An α -carbonic anhydrase from the thermophilic bacterium *Sulfurihydrogenibium azorense* is the fastest enzyme known for the CO₂ hydration reaction

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ABSTRACT

We report the biochemical characterization of a new carbonic anhydrase (CA, EC 4.2.1.1), named SazCA, identified by translated genome inspection in *Sulfurihydrogenibium azorense*, a thermophilic bacterium from terrestrial hot springs of the Azores. SazCA is an α -CA showing kinetic parameters that make it the fastest enzyme of the CA family described so far. The biochemical properties, thermostability and inhibition of SazCA were compared with those of the thermophilic and mesophilic counterparts, demonstrating the special features of this unique enzyme.

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1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc-containing enzymes that catalyze the reversible conversion of carbon dioxide to bicarbonate and protons.^{1–3} The evolutionary analysis of CAs revealed the existence of five distinct classes, the α , β and γ classes and the lately discovered, δ and ζ classes in marine diatoms.^{4–8} In bacteria the existence of genes encoding CAs from all the three classes α , β and γ , suggests that CO₂ hydratase activity plays an important role in prokaryotic physiology. Several roles were proposed for prokaryotic CAs, such as the fixation of CO₂ for photosynthesis, the transport of CO₂ or HCO₃[–]; the supply of CO₂ or HCO₃[–] for the enzymatic reactions; the involvement in cyanate degradation; and the survival of intracellular pathogens within their hosts.^{6,7}

Bacteria can be classified into psychrophiles, mesophiles, and thermophiles. The last group can be subdivided into moderate thermophiles with an optimal growing temperature in the range of 55–65 °C, facultative thermophiles that are able to grow over a wide temperature span, and extreme thermophiles (hyperthermophiles) that grow only above 80 °C.⁹ Owing to the temperature range at which their biomolecules function, thermophiles have

also been considered a valuable source of thermostable enzymes. The large number of sequences in the databases, the tools to search for desired sequences, and the possibility to synthesize genes for expression in conventional host cells provided us to examine genes encoding novel CAs. Recently, our group described the biochemical properties of a bacterial α -CA from *Sulfurihydrogenibium yellowstonense* YO3AOP1.^{10,11} Here, we report the biochemical characterization of a novel CA, named SazCA, identified by translated genome inspection in *Sulfurihydrogenibium azorense*, another recently discovered thermophilic bacterium living in the terrestrial hot springs of the Azores. Intriguing, unlike other CAs identified earlier in thermophiles, which belonged to the γ CA class¹², SazCA belongs to the α class, and showed kinetic parameters that make this new enzyme the fastest one among the CAs described so far. Superoxide dismutase (SOD) and some CAs (hCA II and IX, h = human isoform) are among the fastest enzymes known to date, possessing k_{cat}/K_M values in the range of 1.5×10^8 – 7×10^9 M^{–1} s^{–1}.^{13–16}

SazCA, the enzyme reported and characterized here, is the most effective CA ever investigated, and the second most efficient enzyme known to date (see later in the text). The biochemical properties of SazCA were compared with those of thermophilic and mesophilic CAs. The thermophilic counterpart was represented by the highly thermostable α -CA from *S. yellowstonense* YO3AOP1 (SspCA), while members of the mesophilic counterpart were showed by the carbonic anhydrase from *Helicobacter pylori*

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(hp α CA), the highly active human CA (hCA II), the human isoenzyme I (hCA I) and the bovine CA (bCA II).

2. Experimental

2.1. Gene identification, construct preparation, protein expression and purification

The identification of SazCA was performed with the 'FASTA' program. The nucleotide sequence encoding for the carbonic anhydrase of *Sulfurihydrogenibium yellowstonense* YO3AOP1 (Accession number: ACD66216.1), was used as reference sequence. Among others, the CA sequence from *Sulfurihydrogenibium azorense* (SazCA) was screened. The GeneArt Company (Life Technologies, UK), specialized in gene synthesis, designed the synthetic *S. azorense* gene encoding for the α -carbonic anhydrase lacking the peptide signal (first 20 amino acid of the peptide sequence). Details concerning the construct preparation, protein expression and purification of SazCA are the same described for the preparation on the recombinant SspCA described by Capasso C et al.¹⁰

2.2. Assay for carbonic anhydrase with CO₂ as substrate

CA activity assay was a modification of the procedure described by Chirica.¹⁷ The assay was based on the monitoring of pH variation due to the catalyzed conversion of CO₂ to bicarbonate. For details see Capasso et al.¹⁰

2.3. Esterase activity

Activity for *p*-nitrophenylacetate (p-NpA) hydrolysis was determined at 0 °C using a modification of the method proposed by Armstrong et al.¹⁸ The reaction mixture contained 0.3 mL of freshly prepared 3 mM p-NpA and 0.7 mL of 15 mM Tris sulphate buffer, pH 7.6. For details see Capasso et al.¹⁰

2.4. Enzyme kinetics and inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO₂ hydration activity. 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 NaBF₄ for maintaining constant ionic strength, following the initial rates of the CA-catalyzed CO₂ hydration reaction for a period of 10–100 s. The CO₂ 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^{19,20}, and represent the mean from at least three different determinations.

2.5. Temperature studies

2.5.1. Effect of temperature on the SazCA, SspCA and bCA II activity

The temperature dependence activity was measured using the p-NpA as substrate. Protein concentration used in the assay was

300 ng. The activity was measured in the temperature range from 25 to 100 °C. All the reactions were performed in triplicate.

2.5.2. Effect of temperature on the Saz, SspCA and bCA II stability

To compare the stability of SazCA, SspCA and bCA II at different temperatures, enzymes at the concentration of 3 μ g/mL in 10 mM Tris/HCl, pH 8.3 were incubated at 40, 50, 60, 70, 80, 90 and 100 °C for different time (30, and 180 min). Enzyme aliquots (30 ng) were withdrawn at appropriate times and the residual activity was measured at 0 °C using CO₂ as substrate.

3. Results and discussion

Analysis by SDS–PAGE of the SazCA showed a single band of about 26 kDa (data not show).

Alignment of the SazCA sequence with that from different microorganisms and several other species (Fig. 1) indicated that the bacterial polypeptide chain showed all the typical features of an α -CA: (a) the three histidines (His94, 96 and 119); (b) the proton shuttle residue (His64); (c) the gate-keeping residues (Glu106 and Thr199) involved in the orientation of the substrate and in the binding of the α -CA inhibitors. We observed that in the primary structure of the bacterial CAs (both from thermophilic and mesophilic species) four amino acid loops are absent, whereas they are present in the mammalian sequences (Fig. 1). This feature has not been noticed earlier. The phylogenetic tree (Fig. 2) showed that the thermophilic enzymes (SazCA and SspCA) formed a cluster closer to the mammalian counterpart than to the others α -CAs of bacterial origin, such as the *Neisseria gonorrhoeae*, *Helicobacter pylori*, and *Streptococcus salivarius* CAs. In contrast, the mammalian α -CAs formed a distinct paraphyletic group.

The specific activities for SazCA, SspCA, hp α CA and hCA II were determined using CO₂ as substrate, by a stopped-flow assay method.²¹ As reported in Table 1, the CO₂ hydrase activity of SazCA was the highest among all CAs known to date, being 2.33 times higher than that of hCA II (considered the most active CA up until now). α CAs also catalyze the hydrolysis of esters.^{18,22,23}

With *p*-nitrophenyl acetate (p-NpA) as substrate, the presence of esterase activity was investigated at 0° for SazCA and other such enzymes.¹² SazCA showed a rather low specific activity towards pNpA. The SazCA esterase activity (62 U/mg protein) was 5 and 21 times lower than that of SspCA and bovine bCA II, 'respectively'. We then performed thermoactivity and thermostability studies which showed that SazCA is active in the temperature range from 0 to 100 °C, unlike the mammalian enzymes which are denatured at temperatures higher than 60 °C. The temperature dependence activity of SazCA was assayed following the esterase activity and measurements were made in parallel for the thermophilic (SspCA) and mesophilic (bCA II) counterparts. The optimal temperature for the bovine enzyme activity was found to be 60 °C, while SazCA and SspCA, as expected for thermophilic enzymes, showed an optimum of temperature at 80–90 °C, being still active at 100 °C (Fig. 3A).

Stabilities of SazCA, SspCA and bCA II were compared at the temperatures indicated in Figure 3B. After 30 min, and 180 min of incubation, the activities of the enzymes were determined using CO₂ as substrate. The bovine enzyme was inactivated at temperature higher than 60 °C (Fig. 2B) for all incubation times. Noticeably, the bacterial enzymes were active at the temperature of 100 °C, with the exception that SspCA maintained most of its activity at 100 °C while, SazCA was more stable than the mesophilic enzyme and slightly less stable than SspCA. Our studies indicated that the bacterial CAs were more stable at high temperature and retained their activity for a longer time (e.g., 180 min) when compared to the mammalian enzyme.

Figure 1. Multialignment of the amino acid sequences of α -CAs from different organisms performed with the program Clustal W, version 2.1. Legend: hCA I, *Homo sapiens*, isoform I (Accession n.: NP_001158302.1); hCA II, *Homo sapiens*, isoform II (Accession n.: AAH11949.1); bCA II, *Bos Taurus*, isoform II (Accession n.: NP_848667.1); hp α CA, *Helicobacter pylori* J99 (Accession n.: NP_223829.1) CA; NgCA, *Neisseria gonorrhoeae* enzyme (Accession number: CAA72038.1); SsCA, *Streptococcus salivarius* PS4 CA, (Accession number: EIC81445.1). SspCA, *Sulfurihydrogenibium* sp. YO3AOP1 (Accession n.: ACD66216.1); SazCA, *Sulfurihydrogenibium* azorensis (Accession n.: ACN99362.1) 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 CO₂ hydratase reaction are indicated in bold. The zinc ligands (His94, 96 and 119 in bold), the proton shuttle residue (His64, in bold) and the gatekeeper residues (Glu106 and Thr199, in bold) are conserved in all these enzymes. The four loops absent in the bacterial enzymes are highlighted in grey. The hCAI numbering system was used.

Figure 2. Phylogenetic analysis of some α -CAs discussed here. The tree was constructed using the program PhyML 3.0. Branch support values are reported at branch points. Legend: hCAII, *Homo sapiens*, isoform II (Accession n.: AAH11949.1); bCA II, *Bos Taurus*, isoform II (Accession n.: NP_848667.1); hCAI, *Homo sapiens*, isoform I (Accession n.: NP_001158302.1); SazCA, *Sulfurihydrogenibium* sp. YO3AOP1 (Accession n.: ACD66216.1); hp α CA, *Helicobacter pylori* J99 CA (Accession n.: NP_223829.1); SsCA, *Streptococcus salivarius* PS4 CA, (Accession number: EIC81445.1); NgCA, *Neisseria gonorrhoeae* CA (Accession number: CAA72038.1).

Table 1

Comparison of the CO₂ hydrase activities of SazCA, SspCA (from thermophilic bacteria), hp α CA (from *Helicobacter pylori*) and hCA II

Enzyme	k_{cat} (s ⁻¹)	K_M (mM)	k_{cat}/K_M (M ⁻¹ s ⁻¹)
SazCA	4.40×10^6	12.5	3.5×10^8
SspCA	9.35×10^5	8.4	1.1×10^8
hCA II	1.40×10^6	9.3	1.5×10^8
hp α CA	2.5×10^5	16.6	1.5×10^7

It is important to note that there is enormous industrial/bio-technological interest in using CAs as bio-catalysts for carbon sequestration and biofuel production,¹ and recent work from McKenna's laboratory reported mutants of the mammalian hCA II which showed a better thermostability compared to the native protein. This thermostability has been induced by mutation of

Figure 3. (A and B). (A) Determination of optimal activity temperature of SazCA, SspCA and bCA II. The enzyme activity was measured at the temperatures in the range from 25 to 100 °C using p-NpA as substrate. The results are representative of three independent sets of measurements for which the maximal difference in absorbance at each temperature was 0.20%. (B) Thermostability of SazCA, SspCA and bCA II. The enzymes were incubated for 30 and 180 min at the indicated temperatures on the X-axis and assayed using CO₂ as substrate. Each point is the mean $\pm$ SEM of three independent determinations.

amino acids on the surface of the protein.¹ The CA investigated here, SazCA, similar to the related SspCA from another *Sulfurihydrogenibium* species reported earlier,¹⁰ shows a very high stability at various temperature, and survive to denaturation even after various cycles of heating at temperatures as high as 100 °C. Thus, these robust enzymes from extremophilic bacteria may have important applications in biotechnologic processes dealing with CO₂ fixation, due to their extremely high catalytic activity (we stress again, SazCA is the most active CA known to date) and because they are highly stable in harsh environments such as high temperatures or in the presence of high concentrations of anions (e.g., bicarbonate, see discussion later in the text) which normally act as inhibitors.²⁴

The main classes of CA inhibitors (CAIs) known to date are the inorganic anions with affinity for metal ions,^{24–27} the sulfonamides and their isosteres,^{28–31} the dithiocarbamates,^{32–34} as well as some diverse chemotypes (e.g., coumarins, thioxocoumarins, tio- and dithiocoumarins)^{35–40} which unlike the previously mentioned CAIs, do not bind directly to the metal ion from the enzyme active site but at the entrance of the cavity, being anyhow mechanism-based inhibitors acting as prodrugs and needing the esterase CA activity for the generation of the inhibitory species.^{35,38}

We have investigated here the inhibitory profile of the new enzyme SazCA with some of the main representatives of the inhibitor classes mentioned above, that is, some inorganic anions, a dithiocarbamate, a sulfonamide and several small molecules incorporating zinc-binding moieties of the sulfonamide/ dithiocarbamate type (Table 2). As the bacteria belonging to the genus *Sulfurihydrogenibium* belong to the chemosynthetic bacterial communities living in hot springs, at temperatures up to 110 °C, and in the presence of rather high hydrogen sulfide concentrations (between 1–100 μ M),^{19,20} it is important to understand whether the CA cloned and characterized here is inhibited by sulfur-containing compounds such as hydrogen sulfide, hydrogen sulfite and the sulfate anions.

Furthermore, halides and bicarbonate (the reaction product of CO₂ hydration) may also be present in relevant concentrations in the environment in which these extremophiles live. Thus, these and similar inorganic anions were included in the study. (Table 2) Data of Table 2 show that the halides were submillimolar inhibitors of SazCA (inhibition constants in the range of 0.87–0.98 mM with the four halide anions). SazCA has much higher affinity for

Table 2

Inhibition of hCA II, SspCA, SazCA and hp α CA with several anions, sulfonamides and a dithiocarbamate. AAZ = acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide

Inhibitor	hCA II ^{a,b}	K _i (μ M) SspCA ^{a,b}	SazCA ^b	hp α CA ^b
F ⁻	> 300,000	417,000	980	4080
Cl ⁻	200,000	8300	850	2700
Br ⁻	63,000	49,000	940	2410
I ⁻	26,000	860	870	6050
HCO ₃ ⁻	85,000	33,200	15,700	750
HSO ₃ ⁻	89,000	21,100	950	990
HS ⁻	40	580	380	690
SCN ⁻	1600	710	580	4100
SeCN ⁻	86	70	490	730
CS ₃ ²⁻	8.8	60	39	380
Et ₂ NCS ₂ ⁻	3100	4.0	3.1	5.0
SO ₄ ²⁻	> 300,000	820	10,000	820
H ₂ NSO ₃ H	390	42	4.8	80
AAZ	0.012	0.004	0.0009	0.021

^a Data from ref. 10.

^b Errors in the range of $\pm$ 5% of the reported data from three different assays.

these anions compared to SspCA isolated from a related bacterium living in the Yellowstone National Park¹⁰, or the human isoform hCA II (Table 2), which were scarcely inhibited by these anions. The 'extremo- α -CAs' were strongly inhibited by the iodide ion respect to the mammalian hCA II and bacterial hp α CA (Table 2). In literature, has been described a structural study indicating that in the β -CA from *Coccomyxa*, a unicellular green alga, the iodide ion is bound to the zinc ion via a bridging water molecule.⁴¹ Moreover, in hCAI has been reported a different binding of this anion. The iodide, in fact, is tetrahedrally coordinated directly to the zinc, replacing the zinc-bound water molecule and rendering the enzyme inactive.⁴² The structural modifications in the active site of the 'extremo- α -CAs' constitute the possible explanation for the difference in binding of the iodide ion. Bicarbonate was also a better SazCA than SspCA or hCA II inhibitor, whereas hydrogen sulfide inhibited better hCA II (K_i of 40 μ M) compared to the two bacterial enzymes (K_i s in the range of 380–580 μ M). This may be an evolutionary adaptation of the bacterial enzymes from these extremophiles which survive in the presence of high concentrations of H₂S, and the corresponding anions is thus less inhibitory for their catalytic activity compared to the human isoform hCA II. Thiocyanate and selenocyanate were also medium potency SazCA

inhibitors (K_i s in the range of 490–580 μM). Sulfate on the other hand was a very weak SazCA inhibitor (the weakest among the compounds investigated here), with inhibition constants of 10 mM (but this anions inhibits better SspCA). Tritiocarbonate, diethylditiocarbamate and sulfamate were on the other hand quite effective SazCA inhibitors (K_i s in the range of 3.1–39 μM), whereas the clinically used sulfonamide acetazolamide (AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide) showed an excellent inhibitory activity, with a K_i of 0.9 nM (inhibiting SazCA much more than SspCA or hCA II, which showed K_i s of 4.0 and 12.0 nM, 'respectively', Table 2).

In conclusion, we report here the cloning and characterization of an α -CA from the recently discovered thermophilic bacterium *Sulfurihydrogenibium azorense*, which resulted to be the most catalytically effective CA ever investigated, and as far as we know, the second fastest enzyme after human SOD. Indeed, SazCA, was 2.33 times a better catalyst for the CO₂ hydration reaction compared to hCA II, considered up to now the best catalyst in this family of enzymes, possessing a k_{cat} of $4.40 \times 10^6 \text{ s}^{-1}$ and a k_{cat}/K_M of $3.5 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. SazCA was less effective as an esterase with 4-nitrophenyl acetate as substrate, but was highly thermostable, surviving to incubation at 90–100 °C for several hours, without any loss of the catalytic activity. Similar to other α -CAs, SazCA was sensitive to anion, sulfonamide and dithiocarbamate inhibitors, with acetazolamide being a subnanomolar SazCA inhibitor. The robustness and excellent activity of this enzyme make it an interesting candidate for biotechnologic applications in the field of CO₂ fixation and biofuel production.

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