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The alpha-carbonic anhydrase from the thermophilic bacterium Sulfurihydrogenibium yellowstonense YO3AOP1 is highly susceptible to inhibition by sulfonamides

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ABSTRACT

The α-carbonic anhydrase (CA, EC 4.2.1.1) from the newly discovered thermophilic bacterium *Sulfurihydrogenibium yellowstonense* YO3AOP1 (SspCA) was investigated for its inhibition with a large series of sulfonamides and a sulfamate, the classical inhibitors of these zinc enzymes. SspCA showed an inhibition profile with these compounds very similar to that of the predominant human cytosolic isoform hCA II, and not to that of the bacterial α-CA from *Helicobacter pylori*. Some clinically used drugs such as acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, topiramate, celecoxib and sulthiame were low nanomolar SspCA/hCA II inhibitors (K_Is in the range of 4.5–12.3 nM) whereas simple aromatic/heterocyclic sulfonamides were less effective, micromolar inhibitors. As this highly catalytically active and thermostable enzyme may show biotechnological applications, its inhibition studies may be relevant for designing on/off systems to control its activity.

1. Introduction

The genus *Sulfurihydrogenibium*, reported within the order Aquificales, belongs to the chemosynthetic bacterial communities living in hot springs, at temperatures of up to 110 °C, and in the presence of high hydrogensulfide concentrations (between 1 and 100 μM).[1] *Sulfurihydrogenibium* are chemolithotrophic, sulfide-oxidizing species and were discovered for the first time in 2003, and found in hot springs all over the world, from the Yellowstone National Park, to the Azores Islands and Japan.[1,2] As many bacteria, it is presumed that they express carbonic anhydrases (CAs, EC 4.2.1.1), enzymes involved in the CO_2 fixation and biosynthetic processes. In many bacteria, as well as algae and plants, the role of the various classes of CAs is well established.[1–4] In fact, recently, our groups reported[2] the expression and purification of a bacterial α-CA from *Sulfurihydrogenibium yellowstonense* YO3AOP1.[2] The enzyme has been named SspCA and investigated for its catalytic activities: (a) CO_2-hydratase activity, the physiological reaction used by the bacterium to transform CO_2 to bicarbonate and a proton; (b) esterase activity, measured using 4-nitrophenylacetate as substrate.[2] It has been observed that this highly thermostable enzyme (up to 70 °C) also shows excellent catalytic activity for both reactions mentioned above.[2]

In fact, CAs are a class of enzymes which evolved at least five times independently, to catalyze a simple but physiologically relevant process in all life kingdoms: carbon dioxide hydration to bicarbonate and protons.[5–8] The five genetically distinct CA families known to date, are 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.[9,10] Bacteria, the most successful organisms on earth, encode α-, β-, and/or γ-CAs, with many of them containing several different isoforms, and even enzymes from two or three distinct families.[4–10] These enzymes started to be investigated in detail recently 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.[10a] Thus, the α-CAs from *Neisseria spp.* and *Helicobacter pylori* as well as the β-class enzymes from *Escherichia coli, Helicobacter pylori, Mycobacterium tuberculosis*, *Brucella spp., Streptococcus pneumoniae*, *Salmonella enterica* and *Haemophilus influenzae* have been cloned and characterized in detail in the last few years.[10a] For some of them, X-ray crystal structures of the encoded CAs were also determined, and in vitro and in vivo inhibition

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studies with various classes of inhibitors, such as anions, sulfonamides and sulfamates have been reported.[6,10a] 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. Here we continue our investigations on bacterial CA inhibitors, and report the first inhibition study of SspCA with a series of sulfonamides and sulfamates, which represent the classical CA inhibitors (CAIs), some of which possess pharmacologic applications as drugs (diuretics, antiglaucoma, antiepileptic and anticancer agents).[6]

2. Results and discussion

We have previously reported some evidence[3] that SspCA has a high catalytic activity for the CO_2 hydration reaction (Table 1), with k_{cat} of 9.35×10^5 s^{-1}, K_m 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). As seen from the data of Table 1, where the kinetic parameters for two mammalian isoforms (the human (h) enzymes hCA I and II) as well as those of the bacterial best investigated α-CA, that is hpαCA from the gastric pathogen *H. pylori*, SspCA has a relevant catalytic activity for the physiologic reaction. Whereas hCA II is three times more effective as a catalyst compared to hCA I, SspCA is in fact more than two times more effective than hCA I and more than seven times than hpαCA (based on the k_{cat}/K_m values).

Thus, we have aligned the amino acid sequence of SspCA and that of several other investigated α-CAs from various organisms, in order to try to rationalize the kinetic data mentioned above. In Figure 1 the amino acid sequences of two mammalian (hCA I and II), one nematode (Cah-4b from *Caenorhabditis elegans*),[11] two coral (STPCA and STPCA-2),[12] and two bacterial (hpαCA[13] and SspCA[3]) were aligned. It may be observed that similar to the other investigated α-CA, SspCA has the conserved three His ligands, which coordinate the Zn(II) ion crucial for catalysis (His94, 96 and 119, hCA I numbering system). Except for the nematode enzyme, the proton shuttle residue (His64) is also conserved in all these enzymes. This residue assists the rate-determining step of the catalytic cycle transferring a proton from the water coordinated to the Zn(II) ion to the environment, with formation of zinc hydroxide representing the nucleophilic species of the enzyme. SspCA also 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.[5–10] All these residues are in fact conserved in all these α-CAs of mammalian or bacterial origin.

In Figure 2, a phylogenetic analysis of the bacterial/coral/nematode/mammalian α-CAs mentioned above is shown. It may be observed that SspCA is phylogenetically more related to the other bacterial α-CAs considered here, that is the enzyme from *H. pylori*. This is not unexpected, but it should be mentioned that the two enzymes differ significantly for their catalytic activity. This is, in fact, well known for the human isoforms, which even if clustering together, possess a variable catalytic activity.

As sulfonamides/sulfamates constitute the main class of CAIs, and such compounds have not been investigated for their interaction with SspCA, we report here a comprehensive such study. Simple sulfonamides investigated here for the inhibition of SspCA of types **1**–**24** are shown below, and they incorporate the mostly used scaffolds in the drug design of sulfonamide CAIs.[14–18] Derivatives **AAZ**–**HCT** are clinically used drugs: acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA** and dichlorophenamide **DCP**, are the classical, systemically acting CAIs.[6] Dorzolamide **DZA** and brinzolamide **BRZ** are topically-acting antiglaucoma agents,[6] benzolamide **BZA** is an orphan drug belonging to this class of pharmacological agents, whereas topiramate **TPM** is a widely used antiepileptic drug.[6] Sulpiride **SLP**, indisulam **IND**, valdecoxib **VLX**, celecoxib **CLX**, sulthiame **SLT**, saccharin **SAC** and hydrochlorothiazide **HCT** were recently shown by this group to belong to this class of pharmacological agents.[6] Compounds **1, 2, 4**–**6**, **11, 12**, **18**–**20, 23** and **AAZ**–**SLP** are commercially available or were a gift from the company producing them (see Experimental section for details), whereas **7**–**10,**[14] **13**–**17**,[15] **21,**[16] **22**[17] and **24**,[18] were prepared as reported earlier by this group. Inhibition data of these sulfonamides and the sulfamate **TPM** against isozymes hCA I, hCAII, hpαCA and SspCA are shown in Table 2 (data for the previously reported enzymes, hCA I, hCAII and hpαCA are reported for comparison reasons).

The following structure–activity relationship (SAR) may be observed by comparing the inhibition data of SspCA with these compounds:

(i) A group of compounds, among which derivatives **1**–**5, 13, 14, 21, 22, 24, SZN** and **SAC**, showed modest inhibitory activity against SspCA, with inhibition constants in the range of 137–876 nM (Table 2). It may be observed that they include the simple benzenesulfonamides incorporating *ortho*- or *para*- compact substituents of the amino, hydrazino, methyl or amino/hydroxyl-methyl/ethyl type, the simple heterocyclic sulfonamides **13** and **14** incorporating the thiadiazole or thiadiazoline rings as well as the two clinically used compounds with structural particularities: zonisamide the only aliphatic compound among the clinically used sulfonamides, and the sweetener saccharin, which is a secondary acylated sulfonamide. It should be noted that most these compounds are much weaker hCA I inhibitors, some of them inhibit hCA II better than SspCA, whereas they are modest hpαCA inhibitors (Table 2).

(ii) Derivatives **6**–**12, 19, 20, 23**, as well as **SLP** and **IND**, were more effective CAIs compared to the previously discussed sulfonamides, showing inhibition constants in the range of 41.2–84.1 nM. It may be observed that the longer aminoalkyl tail present in **6** leads to a marked increase of affinity for SspCA compared to **5** and **2**, which have one and

Table 1
Kinetic parameters for CO_2 hydration reaction catalyzed by 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. Inhibition data with acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a clinically used drug

Enzyme	Activity level	kcat (s^{-1})	Km (mM)	kcat/Km ($M^{-1} \times s^{-1}$)	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 Ref. 2a,11a.
[b] From Ref 11a.
[c] Recombinant enzyme, stopped flow CO_2 hydrase assay method, this work.

```
hCAI            ----------------------MASPDWGY-DDKNG---PEQWSKLYP---IAN-GNNQSP
hCAII           ----------------------MSHHWGY-GKHNG---PEHWHKDFP---IAK-GERQSP
CAH-4b          ------------------------------------------------MAA-AQRQSP
STPCA           MKLSLFISSLLAMIVACPNLAESAGSWTY-RDPEG---PDTWKHHYK---DCE-GHEQSP
STPCA-2         ---------MYFLWLISMIAAAMCQEYGY-MSEQGVPTPSNWSKVFP---LCG-GKFQSP
hpαCA           ----MKKTFLIALALTASLIGAENTKWDYKNKENG---PHRWDKLHKDFEVCKSGKSQSP
SspCA           ---MRKILISAVLVLSSISISFAEHEWSY-EGEKG---PEHWAQLKPEFFWCK-LKNQSP
                                                                .     ***
                                                      64
hCAI            VDIKTSETKHDTSLKP---ISVSYNPA---TAKEIINVGHSFHVNFEDNDNRSVLKGGPF
hCAII           VDIDTHTAKYDPSLKP---LSVSYDQA---TSLRILNNGHAFNVEFDDSQDKAVLKGGPL
CAH-4b          IDIVPQHVCCDTDVCKADALNIDYKSG---DCCDVLVSEGGFLVNVK-RNCGTFLTANHL
STPCA           INIVPKDTFFEPGLAD---LVVNYEKS---VSAKLFNNGHTVQATF--LTGKSNISGGNL
STPCA-2         INIETKKVK-KKSYPD---LKISFDNPCGRVTGELLNAGHSPVVNIDSSKGGAKLSGGPL
hpαCA           INIE--HYYHTQDKAD---LQFKYAAS---KPKAVFFTHHTLKASFEPTNHINYRG----
SspCA           INID-KKYKVKANLPK---LNLYYKTA---KESEVVNNGHTIQINIKEDNTLNYLG----
                ::*          .       : . :           :.          .
                          94 96       106           119
hCAI            S-DSYRLFQFHFHWGSTNEHGSEHTVDGVKYSAELHVAHWNSAKYSSLAEAASKADG-LA
hCAII           D-GTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNT-KYGDFGKAVQQPDG-LA
CAH-4b          PSSKFALAQFHAHWGSNSKEGSEHFLDGKQLSGEVHFVFWNT-SYESFNVALSKPDG-LA
STPCA           T-SHFRALQMHFHWGSENSRGSEHQVGGRKFPLEIHIVHYNAEKYPSVSEAVDKGDG-LA
STPCA-2         DCDEYALQQFHFHFGCENSRGSEHLIDSQAFPAQLHLVFFNK-KYETFQNAVDKPDG-LA
hpαCA           --HDYVLDNVHFH------APMEFLINNKTRPLSAHFVHKDA-------------KGRLL
SspCA           --EKYQLKQFHFH------TPSEHTIEKKSYPLEIHFVHKTE-------------DGKIL
                    :   :.* *          *. :     . . *...               .* :

hCAI            VIGVLMK-VGEANPKLQKVLDALQAIKTKGKRAPF---TNFDPSTLLP------------
hCAII           VLGIFLK-VGSAKPGLQKVVDVLDSIKTKGKSADF---TNFAARGLLP------------
CAH-4b          VVGVFLK-EGKYNDNYHGLIDTVRK--ATGNATPIAMPKDFHIEHLLP------------
STPCA           VLGILVELQVQDNPVFDVMVDNLDKARYKGNEVIL---PSLQPFSFLP------------
STPCA-2         VLGVLITATCPGNRVLGSFAKKLTKIIEEGASANVTAVDGIKLNYLMPYNNKQGDEDEDD
hpαCA           VLAIGFE-EGKENPNLDPILEGIQK------KQNL---KEVALDAFLP------------
SspCA           VVGVMAK-LGKTNKELDKILNVAPA--EEGEKILD---KNLNLNNLIP------------
                *:.:        :     . .                  .    ::*
                                                199
hCAI            -------------------SSLD--FWTYPGSLTHPPLYESVTWIICKESISVSSEQLAQ
hCAII           -------------------ESLD--YWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLK
CAH-4b          -------------------SPDKREFVTYLGSLTTPPYNECVIWTLFTEPVEVSFGQLNV
STPCA           -------------------HDIA-QYYTYRGSLTTPGCFESVQWFVFNHTFPISQAQLDK
STPCA-2         EDIAGDDPDAVEEEEVDAKKKIK--YYTYKGSLTTPPCYESVTWIVFKDKIKISNTQLKK
hpαCA           -------------------KSIN--YYHFNGSLTAPPCTEGVAWFVIEEPLEVSAKQLAE
SspCA           -------------------KDKR--YMTYSGSLTTPPCTEGVRWIVLKKPISISKQQLEK
                                     :  : **** *   * * * :    . :*  *:

hCAI            FRSLL-SNVEGDNAVPMQHNNRPTQPLKGRTVRASF-------------------------
hCAII           FRKLN-FNGEGEPEELMVDNWRPAQPLKNRQIKASFK-------------------------
CAH-4b          LRNII------------PANHRACQDRCDREIRSSFNF-----------------------
STPCA           FRDLFDSEKQDTKKLPLVDNYRPVQPLYGRSVSEASNALLFPVARHQTKLWIAWDSLMTR
STPCA-2         FRKLK-AQYGGAPG-LMCDNIRPVQPLHKRKVYSVLSSRE--------------------
hpαCA           IKKRM-KN---------SPNQRPVQPDYNTVIIKSSAETR--------------------
SspCA           LKSVM-----------VNPNNRPVQEINSRWIIEGF-------------------------
                :..               * *. *      :

hCAI            -----------------
hCAII           -----------------
CAH-4b          -----------------
STPCA           QYFMKQQSICALYQPQ
STPCA-2         -----------------
hpαCA           -----------------
SspCA           -----------------
```

Figure 1. Multialignment of the amino acid sequences of α-CAs from different organisms was performed with the program Clustal W, version 2.1. Legend: hCAI, *Homo sapiens*, isoform I (Accession n.: NP_001158302.1); hCAII, *H. sapiens*, isoform II (Accession n.: AAH11949.1); CAH-4b, *Caenorhabditis elegans* (Accession n.: NP_510264); STPCA, *Stylophora pistillata* (Accession n.: ACA53457.1), STPCA-2, *Stylophora pistillata*, (Accession n.: ACE95141.1); hpαCA, *Helicobacter pylori* J99 (Accession n.: NP_223829.1) CA; SspCA, *Sulfurihydrogenibium sp.* YO3AOP1 (Accession n.: ACD66216.1). The asterisk (*) indicates identity at all aligned positions; the symbol (:) relates to conserved substitutions, while (.) means that semi-conserved substitutions are observed. Residues involved in the enzyme CO_2 hydratase reaction are indicated in bold. hCAI numbering system was used. The zinc ligands (His94, 96 and 119) and the gatekeeper residues (Glu106 and Thr199) are conserved in all these enzymes. The proton shuttle residue (His64) is not present in the nematode carbonic anhydrase (CAH-4b), but conserved in all the other α-CAs considered in the sequence alignment.

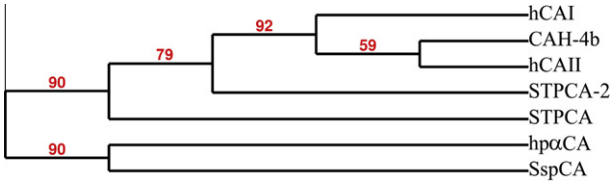

Figure 2. Phylogenetic tree was constructed using the program PhyML 3.0. Branch support values, displayed in %, are reported at branch points. Legend: hCAI, *Homo sapiens*, isoform I (Accession n.: NP_001158302.1); hCAII, *H. sapiens*, isoform II (Accession n.: AAH11949.1); CAH-4b, *Caenorhabditis elegans* (Accession n.: NP_510264); STPCA, *Stylophora pistillata* (Accession n.: ACA53457.1), STPCA-2, *Stylophora pistillata*, (Accession n.: ACE95141.1); hpαCA, *Helicobacter pylori* J99 (Accession n.: NP_223829.1) CA; SspCA, *Sulfurihydrogenibium sp.* YO3AOP1 (Accession n.: ACD66216.1).

Table 2
Inhibition constants of sulfonamide 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	KI* (nM)			
	hCA I[a]	hCA II[a]	hpαCA[b]	SspCA[c]
1	45400	295	426	707 ± 70
2	25000	240	454	652 ± 51
3	28000	300	316	803 ± 64
4	78500	320	430	848 ± 76
5	25000	170	873	350 ± 17
6	21000	160	1150	73.0 ± 6
7	8300	60	1230	68.3 ± 3
8	9800	110	378	41.2 ± 4
9	6500	40	452	75.7 ± 6
10	6000	70	510	44.8 ± 2
11	5800	63	412	61.2 ± 6
12	8400	75	49	87.3 ± 7
13	8600	60	323	137 ± 9
14	9300	19	549	433 ± 31
15	6	2	268	5.5 ± 0.5
16	164	46	131	7.8 ± 0.7
17	185	50	114	9.7 ± 0.3
18	109	33	84	6.5 ± 0.2
19	95	30	207	66.4 ± 3
20	690	12	105	70.1 ± 5
21	55	80	876	493 ± 19
22	21000	125	1134	162 ± 10
23	23000	133	1052	72.4 ± 7
24	24000	125	541	695 ± 65
AAZ	250	12	21	4.5 ± 0.3
MZA	50	14	225	8.2 ± 0.6
EZA	25	8	193	9.3 ± 0.7
DCP	1200	38	378	8.5 ± 0.9
DZA	50000	9	4360	8.0 ± 0.7
BRZ	45000	3	210	12.3 ± 1.1
BZA	15	9	315	8.1 ± 0.4
TPM	250	10	172	6.6 ± 0.5
ZNS	56	35	231	140 ± 11
SLP	1200	40	204	67.4 ± 5
IND	31	15	413	84.1 ± 7
VLX	54000	43	nt	5.3 ± 0.5
CLX	50000	21	nt	6.9 ± 0.7
SLT	374	9	nt	7.8 ± 0.6
SAC	18540	5959	nt	876 ± 53
HCT	328	290	nt	7.2 ± 0.7

[a] Human/hp recombinant isozymes, stopped flow CO_2 hydrase assay method, from Ref. 6.
[b] Recombinant hpCA, stopped flow CO_2 hydrase assay method, from Ref. 13.
[c] This work, mean ± SE (from three different assays). nt = not tested.

respectively two carbon atoms less compared to **6**. The presence of halogen atoms in the sulfanilamide scaffold, as in **7**–**10**, or the 1,3-disulfamoyl moieties present in **11** and **12**, also is beneficial for the SspCA inhibition, when comparing these compounds to the sulfanilamide **2** scaffold.

(iii) A rather large number of the investigated compounds showed excellent SspCA inhibitory action. Indeed, compounds **15**–**18, AAZ, MZA, EZA, DCP, DZA, BRZ, BZA, TPM, VLX, CLX, SLT** and **HCT**, were low nanomolar inhibitors of this enzyme, with inhibition constants in the range of 4.5–12.3 nM (Table 1). It may be observed that the compounds incorporating a longer molecule, such as **15**–**19** which are sulfanylated-aromatic/heterocyclic sulfonamides or pyrimidinyl-substituted sulfanilamide (**19**) as well as the more elaborate heterocyclic or aromatic scaffolds present in most of the clinically used CAIs investigated here, lead to highly effective SspCA inhibitors. It should be also noted that the affinity of many of these sulfonamides/sulfamates to SspCA is many times higher than that for hCA II, a well-known, sulfonamide-'avid' isoform. Furthermore, while hpαCA is significantly inhibited only by acetazolamide (K_I of 21 nM) and is not very sensitive to other sulfonamides, SspCA has a very distinct inhibition profile compared to this and other bacterial enzyme.

3. Conclusion

We investigated sulfonamide/sulfamate inhibition of the α-CA from the thermophilic bacterium *Sulfurihydrogenibium yellowstonense* YO3AOP1 that is catalytically very active for the physiological reaction of CO_2 hydration to bicarbonate and protons, and was shown earlier to be highly thermostable. SspCA showed an inhibition profile with these compounds very similar to that of the predominant human cytosolic isoform hCA II, and not to that of the bacterial α-CA from *Helicobacter pylori*. Some clinically used drugs such as acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brizolamide, topiramate, celecoxib and sulthiame were low nanomolar SspCA/hCA II inhibitors (K_Is in the range of 4.5–12.3 nM) whereas simple aromatic/heterocyclic sulfonamides were less effective, but still micromolar inhibitors of this new enzyme. As this highly catalytically active and thermostable CA may show biotechnological applications, its inhibition studies may be relevant for designing on/off systems to control its activity.

4. Experimental

4.1. Chemistry

Sulfonamides **1**–**24** and **AAZ**–**HCT** were either commercially available (Sigma-Aldrich, Milan, Italy) or prepared as reported earlier by this group.[14–18]

4.2. Enzyme preparation

Competent *E. coli* BL21 (DE3) cells were transformed with the plasmid pET15b containing the *Sulfurihydrogenibium sp* gene encoding for the αcarbonic anhydrase and lacking the signal peptide (first 20 amino acid of the peptide sequence). Cells were grown at 37 °C and induced with 1 mM IPTG. After additional growth for 5 h, the cells were harvested and disrupted by sonication at 4 °C. Following centrifugation, the cell extract was heated at 90 °C for 30 min and centrifuged. The supernatant was loaded into His-select HF Nickel affinity gel and the protein was eluted with 250 mM imidazole. At this stage of purification the enzyme was at least 95% pure. All the details concerning the expression and purification are described by Capasso C *et al.* (2012).[3]

4.3. Enzyme kinetics and inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[19] Phenol

red (at a concentration of 0.2 mM) has been used as indicator, working at the maximum absorbance of 557 nm, with 10–20 mM Hepes (pH 7.5) as buffer, and 20 mM $NaBF_4$ for maintaining constant ionic strength, following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated at room temperature for 15 min prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes were obtained from Lineweaver-Burk plots, as reported earlier,[20,21] and represent the mean from at least three different determinations.

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