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Inhibition of the β-carbonic anhydrase from *Streptococcus pneumoniae* by inorganic anions and small molecules: Toward innovative drug design of antiinfectives?

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ABSTRACT

The Gram-positive bacterium *Streptococcus pneumoniae* is a human respiratory tract pathogen that contributes significantly to global mortality and morbidity. It was recently shown that this bacterial pathogen depends on a conserved β-carbonic anhydrase (CA, EC 4.2.1.1) for in vitro growth in environmental ambient air and during intracellular survival in host cells. Hence, it is to be expected that this pneumococcal carbonic anhydrase (PCA) contributes to transmission and pathogenesis of the bacterium, making it a potential therapeutic target. In this study, purified recombinant PCA has been further characterized kinetically and for inhibition with a series of inorganic anions and small molecules useful as leads. PCA has appreciable activity as catalyst for the hydration of CO_2 to bicarbonate, with a k_{cat} of 7.4×10^5 s^{-1} and k_{cat}/K_m of 6.5×10^7 M^{-1} s^{-1} at an optimum pH of 8.4. Inorganic anions such as chloride, bromide, iodide, cyanate, selenocyanate, trithiocarbonate, and cyanide were effective inhibitors of PCA (K_Is of 21–98 μM). Sulfamide, sulfamic acid, phenylboronic, phenylarsonic acid, and diethyldithiocarbamate showed inhibition constants in the low micromolar/submicromolar range (K_Is of 0.61–6.68 μM), whereas that of the sulfonamide acetazolamide was in the nanomolar range (K_Is 89 nM). In conclusion, our results show that PCA can effectively be inhibited by a range of molecules that could be interesting leads for obtaining more potent PCA inhibitors. PCA might be a novel target for designing antimicrobial drugs with a new mechanism of action.

1. Introduction

The respiratory tract bacterium *Streptococcus pneumoniae*, or pneumococcus, is one of the most frequently encountered pathogens in humans, and is responsible for the death of approximately 1 million children under the age of five each year.[1] It causes various infections, such as meningitis, septicemia, otitis media, sinusitis, and pneumonia. *S. pneumoniae* is a facultative anaerobe capable to adapt to the different environments it encounters during transmission, host colonization, and disease. An important variable in these niches is the level of carbon dioxide (CO_2), which could be as low as 0.038% in environmental ambient during transmission and over 5% in the lungs and infected tissues. It is known for a long time that the complete depletion of environmental CO_2 inhibits pneumococcal growth,[2] suggesting that CO_2 needs to be retained inside *S. pneumoniae* to support cellular processes. Recently, one of our groups showed that a mutant in the single, highly conserved pneumococcal β-carbonic anhydrase (PCA) renders in vitro growth of *S. pneumoniae* dependent on elevated CO_2-levels or on supplementation with unsaturated fatty acids. These observations linked PCA-mediated CO_2-fixation to fatty acid metabolism, and suggested that PCA is important for pneumococcus to cope with changes in nutrient availability during niche transition.[3] Furthermore, we also observed a reduced survival of *S. pneumoniae pca* mutant strains inside host cells suggesting that PCA is also needed for pneumococcal adaptation to pathogenesis-related processes.[3]

Similar to PCA, carbonic anhydrases (CAs, EC 4.2.1.1) of various microbial pathogens have been implicated in cell survival and pathogenesis.[4,5] It is therefore not surprising that CAs are considered as an emerging class of potential therapeutic targets also for the design of antiinfectives.[5] CAs, which catalyze the reversible hydration of carbon dioxide to bicarbonate ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$), are ancient enzymes that were previously shown to be crucial in the regulation of acid–base balance in organisms all over the phylogenetic tree.[4–11] In addition, CAs participate in many other physiological processes, such as lipogenesis, gluconeogenesis, ureagenesis, bone resorption, production of body fluids, and transport of CO_2 and HCO_3^-, tumorigenesis, etc. (in mammals),[5,8] as well as CO_2 concentrating mechanisms and photosynthesis (in plants, algae, some bacteria, and diatoms).[6,7] Five genetically distinct classes of these enzymes have been identified so far: the α, β, γ, δ, and ζ-CAs.[4–8]

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Among them, the β-CAs seem to be the most common class,[4,5] in various organisms all over the phylogenetic tree but absent in humans.[4] The identification and characterization of β-CAs in a number of human pathogens, such as fungi/yeasts (e.g., *Candida* species, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae*),[9–12] and bacteria (e.g., *Brucella suis*, *Helicobacter pylori*, *Mycobacterium tuberculosis*, and *Haemophilus influenzae*),[13–18] has already lead to novel antimicrobial compounds, with a new mechanism of action, as recently shown for a β-CA from *B. suis*,[18] or for the enzymes from *H. pylori*.[13]

Continuing our interest in finding and characterizing members of the β-CA family in pathogenic organisms for the development of novel antimicrobial compounds, we report the kinetic characterization PCA from *S. pneumoniae*. We also report the first inhibition study of this enzyme with inorganic anions, various small compounds known to target the metal ion in metalloenzymes like CAs, and the broad-range CA inhibitory compound acetazolamide (AZA).

2. Results and discussion

2.1. Catalytic activity of PCA

In the previous work[3] the catalytic activity for the physiologic reaction, that is, CO_2 hydration to bicarbonate and protons, has been evidenced for PCA, but the kinetic parameters were not determined. Thus, we investigate here in detail the properties of this new and interesting representative of the β-CA class.

In Figure 1, we have aligned the PCA protein sequence with various β-CAs from archaeal, bacterial or fungal origin.[7,9,12–18] It may be observed that PCA has all the amino acids residues typical of catalytically active β-CAs, which are involved in the catalytic cycle of this class of enzymes:[4] (i) the Zn(II) binding residues Cys36, His89, and Cys92;[4,5,11] and (ii) the Asp38-Arg40 catalytic dyad, involved in the opening/closing of the active site.[14] Indeed, unlike α-, γ-, and δ-CAs, for which the Zn(II) is coordinated by three His residues and a water molecule/hydroxide ion, in the case of the β-class CAs the metal ion coordination is more complicated, being generally achieved by one His and two Cys residues.[4,5,11,14] However, some β-class enzymes have four protein zinc ligands, that is, one His, two Cys, and one Asp coordinated to Zn(II).[14] For these enzymes no water coordinated to the metal ion is present at pH values <8, as shown in an excellent crystallographic work from Jones' group on the mycobacterial enzymes Rv3558c and Rv1284.[14] However, at pH values >8, a conserved Arg residue in all β-CAs investigated so far (belonging to the above-mentioned catalytic dyad)[14] makes a salt bridge with the Asp coordinated to Zn(II), liberating the fourth Zn(II) coordination position, which is then occupied by an incoming water molecule/hydroxide ion, acting thereafter as nucleophile in catalyzing CO_2 hydration to bicarbonate.[14]

Based only on the amino acid sequence, it is however impossible to predict whether PCA will have a open or closed active site. In the open form, Cys36, Cys92, His89, and a catalytic water molecule/hydroxide ion would coordinate the Zn(II) ion, allowing the enzyme to be active over a broad pH-range. In the closed form, Aps38 would additionally coordinate to the Zn(II) ion as well, thereby replacing the water molecule/hydroxide ion needed for the catalytic activity. Only at pH values >8, when the conserved Arg40 residue makes a salt bridge with Asp38, the fourth Zn(II) coordination position may be available for the catalytic water molecule/hydroxide ion.[14] To test if PCA has an open or closed active site, we accurately measured its catalytic activity at different pH values (Table 1). In Table 1 we show that the catalytic activity of PCA is absent at pH of 6.5 and 7.5, being very low at pH 8, and steadily increasing at higher pH values (8.2 and 8,4), which implies that PCA probably has a closed active (with Asp38 is coordinated to the metal ion) at pH values under 8. It should be noted that the optimum pH for the hydration of CO_2 correlates very well physiological conditions in which PCA is optimally expressed. Expression of the *pca* gene is induced in the pneumococcal X-state,[19a] which is a physiological condition that allows for genetic transformation, biofilm formation, and an adequate response to environmental stress.[19b,c] During the X-state, the intracellular pH in *S. pneumoniae* rises to about pH 8.3 as result of increased glycolysis and sodium (Na^+) fluxes.[19d,e] Whether PCA is needed in these conditions to compensate for further alkalinization of the cytosol, providing HCO_3^- to match with intracellular levels of Na^+, or to sustain metabolic pathways needed in the X-state remains to be investigated. It should be anyhow mentioned that the CO_2 hydrase activity of PCA and of any other CA belonging to the various enzyme classes is increasing with the pH as the alkaline medium is favorable to the formation of the zinc hydroxide species of the enzyme. However at pH values >8.5 the uncatalyzed CO_2 hydrations starts to drastically compete with the reaction catalyzed by the enzymes. These are the two reasons why we did not measure PCA catalytic activity at pH values higher than 8.4.

Next, we zoomed in at the PCA kinetic parameters for the CO_2 hydration reaction at pH 8.4, and compared it to that of α-CAs of human (h) origin (hCAI and hCAII) and β-CAs from the archaeon *Methanobacterium thermoautotrophicum* (Cab)[20] and the pathogenic fungus *C. neoformans* (Can2)[11] investigated earlier (Table 1). These data showed that PCA has a significant activity as catalyst for the conversion of CO_2 to bicarbonate, with a k_{cat} of 7.4×10^5 s^{-1}, and k_{cat}/K_m of 6.5×10^7 M^{-1} s^{-1}. Thus, PCA is a better catalyst for the physiological reaction than the human isoforms hCA I or the other two β-class enzymes with which it has been compared in the present work.

2.2. Inhibition of PCA with inorganic anions and other small molecules

Since CAs bind HCO_3^-, a substrate and inhibitor of these enzymes,[4–8] inorganic anions do have a profound impact on their catalytic activity. Indeed, many classes of CAs, including the β one, have been investigated for their interaction with anion inhibitors,[5,6,8,16,20–23] which, as in solution (e.g., cyanide, thiocyanate, azide, and other such metal complexing species), directly bind to the metal ion from the enzyme active site, in tetrahedral or trigonal bipyramidal geometry of the Zn(II) ion.[5,6,8,11]

Inhibition data of PCA with small inorganic anions was compared with inhibition data of the α-CA isoforms hCA I and II (Table 2), as well as the β-class enzymes Cab and Can2 reported earlier.[12,19] Simple inorganic anions, including the physiological ones (such as chloride, bicarbonate, carbonate, and sulfate), as well as 'metal poisons' (cyanide, cyanate, thiocyanate, azide, hydrogen sulfide, bisulfite, nitrite, etc.) or anions with less affinity for metal ions in solution (tetrafluroborate, perchlorate, nitrate, fluoride, and heavier halides, among others) were included in this study. On the other hand, less investigated inorganic anions, such as stannate(IV), selenate(VI), tellurate(VI), perosmate(VIII), persulfate, pyrophosphate(V), pyrovanadate(V), tetraborate, perrhenate(VII), perrutenate(VII), persulfate, selenocyanate, iminodisulfonate, fluorosulfate, and trithiocarbonate were also included in this study, as they recently allowed us to discover new zinc-binding groups for designing potent α-CA inhibitors.[22]

Similar to all other α- and β-CAs investigated so far, PCA was not sensitive to inhibition by perchlorate (K_I > 200 mM). Also sulfate was not very inhibitory (inhibition constant of 4.15 mM), but fluoride, thiocyanate, azide, carbonate, bicarbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, pyrophosphate, and tetraborate were weak inhibitors, with K_Is in the range of 0.32–0.85 mM. Halides other than fluoride, cyanate, and cyanide, were more potent inhibitors of PCA, with K_Is in the range of 41–98 μM. Thus, for the ha-

```
PCA             -------------------------MSYFEQFMQANQAYVAL---HGQLN------LPL 25
Rv1284          -------------------------MTVTDDYLANNVDYASG---FKGP-------LPM 24
CAB             ----------------------MRFVSMIIKDILRENQDFRFR---DLSD---------L 26
CAN2            MPFHAEPLKPSDEIDMDLGHSVAAQKFKEIREVLEGNRYWARK---VTSEEPEFMAEQVK 57
Rv3588c         --------------------MPNTNPVAAWKALKEGNERFVAGRPQHPSQSVDHRAGLAA 40
                                                 *  :

PCA             KPKTRVAIVTCMDSRLH--VAQALGLALGDAHILRNAG---GRVTEDMIRSLVISQQQMG 80
Rv1284          PPSKHIAIVACMDARLD--VYRMLGIKEGEAHVIRNAG---CVVTDDVIRSLAISQRLLG 79
CAB             KHSPKLCIITCMDSRLIDLLERALGIGRGDAKVIKNAG---NIVDDGVIRSAAVAIYALG 83
CAN2            GQAPNFLWIGCADSRVP--EVTIMARKPGDVFVQRNVANQFKPEDDSSQALLNYAIMNVG 115
Rv3588c         GQKPTAVIFGCADSRVA--AEIIFDQGLGDMFVVRTAG---HVIDSAVLGSIEYAVTVLN 95
                        . Z *:*:       :    *:  : :...       .        :   :.

PCA             TREIVVLHHTDCG--AQTFENEPFQE----------YLKEELGVDVSDQDFLPFQDIE-E 127
Rv1284          TREIILLHHTDCG--MLTFTDDDFKR----------AIQDETGIRPT-WSPESYPDAV-E 125
CAB             VNEIIIVGHTDCG--MARLDEDLIVSRMRE-----LGVEEEVIENFSIDVLNPVGDEE-E 135
CAN2            VTHVMVVGHTGCGGCIAAFDQPLPTEENPGGTPLVRYLEPIIRLKHSLPEGSDVNDLIKE 175
Rv3588c         VPLIVVLGHDSCGAVNAALAAINDGTLPGGYVRDVVERVAPSVLLGRRDGLSRVDEFEQR 155
                .  :::: Z .Z*     :                                    :   .

PCA             SVREDMQLLIE-SPLIP-----------DDVIISGAIYNVDTGSMTVVEL---------- 165
Rv1284          DVRQSLRRIEV-NPFVT-----------KHTSLRGFVFDVATGKLNEVTP---------- 163
CAB             NVIEGVKRLKS-SPLIP-----------ESIGVHGLIIDINTGRLKPLYLDED------- 176
CAN2            NVKMAVKNVVN-SPTIQGAWEQARKGEFREVFVHGWLYDLSTGNIVDLNVTQGPHPFVDD 234
Rv3588c         HVHETVAILMARSSAISERIAG------GSLAIVGVTYQLDDGRAVLRDHIGNIGEEV-- 207
                 *   :  :   .. :                : *   ::  *

PCA             ----- 165          (100% / 100%)
Rv1284          ----- 163          ( 37% /  74%)
CAB             ----- 176          ( 30% /  67%)
CAN2            RVPRA 239          ( 16% /  49%)
Rv3588c         ----- 207          ( 17% /  51%)
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Figure 1. Alignment of *S. pneumoniae* PCA, *M. tuberculosis* Rv1284, *M. thermoautotrophicum* Cab, *C. neoformans* Can2, and *M. tuberculosis* Rv3588c amino acid sequences. The three zinc ligands (Cys36, His89, and Cys92, PCA numbering) are evidenced in blue (and the 'Z' sign) whereas the other conserved amino acid residues between the five β-CAs are evidenced by an asterisk. The two conserved residues Asp38, Arg40, thought to be involved in the β-CA catalytic cycle are shown in red. Amino acids where conserved substitutions were identified are represented with the ':' sign, while semi-conserved substitutions are represented by the '.' symbol. Identity and similarity of the five β-CAs to PCA are given in parentheses.

Table 1
Kinetic parameters for the CO_2 hydration reaction catalyzed by the human cytosolic isozymes hCA I and II (α-class CAs) at 20 °C and pH 7.5 in 10 mM HEPES buffer and 20 mM Na_2SO_4, and the β-CAs Cab (from *M. thermoautotrophicum*), Can2 (from *C. neoformans*), and PCA (*S. pneumoniae*), respectively, measured at 20 °C, pH 5.6–8.4 in 10 mM Tris, HEPES or TAPS buffers and 20 mM $NaClO_4$[21]

Isozyme	Activity level	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	pH
hCA I[a]	Moderate	2.0×10^5	5.0×10^7	7.5
hCA II[a]	Very high	1.4×10^6	1.5×10^8	7.5
Cab[b]	Low	3.1×10^4	1.8×10^6	8.4
Can2[b]	Moderate	3.9×10^5	4.3×10^7	8.4
PCA[c]	Inactive	0	0	6.5
PCA[c]	Inactive	0	0	7.5
PCA[c]	Very low	1.1×10^4	1.9×10^5	8.0
PCA[c]	Moderate	6.1×10^5	5.4×10^7	8.2
PCA[c]	Moderate	7.4×10^5	6.5×10^7	8.4

[a,b] Data from Refs. 4,11,20.
[c] This work.

lides the inhibition power increased from fluoride to bromide and then slightly decreases again for iodide. This observation is very relevant, as, especially, chlorides are commonly used for buffer preparation. In fact, our first batch of purified PCA enzyme was only poorly active as a consequence of the presence of 50 mM Tris–HCl in the final preparation (data not shown).[3] It is also interesting to note that cyanide and cyanate are much more inhibitory than thiocyanate, although for other β-CAs, such as Cab and Can2, thiocyanate was a better inhibitor compared to cyanate. Cyanide showed weak inhibition of the β-CAs Cab and Can2 (K_Is of 13.56–27.8 mM) while being an effective PCA inhibitor (K_I of 41 μM). The less investigated inorganic anions incorporating heavy metal ions, as well as persulfate, peroxydisulfate, selenocyanate, fluorosulfate, and trithiocarbonate also showed significant PCA inhibitory activity, with inhibition constants in the range of 21–66 μM (Table 2). In this series of inorganic anions selenocyanate and trithiocarbonate were the best PCA inhibitors, with K_Is of 21–22 μM. As the last anion incorporates a moiety amenable to the development of organic inhibitors, as already showed earlier,[21] we investigated diethyldithiocarbamate, a compound incorporating the CS_2^- moiety present in trithiocarbonate as PCA inhibitor, together with several lead compounds that could serve as basis for the design of even more effective inhibitors (Table 3). Sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic acid are potent zinc-binding molecules that were previously shown to target several CAs in the low micromolar range.[24,25]

Data of Table 3 show that PCA was also inhibited in the low micromolar range by these compounds, with K_Is in the range of 0.61–28.1 μM. Relevant for further research is the fact that phenylboronic acid and phenylarsonic acid inhibited rather well PCA and the other investigated β-CAs but not the human isoforms hCA I and

Table 2
Inhibition constants of anionic inhibitors against isozymes hCA I, and II (α-CA class), and β-isozymes Cab (from the archaeon *M. thermoautotrophicum*) as well as Can2 (from *C. neoformans*) and PCA (from *S. pneumoniae*), for the CO_2 hydration reaction, at 20 °C[21]

Inhibitor[a]	K_I[b] (mM)				
	hCA I[c]	hCA II[c]	Cab[d]	Can2[d]	PCA[e]
F^-	>300	>300	>1000	0.86	0.85
Cl^-	6	200	152	0.92	0.052
Br^-	4	63	42.1	1.00	0.046
I^-	0.3	26	13.2	1.11	0.054
CNO^-	0.0007	0.03	11.2	1.01	0.098
SCN^-	0.2	1.60	0.52	0.94	0.38
CN^-	0.0005	0.02	27.8	13.56	0.041
N_3^-	0.0012	1.51	55.7	0.73	0.35
HCO_3^-	12	85	44.9	0.75	0.33
CO_3^-	15	73	9.6	0.60	0.53
NO_3^-	7	35	7.8	0.92	0.39
NO_2^-	8.4	63	44.8	0.96	0.66
HS^-	0.0006	0.04	0.70	0.60	0.35
HSO_3^-	18	89	45.1	0.71	0.57
SnO_3^{2-}	0.57	0.83	nt	nt	0.066
SeO_4^{2-}	118	112	nt	nt	0.044
TeO_4^{2-}	0.66	0.92	nt	nt	0.049
OsO_5^{2-}	0.92	0.95	nt	nt	0.060
$S_2O_7^{2-}$	0.99	0.97	nt	nt	0.048
$P_2O_7^{4-}$	25.77	48.50	nt	nt	0.45
$V_2O_7^{4-}$	0.54	0.57	nt	nt	0.038
$B_4O_7^{2-}$	0.64	0.95	nt	nt	0.32
ReO_4^-	0.110	0.75	nt	nt	0.039
RuO_4^-	0.101	0.69	nt	nt	0.036
$S_2O_8^{2-}$	0.107	0.084	nt	nt	0.046
$SeCN^-$	0.085	0.086	nt	nt	0.022
FSO_3^-	0.79	0.46	nt	nt	0.060
CS_3^{2-}	0.0087	0.0088	nt	nt	0.021
SO_4^{2-}	63	>200	>200	0.86	4.15
ClO_4^-	>200	>200	>200	>200	>200

[a] As sodium salt.
[b] Errors were in the range of 3–5% of the reported values, from three different assays.
[c,d] From Refs. 12,20.
[e] This work.

Table 3
Inhibition constants of small molecule compounds against isozymes hCA I and II (α-CA class), and β-isozymes Cab (from the archaeon *M. thermoautotrophicum*) as well as Can2 (from *C. neoformans*) and PCA (from *S. pneumoniae*), for the CO_2 hydration reaction, at 20 °C[21]

Inhibitor[a]	K_I[b] (μM)				
	hCA I[c]	hCA II[c]	Cab[d]	Can2[d]	PCA[e]
$NH(SO_3)_2^{2-}$	0.31	0.76	nt	nt	28.1
$H_2NSO_2NH_2$	0.31	1.13	103	0.99	4.25
H_2NSO_3H	0.021	0.39	44.0	8.22	6.68
Ph-$B(OH)_2$	58.6	23.1	0.20	0.81	6.47
Ph-AsO_3H_2	31.7	49.2	0.33	0.87	5.86
$Et_2NCS_2^-$	0.79	3.1	nt	nt	0.61
AZA	0.250	0.012	12.1	0.0105	0.089

[a] As sodium salt.
[b] Errors were in the range of 3–5% of the reported values, from three different assays;
[c,d] From Refs. 5,12,20.
[e] This work.

II. Sulfamide and sulfamic acid were also effective PCA inhibitors (K_Is of 4.25–6.68 μM), whereas iminodisulfonate was the least effective one among this small group of molecules, with an inhibition constant of 28.1 μM (Table 3). However diethyldithiocarbamate was a very effective PCA inhibitor, with a K_I of 0.61 μM, whereas it has a roughly 5 times less affinity for hCA II, the physiologically dominant human isoform. Thus, this simple molecule may be considered as an excellent lead for developing better PCA inhibitors.

The best inhibitor for PCA investigated so far, appeared to be the clinically used sulfonamide acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide (AZA). Unsubstituted sulfonamide inhibitors are known to bind with high affinity only to CAs.[5] AZA showed efficient binding to the pneumococcal enzyme, with a K_Is of 89 nM (Table 3), which is better than the K_Is of the slow human isoform hCA I (250 nM), but higher than that of the physiologically dominant hCA II, which is inhibited with a K_I of 12 nM. Furthermore, inhibition of other β-CAs with this sulfonamide are quite variable, with their archeal enzyme Cab having only micromolar affinity for the compound (K_I of 12.1 μM), whereas the fungal one Can2 showing nanomolar affinity (K_I of 10.5 nM).[11] Interestingly, this data differ from the previous report[3] in which AZA and ethoxzolamide (EZA) were shown to be unable to inhibit PCA (in vitro).[3] However, in that study we were unaware of the inhibitory effect of chloride (present in the buffer solution), which might have interfered with the readout of our assays.[3] Still, preliminary in vivo pneumococcal growth inhibition experiments with AZA and EZA did not render the wild type strain dependent on CO_2-enriched medium (data not shown). This demonstrates that additional work on sulfonamides and other lead molecules with increased power to penetrate through the bacterial cell envelope as PCA inhibitors is a requisite for the successful use of this approach as a therapeutic tool to fight pneumococcal infection.

2.3. Phylogenetic analysis

PCA belongs to the β-CA class, which is a very divergent class of proteins, compared for example with the α-CAs.[4] Interestingly, the PCA gene that is present in the genomes of all *S. pneumoniae* strains available in the public databases and is very conserved (data not shown). This suggests that the gene is essential for pneumococcal dispersal throughout the population, and that effective PCA inhibitors might target most if not all pneumococcal strains. A phylogenetic tree build from an alignment of the amino acid sequences of β-CAs from various other bacterial, archeal, fungal, yeast, and nematode species (Fig. 2), revealed that PCA has only few close homologues. Pneumococcal CA is in the same phylogenetic branch as CAs of related streptococcal species, such as *Streptococcus pyogenes*. Besides, it clusters in the same clade[4] as one type of CAs found in Mycobacterium and Candida species. This suggests that inhibitors that were recently designed for the *M. tuberculosis* Rv1284 CA,[27] could be effective against PCA as well.

3. Conclusion

The β-CA from the bacterial pathogen *Streptococcus pneumoniae* was shown to be an efficient catalyst for the hydration of CO_2 to bicarbonate with an optimum pH at 8.4. These characteristics are physiologically relevant, and helpful to determine the precise role of this protein for growth and survival of this respiratory tract pathogen during transmission and disease. The inhibition profile for PCA was different from that of other α- and β-class CAs investigated earlier for their interactions with anions and similar small molecule inhibitors. It is thus envisageable that stronger PCA inhibitors can be detected by using as lead molecules the most potent zinc-binding groups evidenced here (trithiocarbonate, selenocyanate, dithiocarbamates, and sulfonamides) and by exploring a larger array of sulfonamides. Work is in progress in these laboratories to assess a large number of such derivatives to detect inhibitors with possible in vivo use.

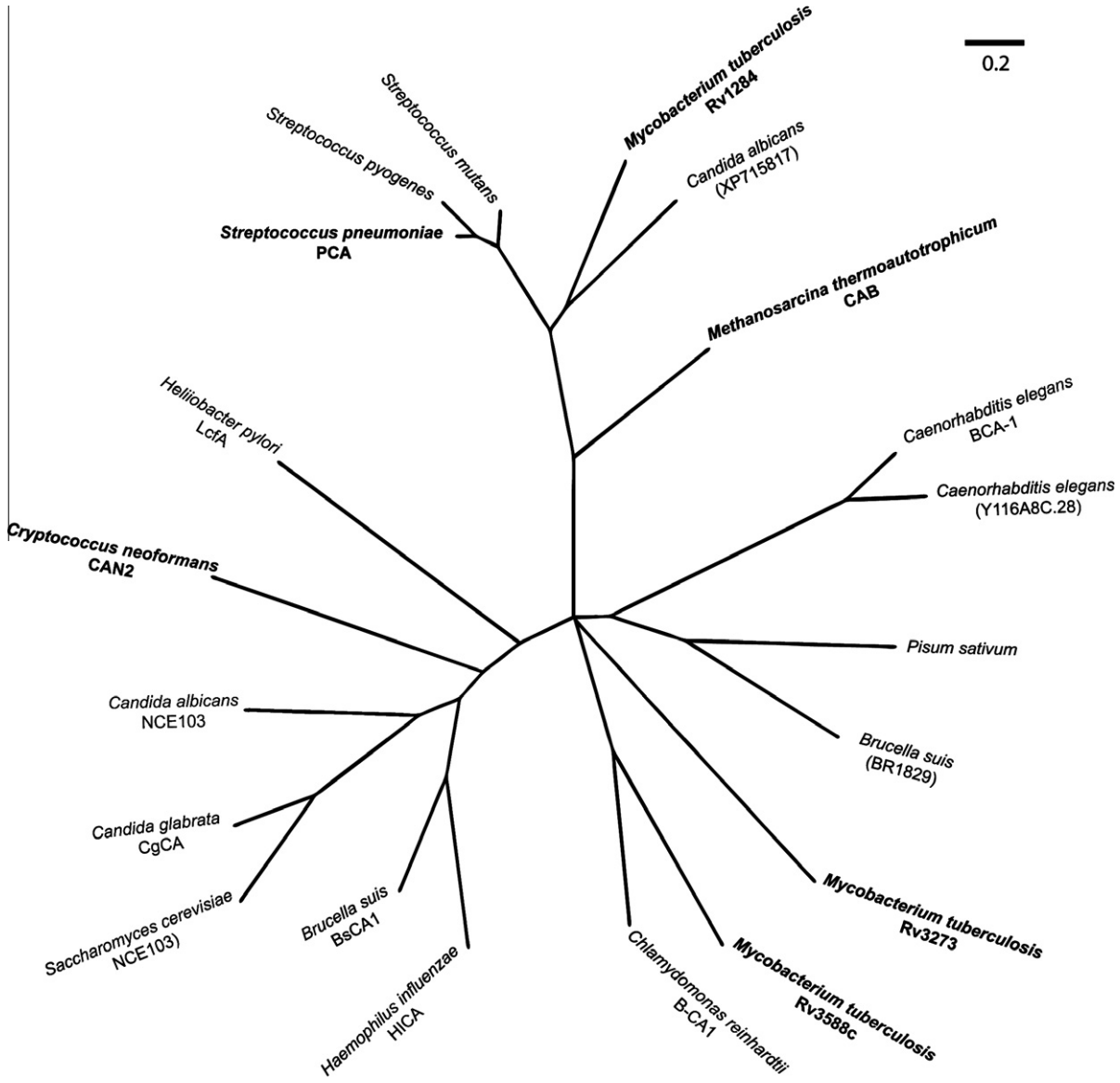

Figure 2. Phylogenetic tree of relevant β-class carbonic anhydrase proteins. The amino acid sequences of the selected β-CAs were imported in the MEGA program[26] and aligned by ClustalW. Phylogeny was calculated by the MEGA program's neighbor joining method with the poisson substitution model and a gamma distribution parameter of 2.0. Displayed is the consensus tree after 1000 bootstrap replicates. The β-CAs used for alignment in Fig. 1 are indicated bold. The bar scale indicates the expected number of amino acid replacements per site.

4. Experimental

4.1. Chemistry

Inorganic sodium salt of anions from Tables 2 and 3, buffers, sulfamide, sulfamic acid, phenylarsonic acid, phenylphosphonic acid, sodium diethyldithiocarbamate, and other reagents were the highest grade purity available, from Sigma–Aldrich (Milan, Italy).

4.2. PCA cloning, preparation, and purification

Production and purification of PCA as a glutathione *S*-transferase fusion protein (GST-PCA) was performed as earlier described[3] with only minor modifications. Briefly, an *Escherichia coli* BL21 (pWA4) culture was grown in 2× Luria Bertani broth at 37 °C to an optical density at 600 nm of 0.6–0.8, shifted to room temperature, and induced for GST-PCA production by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside. After 4 h, cells resuspended in lysis buffer (50 mM Tris–HCl pH 7.5, 300 mM NaCl, 0.5 mM dithiothreitol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% Triton X-100) with 1× protease inhibitor mixture (Complete Mini; Roche Applied Science), lysed by sonication, and GST-PCA was affinity purified from the soluble fraction with Glutathione Sepharose 4 Fast Flow beads (GE Healthcare) at 4 °C. Eluted GST-PCA was dialyzed against 10 mM Tris–HCl pH 7.5. The protein concentration in the solution was determined with a BCA Protein Assay Kit (Pierce).[3]

4.3. CA activity and inhibition measurements

An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity by the method of Khalifah.[21] Phenol red or *o*,*o*′-phenanthroline (at a concentration of 0.2 mM) have been used as indicators, working at the absorbance maximum of 557 nm and 554, respectively, with 10–20 mM HEPES (pH 7.5, for α-CAs) or Tris, HEPES or TAPS (pH

5.6–8.4 for β-CAs) as buffers, and 20 mM Na_2SO_4 (for α-CAs) or 10–20 mM $NaClO_4$—for β-CAs (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s.[21] 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 μM were done thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature 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 from Lineweaver–Burk plots, as reported earlier,[11–13] and represent the mean from at least three different determinations.

4.4. Alignment of protein sequences and phylogenetic analysis

The amino acid sequences of the selected β-CAs were imported in the MEGA program[26] and aligned by ClustalW. Phylogeny was calculated by the MEGA program's neighbor joining method with the poisson substitution model and a gamma distribution parameter of 2.0. Displayed is the consensus tree after 1000 bootstrap replicates. The β-CAs used for alignment in Figure 1 are indicated bold. The bar scale indicates the expected number of amino acid replacements per site.

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