Biochemical, structural, and computational studies of a γ-carbonic anhydrase from the pathogenic bacterium *Burkholderia pseudomallei*

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A B S T R A C T

Melioidosis is a severe disease caused by the highly pathogenic gram-negative bacterium *Burkholderia pseudomallei*. Several studies have highlighted the broad resistance of this pathogen to many antibiotics and pointed out the pivotal importance of improving the pharmacological arsenal against it. Since γ-carbonic anhydrases (γ-CAs) have been recently introduced as potential and novel antibacterial drug targets, in this paper, we report a detailed characterization of BpsγCA, a γ-CA from *B. pseudomallei* by a multidisciplinary approach. In particular, the enzyme was recombinantly produced and biochemically characterized. Its catalytic activity at different pH values was measured, the crystal structure was determined and theoretical pKa calculations were carried out. Results provided a snapshot of the enzyme active site and dissected the role of residues involved in the catalytic mechanism and ligand recognition. These findings are an important starting point for developing new anti-melioidosis drugs targeting BpsγCA.

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1. Introduction

Melioidosis is a severe disease that is estimated to provoke 89,000 deaths per year worldwide [1]. It is caused by the highly pathogenic Gram-negative bacterium *Burkholderia pseudomallei* [1], commonly found in soil and surface water of many tropical and subtropical regions [2–4]. Although most cases of melioidosis have been identified in northern Australia and Southeast Asia, increased travel and migration have augmented the incidence in other parts of the world, thus causing significant health and socioeconomic burden.

*B. pseudomallei* can infect humans and a wide range of animals, adopting different routes of infection [1]: skin penetration is considered to be the most common mode of transmission [5], whereas its ingestion by contaminated water and inhalation also represent important means to infect hosts [6–8]. Because inhalation of *B. pseudomallei* can lead to severe disease with high mortality, the bacterium is also regarded as a significant potential biothreat agent [9,10].

A large variability of clinical symptoms has been recognized in patients with melioidosis spanning from localized cutaneous manifestations at the bacterial entry site with no systemic manifestations to sepsis and death. Pneumonia is the most prevalent presentation of this disease and is involved in approximately half of all cases, bacteremia occurs in 40–60 % of all patients, whereas septic shock has been observed in ~ 20 % of all cases. Dissemination of the bacteria to internal organs is also common, particularly to spleen, prostate, liver, and kidney [11].

The recommended melioidosis treatment consists of two steps: an initial intensive phase, which should last a minimum of 10–14 days with the administration of intravenous ceftazidime or
meropenem antibiotics, followed by an eradication phase with oral antibiotics recommended for a long variable period (ranging from 3 to 6 months), to avoid recrudescence of the disease or relapse of the patient [12].

Several studies have been reported in literature on the molecular mechanisms responsible for B. pseudomallei pathogenicity highlighting its remarkable intrinsic array of virulence factors [13] and the broad resistance to many antibiotics including penicillin, ampicillin, and first- and second-generation cephalosporins [1,14,15]. Considering that B. pseudomallei infection can involve many people and that its antibiotic resistance will likely increase in the future, the improvement of the pharmacological arsenal against this pathogen is of pivotal importance.

An up-to-date strategy to develop anti-microbial drugs with novel mechanisms of action consists in the identification of new bacterial enzymes involved in cellular pathways crucial for the life cycle and/or the virulence of pathogenic organisms and in the development of molecules able to interfere with their activity [16]. In this context, members of the carbonic anhydrase (CA, EC 4.2.1.1) family have recently emerged as suitable targets; indeed, compelling data in literature strongly indicate that interference in catalyzing the CO2 hydration reaction (kcat = 1.6 \times 10^7 \text{ M}^{-1} \text{s}^{-1}) [23–25]. They are grouped into eight genetically distinct classes, named α, β, γ, δ, ε, η, θ and i-CAs, showing low sequence identity and different catalytic efficiency, inhibition and activation profiles [23,24,26–32]. For most of these classes, it has been shown that the catalytic mechanism occurs in two distinct half-reactions, described by Eqs. (1) and (2) (M is the metal ion, E the enzyme and B the buffer) [24]. The first half-reaction (Eq. (1)), which is reflected in the steady-state parameter kcat/KM, consists of the nucleophilic attack of a metal-bound hydroxide ion on carbon dioxide, yielding bicarbonate that is subsequently substituted by a water molecule. The second half-reaction (Eq. (2)), which is rate limiting and is reflected in the steady-state parameter kcat, is the proton transfer from the metal-bound water molecule to the buffer [33]. It consists of two steps: the intramolecular proton transfer from the metal-bound water to a proton shuttle residue (PSR) and the subsequent intermolecular proton transfer from the PSR to the buffer [34,35].

\[
\begin{align*}
\text{EM}^{2+} + \text{OH}^- + \text{CO}_2 &\rightarrow \text{EM}^{2+} - \text{HCO}_3^- + \text{H}_2\text{O} \\
\text{EM}^{2+} - \text{H}_2\text{O} &\rightarrow \text{EM}^{2+} - \text{OH}^- + \text{B} \\
\end{align*}
\]

Interestingly, CAs belonging to the α-, β- and/or γ-CA classes have been identified in many bacterial pathogens [17,18], whereas of the eight groups only α-CAs are present in humans [23,36]. Based on this observation, β- and γ-CAs have been introduced as potential and novel antibacterial drug targets [37].

B. pseudomallei genome encodes for two CAs belonging to β- and γ-class each, namely BpsCA and BpsγCA. Within a general research project aimed at developing new anti-melioidosis drugs, we have undertaken a detailed characterization of these two enzymes. In particular, both enzymes were produced in Escherichia coli and kinetically characterized, showing to be moderately active in catalyzing the CO2 hydration reaction (kcat = 1.6 \times 10^6 \text{ s}^{-1}, kcat/KM = 3.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1} for BpsCA and kcat = 5.3 \times 10^5 \text{ s}^{-1}, kcat/KM = 2.5 \times 10^7 \text{ M}^{-1} \text{s}^{-1} for BpsγCA) [38–40]; furthermore their inhibition profile with different classes of molecules was deeply investigated [39–48]. However, while the crystallographic structure of BpsβCA was determined [38], to date any structural information on BpsγCA is missing. Here we completed the characterization of this enzyme investigating on its catalytic activity at different pH values, reporting its crystallographic structure and dissecting the role of residues involved in the catalytic mechanism by means of theoretical pKa calculations. These findings provide useful insights into BpsγCA enzyme and represent a significant starting point for the development of new antimelioidosis drugs targeting BpsγCA.

2. Materials and methods

2.1. Cloning, expression and purification

A recombinant BpsγCA enzyme, containing the protein sequence, a His-tag and a linker at the N-terminus (Fig. 1) was heterologously expressed in E. coli as previously described [40]. Before crystallographic studies, BpsγCA was purified on a size exclusion chromatography (SEC) Superdex 75 10/300 in 20 mM MES pH 6.8, 200 mM KCl and 1 mM BME. The main peak was collected, pooled, concentrated at 6.0 mg/mL and stored at 4 °C before use.

2.2. Determination of quaternary structure

Quaternary structure of BpsγCA was investigated by SEC as previously described [49]. In particular, column calibration curve was performed on a Superdex 75 10/300 column (GE Healthcare) connected to an ÄKTA™ System (Cytiva) at room temperature. Running buffer was prepared with 20 mM Tris, 100 mM NaCl, pH 8.0. Calibration was carried out using the following standards (Sigma Aldrich, St. Louis, MO, USA): horse Cytochrome C (Cyt C, 12.4 kDa), Bovine Serum Albumin (BSA, 66.4 kDa) and Carbonic Anhydrase from bovine erythrocytes (CA 29.0 kDa). Blue dextran (2,000,000 Da) was used to calculate the column void volume (V0). The molecular weight of BpsγCA was determined by plotting Kav, calculated from the measured elution volume (Eq. (3)) against the logarithm of the molecular weights of the standard proteins. Prism - GraphPad software was used to generate the graph [50].

\[
K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)}
\]

2.3. Secondary structure and thermal stability

CD analyses were performed with a Jasco J-1500 spectropolarimeter equipped with a Peltier temperature control system using a 4.85 μM sample in 0.37 mM Na2HPO4, 5.0 mM NaCl, 0.1 mM KCl, 0.07 mM KH2PO4, pH 7.4 as previously described [51]. The thermal stability of the sample was evaluated in a temperature range of 25–92 °C with a temperature increase of 1 °C/min and the signal followed at 222 nm. Additionally, three spectra were registered at 25 °C, 92 °C and 25 °C again after the thermal treatment. Finally, the CD signal was converted into mean molar ellipticity per residue (Θ) (deg cm2 dmol−1) and the spectra overlaid. The overlap of the voltage signals recorded at the three temperatures above indicated was also generated. The graphs were obtained using GraphPad software [50]. Data were analyzed using the DICHROWEB website [52], setting CDSSTR as reference for the estimation of the secondary structure content of the protein.

2.4. Crystallographic studies

BpsγCA was crystallized using the hanging-drop vapor diffusion method at 293 K. The droplets were prepared by mixing 1 μL of protein solution at a concentration of 6.0 mg/mL in 20 mM MES pH 6.8, 200 mM KCl and 1 mM BME with 1 μL of precipitant solution consisting of 20 % (w/v) Polyethylene glycol 3350 and 0.15 M α-cyano-4-hydroxycinnamic acid pH 7.0. The drops were equilibrated over a well containing 500 μL of precipitant solution. Crystals grew within a few
days to maximum dimensions of 0.2 × 0.15 × 0.2 mm². Before the
diffraction experiment, crystals were transferred to the precipitant
solution with the addition of 25 % (w/v) glycerol. A complete data-
set was collected at 2.10 Å resolution from a single crystal at the
temperature of 100 K, by using a copper rotating anode generator
developed by Rigaku and equipped with a Rigaku Saturn CCD
detector. Data were processed using HKL2000 [53]. The crystals
belonged to the space group P61 with unit cell dimensions of a =
b = 50.1 Å and c = 48.5 Å. The Matthews coefficient (V_M = 2.53 Å³/
Da) indicated that the crystallographic asymmetric unit con-
tained one molecule according to a solvent content of 51 %. Data
collection statistics are reported in Table 1.

BpsCA structure was solved by the molecular replacement
technique using the program AMoRe [54] and the crystallographic
structure of the γ-CA from Brucella abortus (RiCA) (PDB accession
code 4 N27) as model [55]. The rotation and translation functions
were calculated using data between 15.0 and 3.5 Å resolution,
leading to a solution with a correlation coefficient of 59.1 and a
R-factor of 47.6. At this point, before proceeding with the structure
refinement, data were submitted to Auto-Rickshaw for rounds of
automated model building [56,57]. This approach allowed the
complete reconstruction of the model.

Refinement of the structure was performed with CNS program
[58,59] and model building was performed with O program [60].
Many cycles of manual rebuilding and positional and temperature
factor refinement were necessary to reduce the crystallographic
Rwork and Rfree values (in the 30.4–2.10 Å resolution range) to
19.7 and 23.1, respectively. The final model contains 1279 non-
hydrogen atoms, 1 catalytic zinc ion, 61 solvent molecules and 4
atoms of β-mercaptoethanol (BME). All residues were well defined
in the electron density maps, except for the N-terminal region that
includes the His-tag and the linker region. The refined model pre-

dented a good geometry with root mean square deviations (r.m.s.
d.) from ideal bond lengths and angles of 0.01 Å and 1.5°, respec-

tively. The structure had a good stereochemistry, as tested by PRO-
CHECK [61]. The most favored and additionally allowed regions of
the Ramachandran plot contained 88.4 % and 11.6 %, respectiv-
ely, of the non-glycine residues. Refinement statistics of BpsCA
structure are reported in Table 1. Coordinates and structure factors have
been deposited in the Protein Data Bank (accession code 7ZW9).

### 2.5. pH-dependent activity

An Applied Photophysics Stopped-Flow instrument was used for
assaying the pH-dependent kinetic parameters of the BpsCA-
catalyzed CO2 hydration reaction [62]. The initial rates of the CA-
catalyzed reaction were followed for a period of 10–100 s and
the kinetic parameters were determined by Lineweaver-Burk plots.
The concentration of CO2 was in the range 1.7–17 mM. The uncata-
ylarized rates were identical and detracted from the total observed rates. A 30 nM enzyme concentration was used in the
assays. Buffer-indicator dye pairs used were MOPS and 4-
nitrophenol (at pH 6.5–7.0) measured at a wavelength of
400 nm, HEPES and Phenol Red (at pH 7.0–8.0) measured at a
wavelength of 557 nm and Trizma base and m-cresol purple (at
pH 8.0–9.0) measured at a wavelength of 578 nm.

Apparent enzyme pKa value was obtained from pH profile of
k_cat using a nonlinear least-squares analysis according to Eq. (4).

\[
k_{cat} = \frac{k_{cat,max}}{1 + 10^{pK_a - pH}}
\]

Where k_cat is the observed value of k_cat at a given pH, k_{cat,max}

is the maximal limiting value of k_cat at high pH and Ka is the apparent
acid dissociation constant for the ionizing group controlling the pH
dependence. Data fit was performed with the Prism - GraphPad
software [50].

### 2.6. Theoretical pKa calculations

The PROPKA empirical algorithm for pKa prediction [63] was
employed, as implemented at the APBS/PDB2PQR server [64].
PROPKA is an empirical pKa predicting method, which estimates
the shift in pKa arising from hydrogen bonds, relative burial and

![Fig. 1](image-url)
coulombic interactions [65,66]. These contributions are parameterized to fit experimentally measured values. Calculations were performed using the BpsyCA crystal structure herein reported.

3. Results

3.1. Protein production, purification, and determination of quaternary structure

Recombinant BpsyCA containing a His-tag and a linker at the N-terminus (Fig. 1) was heterologously expressed in E. coli, as previously described [40], and purified to homogeneity by Ni²⁺ affinity chromatography and SEC. Protein purity and homogeneity were evaluated by SDS/PAGE and LC-ESI-MS analysis.

The quaternary structure of the purified protein was investigated by SEC indicating that in our experimental conditions, BpsyCA is trimeric (Fig. 2A), as reported for γ-CAs previously characterized [55,67–74]. Circular dichroism experiments carried out at 25 °C allowed us to estimate a preponderant content of β-sheet secondary structure (29%). Temperature denaturation experiments, carried out between 25 °C and 92 °C, showed that the protein unfolds only partially (Fig. 2B), slightly aggregating, as verified by the increase in the voltage (Fig. 2B inset). Interestingly, the content of β-sheet secondary structure was retained, re-cooling the sample at 25 °C restored some of the initial signals of the dichroic spectrum. Results highlighted the great thermal stability of the protein, which is only slightly affected by temperatures as high as 92 °C.

3.2. Crystallographic studies

Crystallization experiments were carried out on the purified protein concentrated at 6.0 mg/mL. Large well-formed crystals were obtained using the hanging drop vapor diffusion method and polyethylene glycol (PEG) 3350 as precipitant agent. They belonged to the space group P2₁2₁2₁ and contained one molecule per asymmetric unit, according to a solvent content of 51 % (Table 1). The structure was solved by the molecular replacement technique using the crystallographic coordinates of RicA [55] as starting model and refined to 2.10 Å resolution with the CNS program [58,59]. All residues were well defined in the electron density maps, except for the N-terminal His-tag, the linker region and the glycine residue at C-terminus (Fig. 1), which were not included in the final model. Refinement statistics are reported in Table 1.

BpsyCA structure consists of a seven-turn left-handed parallel β-helix (residues 3–134) followed by an antiparallel β-strand (residues 139–143) and a long α-helix (residues 145–170) positioned antiparallel to the axis of the β-helix (Fig. 3A). In agreement with the above reported SEC experiments, it forms a trimer (approximate dimension of 46 x 50 x 54 Å³) with two molecules related by a crystallographic 3-fold rotation axis (Fig. 3B). Interaction between two adjacent monomers in the trimer is very extensive, with a buried surface at the monomer–monomer interface of about 1917 Å². Similarly to the previously characterized γ-CAs family members [55,67–74], there are three active sites per trimer located at the monomer–monomer interfaces (Fig. 3B) in large cliffs characterized by a highly hydrophobic base and polar edges (Fig. 4). Each active site contains a catalytic zinc ion, which is tetrahedrally coordinated by three histidine residues, namely His65’, His87’ and His82’ (hereafter prime indicates residues from one subunit and double prime residues from a second subunit), and a water molecule/hydroxide ion (Fig. 5). The latter is, in turn, hydrogen bonded with Tyr159’OH. It could be assumed that this latter residue plays a role similar to that of the gatekeeper Thr199 in α-CAs [75], opportunely orienting the hydroxide ion for the nucleophilic attack to the carbon dioxide substrate. Interestingly, a BME molecule, deriving from the buffer used for protein purification, is anchored to the zinc-bound solvent molecule through a hydrogen bond, establishing also other polar interactions with residues delimiting the site (Fig. 5). The structural superposition of BpsyCA with the γ-CA from Pyrococcus horkoshii (Zn-Cap) bound with bicarbonate [72] shows that the BME molecule occupies within the active site the same position of the CO₂ reaction product (Fig. S1).

The superposition of BpsyCA with the γ-CAs previously characterized (see Table 2), namely RicA [55], Zn-Cap [72], and the γ-CAs from Methanospirillum hungatiae (Cam) [67], E. coli (YrdA) [71], Thermosynechococcus elongatus (TeCcmM) [70], the Discovery Deep Brine Pool (CA_D) [69], Thermus thermophilus HB8 (TtCA) [68], and Geobacillus kaustophilus (Cag) [74], revealed a substantial conservation of their three-dimensional structure, with the highest similarity detected with Zn-Cap (identity = 43.5 %, r.m.s.d. = 0.6 Å) [72] and RicA (identity = 48.9 %, r.m.s.d. = 0.8 Å) [55]. Indeed, as evident from Fig. 6A and 6B, all these proteins share the central left-handed β-helix and the C-terminal α-helix with some variability observed in the loop regions. In particular, Cam presents two big loops: the first between β1–β2 and the second, characterized by the several acidic residues, between β8–β9 (numbering of secondary structure elements refers to BpsyCA), which are absent in all the other struc-

![Fig. 2.](A) Calibration curve used to estimate the quaternary structure of BpsyCA in solution. The curve was obtained using the horse Cytochrome C (Cyt C, 12.4 kDa), Carbonic Anhydrase from bovine erythrocytes (CA 29.0 kDa) and Bovine Serum Albumin (BSA, 66.4 kDa) as molecular weight standards. (B) CD spectra of BpsyCA at different temperatures: 25 °C (black line), after heating at 92 °C (red line) and after heating and cooling at 25 °C (green line). Inset: voltage as function of the wavelength. The voltage curves relative to the spectra registered at 25 °C before and after heating are perfectly overlapping (black and green lines) whereas the voltage curve relative to the spectrum registered at 92 °C is shown as a red line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
tures, except for TeCcmM, which conserves the \( \beta_1-\beta_2 \) loop and CA_D which contains only the \( \beta_8-\beta_9 \) one even if of reduced sizes. It is worth noting that in previous studies, the presence or absence of the \( \beta_8-\beta_9 \) loop, also known as “the acidic loop”, led to the division of \( \gamma \)-CAs into two subgroups, referred to as Cam and CamH, from the name of their founding members, namely Cam or CamH from *M. thermophila* [33].

### 3.3. pH-dependent activity

To identify the experimental pKa of the Bps\( \gamma \)CA PSR (pKa\(_{PSR}\)), we analyzed the pH-dependent profile of \( k_{cat} \) for the \( \text{CO}_2 \) hydration reaction by stopped-flow spectrophotometry. Indeed, since the
proton transfer step is rate-limiting, the pH profile of \( k_{\text{cat}} \) most likely reflects the pKa of the PSR. Our data show that \( k_{\text{cat}} \) increases with pH (Fig. S2) and fits a single ionizing group titration curve with an apparent pKa of 7.25 ± 0.07, corresponding to the pKaPSR.

### 3.4. Theoretical pKa calculations

To identify the Bps\( \gamma \)CA PSR, pKa theoretical calculations of all the enzyme titratable residues (Asp, Glu, His, Cys, Tyr, Lys, Arg) were performed with the PROPKA method [63,65] and then possible PSR candidates were searched among residues with a computed pKa close to experimental pKaPSR and placed not too far from the zinc ion along the cleft leading to the active site (Fig. 4). Table 3 reports the computed pKa values of each residue. As evident, most of residues exhibits pKas significantly far from the experimentally determined pKaPSR value, with few exceptions represented by His14 (pKa = 6.49), His153 (pKa = 5.88), Glu123 (pKa = 5.62) and Lys125 (pKa = 8.9). By looking at the structure, the two His residues and Lys125 are far from the active site cleft (Fig. 7), thus their involvement in the catalytic mechanism is unlikely. On the contrary, Glu123 is in a favorable position for a putative catalytic proton transfer step since it is placed at the mouth of the hydrophobic cleft leading to the active site, at 9.9 Å (Glu123OE1 – Zn\(^{2+}\) distance) from the catalytic zinc ion, facing the interior channel and pointing towards the active site cleft (Fig. 7). Moreover, Glu123 shows a significant shift toward an elevated pKa value (5.62) with respect to the canonical pKa of a Glu residue (4.5), especially if compared to pKas computed for the other Bps\( \gamma \)CA Glu residues, which range between 3.85 and 4.64 (Table 3). This pKa shift is likely due to the high hydrophobic environment of Glu123 that favors the neutral state. Combining this result with the observation that this residue is well exposed to the solvent and presents rather high B-factor values which are indicative of flexibility, we suggest that Glu123 is the PSR of Bps\( \gamma \)CA. It is worth noting that computed Glu123 pKa is lower than the experimental pKaPSR (5.62 vs 7.25) likely due to some methodological limitations among which the inability to take into account protein flexibility and consequently the structural reorganization due to ionization/deionization of the titratable residues [76–78].

### 4. Discussion

Although widely distributed in diverse species belonging to the three domains of life [33], \( \gamma \)-CAs have been only scarcely investigated so far. The first member of the family to be identified was Cam from the anaerobic methane-producing species *M. thermophila* [34,35,67,79–85], an in vivo iron-dependent enzyme that captures zinc ions when overexpressed in *E. coli* [82]. The structural characterization of Cam [67] revealed for the first time the distinctive \( \gamma \)-CA homotrimeric structure where each monomer adopts a left-handed \( \beta \)-helix fold, whereas kinetic analyses of single-residue Cam variants pointed out several residues as important for the catalysis and/or for the integrity of the active site, and among these Glu84 was identified as the PSR (Fig. 6) [33,34,81,86,87].

In the following years, seven more \( \gamma \)-CAs were biochemically and structurally characterized (see Table 2) [55,68–72,74]; surprisingly, although possessing a significant structural similarity, these proteins showed very different levels of catalytic activities spanning from inactive proteins such as RicA [55], and TeCcmM [68] to very active proteins such as Cam [67,73,81], highlighting that a complete understanding of the molecular mechanisms underlying the catalytic features of \( \gamma \)-CAs is still lacking. To fill this gap and to provide insights into Bps\( \gamma \)CA active site and residues to be targeted in the design of new potential anti-melioidosis drugs, in this paper, we report an extensive characterization of this enzyme. In detail, Bps\( \gamma \)CA was expressed in *E. coli* and purified at a high yield. SEC and CD experiments indicated a very stable trimeric structure in agreement with previous reports on \( \gamma \)-CAs. Accordingly, the crystallographic structure of the enzyme showed the typical trimeric arrangement, with three active sites at the monomer–monomer interface. Unexpectedly, a BME molecule was found in the bicarbonate binding pocket [72], hydrogen-bonded to the zinc-bound water molecule. This finding, together with the observation that anchoring to the zinc-bound water molecule is a well-known inhibition mechanism adopted by several inhibitors of ICAs such as phenols [88,89], carboxylic acids [90,91] and polyamines [23,92], opens exciting perspectives in the design of Bps\( \gamma \)CA selective inhibitors.

The structural superposition of Bps\( \gamma \)CA with the previously characterized \( \gamma \)-CAs revealed a substantial conservation of the three-dimensional structure; however, many residues, described as important for the catalytic activity in the archetypal Cam, are not conserved in Bps\( \gamma \)CA (Fig. 6B). Among these the most striking lack is that of the PSR Glu84. Even more surprising is that this residue is not conserved also in other active members of the family such as Cag [74], CA_D [69] and TeCcmM [70]. Thus, we carried out experimental and theoretical pKa determinations to identify Bps\( \gamma \)CA PSR. Our studies indicated Glu123 as the most likely candidate. Interestingly, as noted previously for the Cam Glu84 residue, also Glu123 is not strictly conserved in the active \( \gamma \)-CAs (Fig. 6B), suggesting that in this enzyme family PSR position may vary according to the residues that delimit the active site cleft. These findings highlight local differences between \( \gamma \)-CA family members and suggest the need to specifically characterize each member of the family to shed light on the molecular determinants responsible for each catalytic activity and mechanism. The molecular knowledge of the features which are responsible of the catalytic activity of pathogenic CAs is of great importance for finely tuning their enzymatic activity and thus interfering with bacteria living. This information will pave the way for the structure-based design of a new generation of molecules to be used as antibacterial drugs.

### Table 2

\( \gamma \)-CAs whose crystal structure has been previously determined.

| Protein | Source | Subclass | PDB Code | Ref. |
|---------|--------|----------|----------|------|
| Bps\( \gamma \)CA | *B. pseudomallei* | CamH | 7ZW9 | [67,73,81] |
| Cam | *M. thermophila* | Cam | 1QQO, 1QRG, 1QRF, 1QRE, 1QRM, 1QR1, 1THJ, 3OUP, 3OW5, 3OTZ, 3OU9 | [55] |
| RicA | *B. abortus* | CamH | 4N27 | [72] |
| Zn-Cap | *P. horikoshii* | CamH | 1V3W, 1V67, 2FKO | [71] |
| Yrda | *E. coli* | CamH | 3TIS, 3TIO | [70] |
| TeCcmM | *T. elongatus* | CamH | 3KWC, 3KWD, 3KWE | [70] |
| CA_D | Discovery Deep Brine Pool | Cam | 6SC4 | [69] |
| TtCA | *T. thermophilus HB8* | CamH | 6IVE | [68] |
| Cag | *G. kiuwoshilis* | CamH | 3VNP | [74] |

*Protein Source Subclass PDB Code Ref.*
Fig. 6. (A) Structural superposition of Bps\textit{c}CA to related \(\gamma\)-CA family proteins showing loop region variability. Bps\textit{c}CA is colored in yellow, Cam in cyan, RicA in salmon, Zn-Cap in red, YrdA in violet, TeCcmM in green, CA\_D in magenta, TtCA in orange, and Cag in dark green. (B) Structure-based sequence alignment of Bps\textit{c}CA with Zn-Cap\[72\], RicA[55], Cag[74], YrdA[71], TtCA[68], CA\_D[69], Cam[67], TeCcmM[70]. Proteins with either no or very low activity are highlighted in red, whereas active proteins are in black. Zn-Cap, whose catalytic activity has not been measured is in blue. Secondary structure of Bps\textit{c}CA is annotated above the amino acid sequence (\(b\)-strands are represented with cyan arrows and \(a\)-helices with yellow cylinders). Secondary structure elements of all \(\gamma\)-CAs are shown highlighting residues in \(b\)-strands and those in \(a\)-helices in cyan and yellow, respectively. ● identifies the three catalytic histidines, whereas * highlights residues which in Cam have been identified as important for the catalysis and/or for the integrity of the active site. All strictly conserved residues are bolded. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
molecular mechanisms underlying the catalytic features of stabilizing interactions with a putative ligand have been identified. Moreover, other amino acids involved into experimental, and computational analysis allowed us to obtain a multidisciplinary approach presented here, combining structural, recently regarded as a significant potential biothreat agent. The pKa predictions for titratable residues of Bps

Table 3

| Res. pKa | Res. pKa | Res. pKa | Res. pKa | Res. pKa | Res. pKa | Res. pKa |
|---------|---------|---------|---------|---------|---------|---------|
| D21 3.42 | E8 4.46 | H14 6.49 | C70 10.15 | K5 10.26 | R44 14.66 |
| D46 3.15 | E15 4.36 | H65 5.88 | C89 12.60 | K28 10.20 | R106 12.84 |
| D67 1.02 | E32 4.47 | H82 3.12 | C113 10.70 | K125 8.92 | R111 12.93 |
| D129 3.38 | E33 4.43 | H87 1.27 | Y4 12.89 | K139 10.35 | R142 12.80 |
| D146 3.54 | E48 3.85 | H153 5.88 | Y139 9.52 | K157 10.37 | R151 13.24 |
| D148 2.14 | E60 4.59 | E93 4.30 | Y165 10.55 | K167 9.62 | R162 11.95 |
| E123 5.62 | E147 3.91 | E168 4.64 |

Fig. 7. Ribbon diagram of the BpsCA trimer. One of the three catalytic sites is visible with residues H14, E123, K125, and H153 as sticks. Their distances with respect to zinc ion are represented as dashed lines and reported in Å.

5. Conclusion

In this paper we have carried out a detailed characterization of BpsCA, one of the two CAs present in B. pseudomallei, which was recently regarded as a significant potential biothreat agent. The multidisciplinary approach presented here, combining structural, experimental, and computational analysis allowed us to obtain a detailed snapshot of the enzyme active site and strongly suggests that Glu123 is the PSR. Moreover, other amino acids involved in stabilizing interactions with a putative ligand have been identified.

Altogether, this information has provided insights into the molecular mechanisms underlying the catalytic features of γ-CAs and represents a starting point for the rational design of new potential anti-melioidosis drugs.

Author contributions

ADF, SMM, CTS, CC and GDS, designed research; ADF, SDP, EL, AN, MB performed research; ADF, EL, SMM and GDS wrote the paper.

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CRediT authorship contribution statement

Anna Di Fiore: Conceptualization, Methodology, Investigation, Writing - original draft. Viviana De Luca: Investigation. Emma Langella: Investigation, Writing - original draft. Alessio Nocentini: Investigation. Martina Buonanno: Conceptualization, Methodology. Simona Maria Monti: Conceptualization, Methodology, Writing - original draft. Claudio T. Supuran: Conceptualization, Methodology. Clemente Capasso: Conceptualization, Methodology. Giuseppina De Simone: Conceptualization, Methodology. Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.07.033.

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