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Inhibition studies of a β-carbonic anhydrase from *Brucella suis* with a series of water soluble glycosyl sulfanilamides

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ABSTRACT

A β-carbonic anhydrase (CA, EC 4.2.1.1) from the bacterial pathogen *Brucella suis*, bsCA 1, has been cloned, purified characterized kinetically and for inhibition with a series of water soluble glycosylated sulfanilamides. bsCA 1 has appreciable activity as catalyst for the hydration of CO_2 to bicarbonate, with a k_{cat} of 6.4×10^5 s^{-1} and k_{cat}/K_m of 3.9×10^7 M^{-1} s^{-1}. All types of inhibitory activities have been detected, with K_Is in the range of 8.9–110 nM. The best bsCA 1 inhibitor were the galactose and ribose sulfanilamides, with inhibition constants of 8.9–9.2 nM. Small structural changes in the sugar moiety led to dramatic differences of enzyme inhibitory activity for this series of compounds. One of the tested glycosylsulfonamides and acetazolamide significantly inhibited the growth of the bacteria in cell cultures.

The genome sequences of pathogenic bacteria belonging to the genus *Brucella* have been recently published.[1,2] *Brucella spp*. are facultative intracellular pathogens responsible of widespread zoonosis, known as brucellosis or Malta fever.[3–5] *Brucellae* are Gram-negative α-proteobacteria, infecting various vertebrates, from fish to primates. *Brucella melitensis* is the least host specific and also the most infectious for humans.[3–5] The other highly pathogenic species are *Brucella suis* (the primary host being the pig) and *Brucella abortus* (cattle), but more recently human cases of infection with the whale pathogen, *Brucella cetaceae*, have also been reported.[5] Brucellosis is difficult to fight, as these bacteria have developed strategies to evade immune recognition by the host.[3–5] The bacterium is able to cause enormous losses in agriculture, and is endemic in several areas such as the Mediterranean Europe, Middle East, and Latin America. The incidence of human brucellosis may be as high as 200 per 100,000 inhabitants.[6] *Brucella* is extremely infectious via the aerosol route (10 bacteria are sufficient to provoke disease) and is a potential bioterroristic agent,[6] especially as strains resistant to antibiotics used in the treatment of human brucellosis may be easily obtained, and no human vaccine is available so far.[7] Furthermore, it is worth to point out the emergence of highly resistant strains to common antibiotics in clinical isolates recently reported in Turkey and Balkanic countries.[7]

More generally, infections caused by bacteria represent indeed one of the main causes of mortality and morbidity worldwide.[8] Antibiotics are successfully used drugs, but the emergence of new pathogens, the reemergence of bacteria whose incidence had previously declined (such as *Mycobacterium tuberculosis*),[9] the antibiotic resistance of many common bacterial strains to several classes of antibiotics, as well as the potential of using bacteria as bioterrorism agents, led to considerable hurdles in fighting bacterial infections in recent years.[8,9] Such facts led to a renewed interest in the discovery of antibacterials able to act on novel molecular targets, circumventing the drug resistance problems.[8]

The growing information resulting from bacterial genomics[1,2] led to new targets for the design of mechanism-based drugs, by considering proteins required for the growth and/or virulence of such pathogens.[10] Among the many proteins encoded in the *Brucella* genome are also the carbonic anhydrases (CAs, EC 4.2.1.1),[11] zinc enzymes present in many other pathogens such as the protozoa *Plasmodium falciparum*[12] which provokes malaria, the bacterium *Helicobacter pylori*[13] which provokes ulcer and gastric cancer, highly pathogenic and drug-resistant fungi such as *Candida albicans* and *Cryptococcus neoformans*[14] and the widespread

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bacterial pathogen *M. tuberculosis*.[15] Many of the α- or β-class enzymes present in these organisms started to be investigated as new drug targets in the search of effective agents, devoid of drug resistance problems.[11–15] By searching for metalloenzyme-specific sequence motifs within the *B. suis* genome,[1b] two CA-encoding genes belonging to the β-class CA family (BR1829 and BRA0788) were identified by us. Considering these β-CAs as possible new targets, we report here the cloning, characterization, and inhibition studies with a panel of sulfonamides of the first β-CA from *B. suis*, denominated here bsCA 1.

bsCA 1 was cloned as hexa-histidyl-tagged fusion protein from the BRA0788 gene (accession number NC_004311) identified as a putative β-CA,[1b] and presents an identical sequence to clones previously deposited in GenBank (accession # NP_699962). This gene encodes a protein of 219 amino acid residues, which has the typical β-CA class metal-binding motif (see later in the text), and has a molecular weight (as a monomer) of 25 kDa. Furthermore, bsCA 1 has 51.0% homology with the *Escherichia. coli* Cyn T2 enzyme, 45.6% homology with the *Haemophilus. influenzae* enzyme, 27.1% homology with the *E. coli* Cyn T β-CA, 27.3% sequence homology with the *H. pylori* β-CA and 27.9% homology with the *Synecoccus elongates* enzyme (Fig. 1).[13,16,17]

Alignment of the amino acid sequence of bsCA 1 with that of other bacterial β-CAs recently investigated by this and other groups (Fig. 2),[13–20] such as the *E. coli* T2 and T enzymes,[16] the CA from *H. influenzae*,[17] as well as *S. elongates* β-CA, evidenced that bsCA 1 possesses the amino acids residues typical of β-CAs and involved in the catalytic cycle of this class of enzymes: (i) the Zn(II) binding residues Cys52, Asp54, His108, and Cys111, and (ii) the Asp54-Arg56 dyad, involved in the opening/closing of the active site.[20] Indeed, in the case of the β-CAs there are two types of metal ion coordination within the enzyme active site:

(i) the open active site, with the Zn(II) ion coordinated by two Cys and one His residues, and the fourth zinc ligand being a water molecule/hydroxide ion, which is responsible for the catalysis;[13–20]

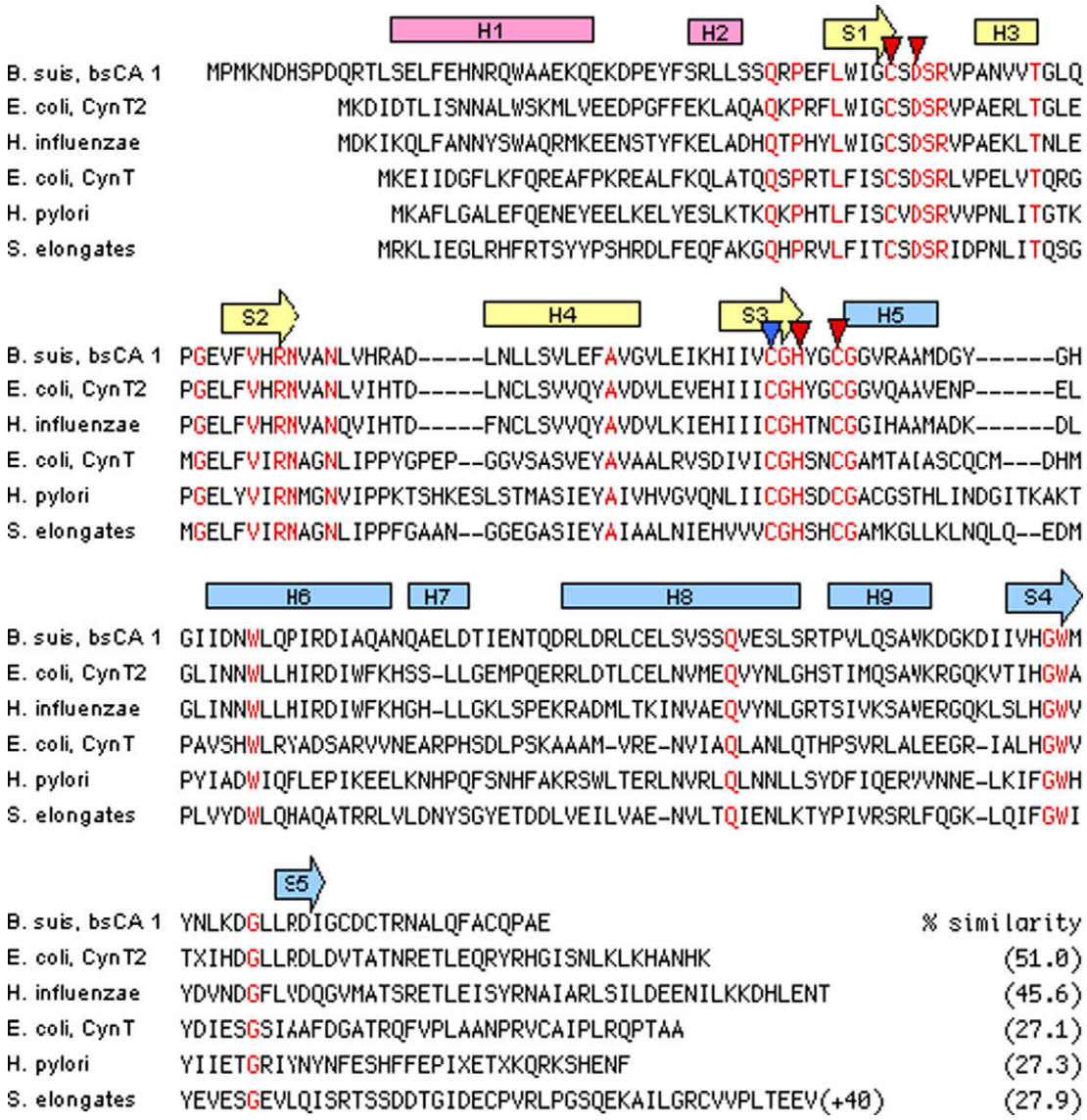


Figure 1. Alignment of bsCA 1 amino acid sequence with that of other bacterial β-CAs, including the two enzymes from *E. coli* (T and T2),[17] *H. influenzae* (P45148),[16] and *S. elongates*. Conserved amino acid residues in these β-CAs are indicated in red. The four zinc-binding residues, Cys42, Asp44, His98, and Cys101 are indicated by a red arrow above the residue (residue numbering is based on the *E. coli* CynT2 numbering system).[17] Another cysteine residue conserved in all these enzymes (Cys96) is evidenced by a blue arrow, but its role is largely unknown at this moment. Putative α-helices (H1–H9) and β-sheets (S1–S5) are also evidenced as bars/arrows above the corresponding sequence.

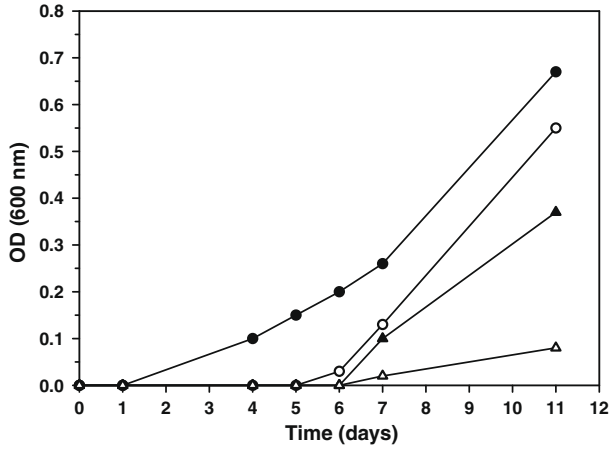


Figure 2. Inhibition of *B. suis* growth in minimal medium with sulfonamide CA inhibitors. Growth of untreated bacteria (●), of bacteria treated with 100 μM acetazolamide **AAZ** (○), and of bacteria treated with 10 μM (▲) or 100 μM (△) compound **7** was monitored over an incubation period of 11 days in minimal medium at 37 °C with shaking as described previously by one of our groups.[26]

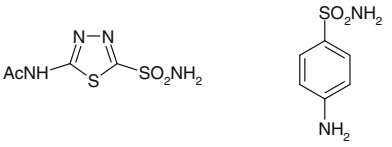
(ii) closed active site enzymes, with the Zn(II) ion coordinated by two Cys, one His, and one Asp residues, in the tetrahedral geometry typical of Zn(II) in metalloenzymes.[13–20] For these enzymes no water coordinated to the metal ion is present at pH values <8, as shown in an excellent crystallographic work from Jones' group on the mycobacterial enzymes Rv3558c and Rv1284.[20] However, at pH values >8, a conserved Arg residue in all β-CAs investigated so far (belonging to the catalytic dyad mentioned above)[18] makes a salt bridge with the Asp coordinated to Zn(II), liberating the fourth Zn(II) coordination position, which is occupied by an incoming water molecule/hydroxide ion.[20] Thus, the catalytic activity of the β-CAs possessing the closed active site can be measured only at pH values >8 (and this is the reason why we measure the catalytic/inhibitory activity of enzymes from this class at pH values of 8.3).[19,21] Based on the amino acid sequence, it is impossible to predict whether a β-CA will have a close or open active site. For example, the enzyme from the fungal pathogen *C. neoformans*, Can2, recently crystallized by Schlicker et al.[14b] was shown to possess and open active site and to be highly effective catalytically for the CO_2 hydration reaction. Based on these facts, we cannot establish what type of active site bsCA 1 possesses, that is, whether Asp 54 is coordinated to the metal ion at pH <8 (closed active site), or whether it is interacting permanently with Arg56 (in the Asp-Arg dyad mentioned above), which is critical for orientating the substrate and generating the nucleophilic species of the enzyme crucial for catalysis.

As shown in Table 1, we have measured the activity of bsCA 1 and compared it to that of other α-class enzymes of human origin, known to be drug targets, such as hCA I, II, VA, and XII. These data show that bsCA 1 has a significant activity as catalyst for the conversion of CO_2 to bicarbonate (at pH 8.3), with a k_{cat} of 6.4×10^5 s^{-1}, and k_{cat}/K_m of 3.9×10^7 M^{-1} s^{-1}. Thus, bsCA 1 was a better catalyst for the physiological reaction than the human isoforms hCA VA and hCA XII (know to be antiobesity[22] and anticancer[11,23,24] drug targets, respectively) and has an activity comparable to that of hCA I. Only hCA II, one of the best catalysts known in nature has a higher turnover number than hCA I and bsCA 1 (Table 1). It can be also observed that all these enzymes were appreciably inhibited by the clinically used sulfonamide compound, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide (see discussion later in the text).

Table 1
Kinetic parameters for CO_2 hydration reaction catalysed by some human α-CA isozymes at 20 °C and pH 7.5, and β-CA enzymes from *Brucella suis* (bsCA 1) at 20 °C, pH 8.3 in 20 mM Tris–HCl buffer and 20 mM NaCl, and their inhibition data with acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a clinically used drug[19]

Enzyme	Class	Activity level	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_I (acetazolamide) (nM)
hCA I[a]	α	Medium	2.0×10^5	5.0×10^7	250
hCA II[a]	α	High	1.4×10^6	1.5×10^8	12
hCA VA[a]	α	Low	2.9×10^5	2.9×10^7	63
hCA XII[a]	α	Low	4.2×10^5	3.5×10^7	5.7
bsCA 1[b]	β	Medium	6.4×10^5	3.9×10^7	63

[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method (pH 7.5), from Ref. 11,13c.
[b] Recombinant bsCA 1, stopped flow CO_2 hydrase assay method (pH 8.3), this work.[19]



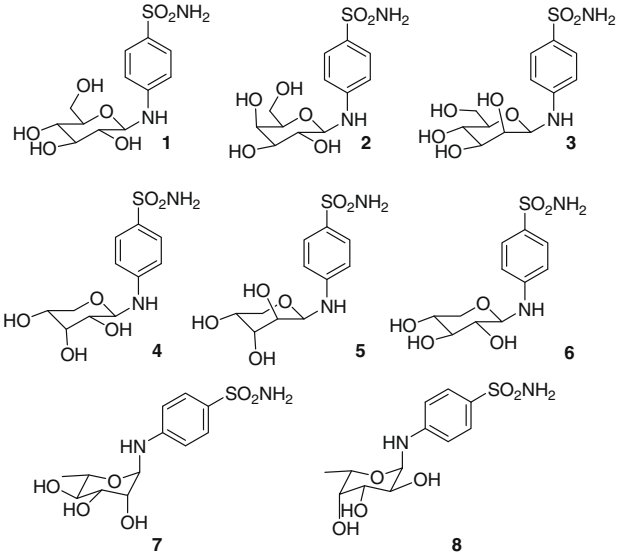
AAZ **SA**

Sulfonamides are indeed the main class of CA inhibitors (CAIs) with many such compounds used clinically.[11] However, one of the main hurdles of these derivatives is their poor water solubility.[22,23] Recently, we have reported a class of sugar conjugates of sulfanilamide, which are highly water soluble and also effective inhibitors of the human widely diffused isoforms hCA I and II.[25] In this Letter we report the investigation of the inhibitory effects of these glycosyl sulfanilamide derivatives, of types **1–8**,[25] against the new enzyme cloned and purified by us, bsCA 1 (Table 2).

The following structure activity relationship (SAR) can be observed from data of Table 2: two glycosyl sulfanilamides, that is, the glucose and mannose derivatives **1** and **3** showed weaker bsCA 1 inhibitory activity, with inhibition constants of 103–110 nM. Two other derivatives, that is, the arabinose **5**, xylose **6** sulfanilamides were similarly to acetazolamide **AAZ** medium potency bsCA 1 inhibitors, with K_Is in the range of 63–67 nM (Table 2). On the other hand, the remaining four sulfonamides, that is, the galactose, ribose, rhamnose, and fucose glycoconjugates of sulfanilamide **2**, **4**, **7**, and **8**, were very effective bsCA 1 inhibitors, with K_Is in the range of 8.9–28 nM. Thus, in this small series of sulfonamides we have identified both compounds with weak bsCA inhibitory activity, derivatives with medium potency as well as very effective, low nanomolar inhibitors. It is thus clear that very small structural changes in the sugar moiety of derivatives **1–8** have dramatic consequences for their enzyme inhibitory activity. For example the glucose and galactose derivatives **1** and **2**, which differ only by the stereochemistry of the OH group in position 4 of the sugar ring, have activities as bsCA 1 inhibitors which differ by a factor of 11.9. The same is true by comparing the mannose derivative **3** with the galactose one **2**, with **2** being a 11.2 times better bsCA 1 inhibitor than **3**. The best bsCA 1 inhibitor detected so far was the ribose glycoconjugate **4** (K_I of 8.9 nM). It must be also observed that sulfanilamide **SA** is a quite weak bsCA 1 inhibitor (K_I of 2500 nM).

It should be noted from the data of Table 2 that all sulfonamides **1–8** were much weaker hCA I than bsCA 1 inhibitors, with selectivity ratios for inhibiting the bacterial over human enzyme of 9.0–108.7. The most *Brucella*-specific inhibitors (over hCA I) were **2** and **3**, with a selectivity ratio of 108.7–109. Acetazolamide **AAZ** was on the other hand a less selective such agent, with a selectivity ratio of only 3.9 in inhibiting bsCA 1 over hCA I. However, not the

Table 2
Human (h) hCA I, II, and *Brucella* enzyme, bsCA I inhibition data with compounds **1**–**8** and the clinically used sulfonamide acteazolamide, **AAZ**



Inhibitor	K_I[a] (nM)			Selectivity ratios	
	hCA I[b]	hCA II[b]	bsCA I[c]	hCA I/bsCA I	hCA II/bsCA II
1	1200	23	110	109	0.21
2	1000	25	9.2	108.7	1.7
3	930	18	103	9.0	0.17
4	840	16	8.9	94.4	1.8
5	630	12	65	9.7	0.18
6	750	15	67	11.2	0.22
7	510	10	19	26.8	0.52
8	680	14	28	24.3	0.50
AAZ	250	12	63	3.9	0.19
SA	25000	240	2500	10.0	0.096

Inhibition data of sulfanilamide (**SA**) is also shown for comparison.
[a] Errors in the range of 5–10% of the shown data, from three different assays.
[b] Human recombinant isozymes, stopped flow CO_2 hydrase assay method, from Ref. 25.
[c] Recombinant bsCA 1, stopped flow CO_2 hydrase assay method, this work.

same is true regarding the physiologically dominant[11] isoform hCA II. In this case, only two sulfonamides had a selectivity ratio for inhibiting the bacterial over host enzyme >1, that is, **2** and **4**, with selectivity ratios of 1.7–1.8. All other glycosylated sulfanilamides, similarly to acetazolamide, were better hCA II than bsCA 1 inhibitors, with selectivity ratios of 0.17–0.52 (Table 2).

We have investigated the effect of some sulfonamide CA inhibitors on the growth of *B. suis* in vivo, in cell cultures.[26] As seen from data of Figure 2, the bacteria showed an exponential growth starting with day 4, in the minimal growth medium (i.e., phosphate buffer, with glutamic acid and glycerol as carbon source, and ammonium sulfate as nitrogen source. No bicarbonate/carbonate is present in this medium).[26] In the presence of 100 μM **AAZ** or compound **7**, a significant inhibition of the bacterial growth has been observed after 8–11 days of culture. Acetazolamide had a much weaker effect compared to the glycosylsulfonamide **7**, which was extremely effective in inhibiting the growth of *B. suis* both at 100 μM as well as at 10 μM concentration of inhibitor in the assay system (Fig. 2). It should be also noted that **7** is a stronger in vitro inhibitor of bsCA 1 compared to **AAZ** (Table 2). These preliminary data show bsCA 1 inhibitors to possess a significant antibiotic effect but the mechanism of action of such compounds warrants further studies. It is known that some CA isozymes in mammals,[27] arthropods[28] or yeasts[29] are involved in biosynthetic processes in which CO_2/bicarbonate are involved (e.g., carboxylation reactions catalyzed by pyruvate carboxylase, acetyl-coenzyme A carboxylase, etc.). Such reactions lead to the biosynthesis of Krebs cycle intermediates and we speculate that in *B. suis* a similar effect may be responsible for the inhibition of growth reported in Figure 2. These data strongly suggest that bacterial β-CA inhibition may represent an alternative strategy for designing agents with a new mechanism of action compared to classical antibiotics.

In conclusion, we report here that a β-CA from the bacterial pathogen *B. suis*, bsCA 1, has appreciable activity as catalyst for the hydration of CO_2 to bicarbonate, with a k_{cat} of 6.4×10^5 s^{-1}, and k_{cat}/K_m of 3.9×10^7 M^{-1} s^{-1}. A number of sulfonamides incorporating sugar moieties have been investigated for inhibition of this new β-CA. All types of activities have been detected, with K_Is in the range of 8.9–110 nM. The best bsCA 1 inhibitors were the galactose and ribose sulfanilamides, with inhibition constants of 8.9–9.2 nM. Small structural changes in the sugar moiety led to dramatic differences of enzyme inhibitory activity for this small series of compounds. One of the tested glycosylsulfonamides and acetazolamide significantly inhibited the growth of the bacteria in cell cultures.

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19. Khalifah, R.G. *J. Biol. Chem.* **1971**, *246*, 2561. An Applied Photophysics stopped flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10–20 mM Hepes (pH 7.5, for α-CAs) or TRIS (pH 8.3 for β-CAs) as buffers, and 20 mM Na_2SO_4 (for α-CAs) or 10–20 mM NaCl—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. 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 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,[15] and represent the mean from at least three different determinations.
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21. The CA-encoding gene *BRA0788* (accession number NC_004311), described here as bsCA 1, was specifically amplified by PCR using *B. suis* 1330 chromosomal DNA as template and OPJ17-foward primer (5′-GCGGGCATATGCCCATGAAGAACGATC-3′) and OPJ18-reverse primer (5′-GCGCGGGATCCTTATTCTGCCGGTTGGCAGG-3′) which contain *Bam*HI and *Nde*I recognition sequences (underlined), respectively. The PCR products were digested with *Bam*HI and *Nde*I and ligated to *Bam*HI- and *Nde*I-digested pET15b (Novagen) prior to introduction into *E. coli* strain DH5α. The integrity of the cloned gene was verified by sequencing, using primers OPJ17 and OPJ18 described above. The construct pET15bCA was then transformed into *E. coli* strain BL21(DE3) for production of the 6x(His)-CA fusion protein. *E. coli* BL21(DE3) harboring pET15bCA was grown at 37 °C in two litres of Luria–Bertani medium supplemented with 50 μg/ml ampicillin. When the culture reached an optical density at 600 nm (OD_{600}) of approximately 0.6, expression of 6x(His)-CA protein was induced by the addition of isopropyl-thio-β-D-galactoside (IPTG) to a final concentration of 1 mM and growth was continued for 5 h. Cells were then harvested by centrifugation at 3500 rpm at 4 °C for 20 min and broken by sonication in buffer A (200 mM KCl, 50 mM Tris–Cl (pH 7.5), 10% glycerol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A) supplemented with 0.5 mM dithiothreitol and 0.2 mM disodium EDTA. All subsequent steps were performed at 4 °C. After centrifugation (13000 rpm, 20 min), the soluble extract was treated with streptomycin sulfate to remove ribosomes and nucleic acids. The suspension was then centrifuged at 13000 rpm for 15 min, and the supernatants were dialyzed against two litres of sonication buffer A for 1 h. The dialyzed lysates were mixed with Talon Co^{2+}-affinity resin (Clontech) that has been equilibrated with buffer I (20 mM Tris–HCl (pH 8.0), 5 mM β-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, and 0.1% Nonidet P-40) supplemented with 150 mM KCl. The resin and bound His-tagged protein were collected by centrifugation and washed with buffer I containing 500 mM KCl and 10 mM imidazole. A subsequent wash was performed with buffer I supplemented with 125 mM KCl and 75 mM imidazole without Nonidet P-40. The 6x(His)-CA protein was eluted with buffer I containing 125 mM KCl and 150 mM imidazole without Nonidet P-40. Elution fractions were free of detectable contaminating proteins as determined by Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels. The fractions containing the His-tagged proteins (estimated purity, >95%) were pooled and dialyzed prior lyophilisation. The purified bsCA 1 was dialyzed sequentially against the following buffer, buffer B (125 mM KCl, Tris–HCl (pH 7.5), imidazole 100 mM), buffer C (50 mM KCl, Tris–HCl (pH 7.5), imidazole 50 mM), and buffer D (10 mM Tris–HCl, pH 8.3). The His_6 tag has been removed in a small sample of the protein by treatment with enterokinase (Sigma–Aldrich). However the catalytic activity and acetazolamide inhibition of the His-tagged and untagged enzymes were identical (data not shown), so that the data of Tables 1 and 2 refer to the enzyme with the (6x)His-tag.
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