complexed with anions that, as SspCA, which as are inhibited more or less in the same range by many of these anions (see Table 2). The same situation is true also by comparing SspCA and of hpαCA,[19c] although the inhibition profiles of the two enzymes are quite distinct. A special mention should be made on hydrogensulfide which is a relatively inefficient

Table 2
Inhibition constants of anionic inhibitors against α-CA isozymes derived from human (hCA I and II), and the bacterial enzymes hpαCA and SspCA, at 20 °C by a stopped flow CO_2 hydrase assay[18]

Inhibitor[a]	K_I[b] (mM)			
	hCA I[c]	hCA II[c]	hpαCA[d]	SspCA[e]
F^-	>300	>300	4.08	41.7
Cl^-	6	200	2.70	8.30
Br^-	4	63	2.41	49.0
I^-	0.3	26	6.05	0.86
CNO^-	0.0007	0.03	0.60	0.80
SCN^-	0.2	1.60	4.10	0.71
CN^-	0.0005	0.02	0.76	0.79
N_3^-	0.0012	1.51	0.83	0.49
HCO_3^-	12	85	0.75	33.2
CO_3^{2-}	15	73	0.66	39.3
NO_3^-	7	35	0.81	0.86
NO_2^-	8.4	63	0.93	0.48
HS^-	0.0006	0.04	0.69	0.58
HSO_3^-	18	89	0.99	21.1
SnO_3^{2-}	0.57	0.83	0.55	0.52
SeO_4^{2-}	118	112	0.72	0.57
TeO_4^{2-}	0.66	0.92	0.34	0.53
$P_2O_7^{4-}$	25.77	48.50	0.66	0.69
$V_2O_7^{4-}$	0.54	0.57	0.27	0.66
$B_4O_7^{2-}$	0.64	0.95	0.56	0.67
ReO_4^-	0.110	0.75	0.88	0.80
RuO_4^-	0.101	0.69	0.36	0.69
$S_2O_8^{2-}$	0.107	0.084	0.92	84.6
$SeCN^-$	0.085	0.086	0.73	0.07
CS_3^{2-}	0.0087	0.0088	0.38	0.06
$Et_2NCS_2^-$	0.79	3.1	0.005	0.004
SO_4^{2-}	63	>200	0.82	0.82
ClO_4^-	>200	>200	10.1	>200
BF_4^-	>200	>200	>200	>200
FSO_3^-	0.79	0.46	0.91	0.73
$NH(SO_3)_2^{2-}$	0.31	0.76	0.54	0.75
$H_2NSO_2NH_2$	0.31	1.13	0.073	0.009
H_2NSO_3H	0.021	0.39	0.080	0.042
Ph-$B(OH)_2$	58.6	23.1	0.097	0.041
Ph-AsO_3H_2	31.7	49.2	0.44	0.005

[a] As sodium salt.
[b] Errors were in the range of 3–5% of the reported values, from three different assays.
[c] From Ref. 20.
[d] From Ref. 19c.
[e] This work.

SspCA and hpαCA (K_Is of 0.58–0.69 mM) whereas this anion is much more inhibitory against hCA I and II (K_Is of 0.6–40 μM). Thus, there is a difference of several orders of magnitude in the sensitivity of the bacterial versus the mammalian isoforms for this anion. This is probably due to the fact that SspCA must live in an environment rich in hydrogen sulfide.[14]

(iv) Several of the investigated anions, such as trithiocarbonate, selenocyanide, diethyldithiocarbonate, sulfamides, sulfamate, as well as phenylboronic acid and phenylarsonic acid, were highly effective, micromolar SspCA inhibitors, with K_Is in the range of 4–70 μM. The most interesting case is constituted by trithiocarbonate, a compound known to coordinate monodentately to the Zn(II) ion from the hCA II active site, as observed by X-ray crystallography,[20b] and which led to the discovery of dithiocarbonates as a new class of CAIs.[21] Also in this case, the 'lead' trithiocarbonate was 15 times a weaker CAI compared to diethyldithiocarbonate, the best inhibitor so far detected for this interesting enzyme, with a K_I of 4 μM (Table 2).

As most classes of CAIs, the anions investigated here presumably coordinate to the metal ion from the enzyme active site. A comprehensive review on the binding of various classes of inhibitors, including the anions, was recently published by our group. Very probably the anions investigated in this paper bind to SspCA in a similar manner as they bind to other α-CAs investigated in more detail (e.g., hCAs) with the Zn(II) ion either in tetrahedral or trigonal bipyramidal geometries.[17,21,22]

In conclusion, we investigated an α-CA from the thermophilic bacterium *Sulfurihydrogenibium yellowstonense* YO3AOP1 which is catalytically very active for the physiological reaction of CO_2 hydration to bicarbonate and protons, and was shown earlier to be highly thermostable. Here we report the inhibition of this enzyme, SspCA, with inorganic and complex anions and other molecules interacting with zinc proteins. SspCA was inhibited in the micromolar range by diethyldithiocarbamate, sulfamide, sulfamic acid, phenylboronic and phenylarsonic acid, trithiocarbonate and selenocyanide (K_Is in the range of 4–70 μM) and in the submillimolar one by iodide, cyanide, (thio)cyanate, hydrogensulfide, azide, nitrate, nitrite, many complex anions incorporating heavy metal ions and iminodisulfonate. SspCA was not substantially inhibited by bicarbonate and carbonate, and hydrogensulfite (K_Is in the range of 21.1–39.3 mM). The exceptional thermostability and lack of strong affinity for bicarbonate, carbonate and also hydrogensulfide, make this enzyme an interesting candidate for biotechnological applications of enzymatic CO_2 fixation

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