Crystal Structure of β-Carbonic Anhydrase CafA from the Fungal Pathogen Aspergillus fumigatus

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INTRODUCTION

Carbonic anhydrases (CAs) are widely distributed zinc metalloenzymes that catalyze the interconversion of carbon dioxide to bicarbonate and a proton (Supuran, 2016). They are currently divided into seven families (α, β, γ, δ, ζ, η, θ), which are evolutionarily unrelated in amino acid sequence and structure (Del Prete et al., 2014a; 2014b; Iverson et al., 2000; Kikutani et al., 2016; Meldrum and Roughton, 1933; Mitsuhashi et al., 2000; Xu et al., 2008). Of the different classes, α- and β-CAs have been well-characterized biophysically and structurally. α-CAs are found in mammals, fungi, prokaryotes, and plants. All known bacterial α-CAs are dimers (Kim et al., 2019b; Kimber and Pai, 2000), while most mammalian CAs are monomers, with some also possessing dimeric α-CA forms (Alterio et al., 2009; Pilka et al., 2012; Whittington et al., 2001). α-CAs have a catalytic zinc ion coordinated by three conserved histidine residues and a water molecule (or hydroxide ion) in their active sites (Nair et al., 1995). Extensive structural and biochemical analysis has led to the two-step CO₂ hydration model for the α-CA family (Silverman and Lindskog, 1988). In this model, the first step is initiated by zinc ion, which promotes the ionization of bound H₂O and results in nucleophilic attack on CO₂ to generate hydrated carbon dioxide (bicarbonate, HCO₃⁻) ions, with its functional intolerance of nitrate (NO₃⁻) ions, could be exploited to develop new antifungal agents for the treatment of invasive aspergillosis.

Keywords: β-class carbonic anhydrase, Aspergillus fumigatus, CafA, X-ray crystallography, zinc metalloenzyme
α-CA enzymes from mycobacteria and algae have attracted great attention for mitigating global warming because biomineralization of CO$_2$ using these enzymes is one of the most economical ways of permanently storing carbon (Capasso et al., 2012b; Jo et al., 2014; Luca et al., 2013; Russo et al., 2013).

β-CAs were first discovered in plant leaf chloroplasts, but have since been found in bacteria, fungi, and algae (Neish, 1939; Smith et al., 1999). Based on the amino acid sequence and structure, they are divided into two main subgroups: plant-type tetramers such as that in _Pisum sativum_, and the cab-type dimer such as that in _Methanobacterium thermautotrophicum_ (Kimber and Pai, 2000; Smith and Ferry, 1999; Strop et al., 2001). In the fungal kingdom, members of the β-class of carboxic anhydrases play a key role in growth, development, virulence, and survival, although some fungi also possess α-CAs (Elleuche and Pöggeler, 2010). In hemiascomycetes yeasts, the Nce103 gene encoding a single copy of a plant-type β-CA is required for growth under ambient conditions but is dispensable for pH homeostasis at high CO$_2$ levels (Götz et al., 1999; Innocenti et al., 2009; Kengel et al., 2005). By contrast, basidiomycetes and filamentous ascomycetes possess multiple CA isoforms with different cellular locations and catalytic efficiency. For example, two closely related β-CAs, Can1 and Can2, have been identified in _Cryptococcus neoformans_ and _Cryptococcus gatti_, but only Can2 has been shown to be crucial for growth under ambient CO$_2$ conditions (Bahn et al., 2005; Ren et al., 2014). In _Sordaria macrospora_, four β-CAs have been identified (CAS1-4), and only deletion of CAS2 had a severe effect on vegetative growth and ascospore germination, indicating that CAS2 is the major β-CA (Elleuche and Pöggeler, 2009).

The functions of four β-CAs (CafA-D) also have been determined in _Aspergillus fumigatus_, the main causative agent of invasive aspergillosis (Han et al., 2010). CafA and CafB are closely related proteins that belong to plant-type tetrameric β-CAs, whereas CafC and CafD are classified as cab-type dimeric enzymes. *In silico* sequence analysis predicted that CafA and CafD are translocated into mitochondria, whereas CafB and CafC are cytoplasmic. Analysis of deletion mutants showed that CafA and CafB genes are constitutively and strongly expressed, whereas CafC and CafD are weakly expressed but induced by high CO$_2$ concentrations. Furthermore, only the double deletion mutant lacking CafA and CafB was unable to grow under ambient CO$_2$ concentrations. These results suggest that CafA and CafB are the major β-CAs in _A. fumigatus_, whereas CafC and CafD are minor β-CAs in this organism.

Recently, we solved the crystal structures of CafC and CafD by X-ray crystallography (Kim et al., 2019a). Using these structures, combined with our biochemical data, we revealed the molecular basis underlying their low activities. In CafC, access of CO$_2$ to the active site is limited by a narrow opening. Meanwhile, CafD exists predominantly in an inactive form because a conformational change of an aspartic acid induced by formation of an Asp-Arg pair acts as an on/off switch, which is inhibited by substitution of a glycine for an arginine.

In the present study, the crystal structures of CafA in the absence and presence of the potent inhibitor acetazolamide were determined at 1.8 Å and 2.0 Å resolution, respectively. Together with our biochemical results, our findings establish the molecular basis for understanding the catalytic mechanism of the major β-CAs in _A. fumigatus_, as well as a possible strategy for developing new antifungal agents for the treatment of invasive aspergillosis.

**MATERIALS AND METHODS**

**Cloning, expression, and purification of CafA**

A codon-optimized gene encoding CafA (residues 75-287) of _A. fumigatus_ was cloned between the EcoRI and NotI sites of the pGEX4T3 vector. This vector harbors a thrombin-cleavable N-terminal glutathione S-transferase. The resulting construct was transformed into _Escherichia coli_ BL21 (DE3) cells, grown in Lysogeny Broth medium at 37°C. When the optical density at 600 nm (OD$_{600}$) reached 0.6-0.7, protein expression was induced with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) for 16 h at 20°C.

After harvesting cells and lysis using a microfluidizer, CafA protein was purified using glutathione agarose beads (GoldBio, USA). After thorough washing, the protein was eluted by on-column thrombin cleavage in buffer containing 20 mM TRIS-HCl pH 8.0 and 200 mM NaCl. The protein was further purified by HiTrap Q anion exchange chromatography (GE Healthcare, USA) and Superdex 200 gel filtration chromatography (GE Healthcare). All purification steps were performed on ice or at 4°C.

**CO$_2$ hydration activity**

*In vitro* CO$_2$ hydration activity was measured using a modified electrometric method (Carter et al., 1969). Briefly, CO$_2$-saturated water was freshly prepared by bubbling CO$_2$ gas through water for at least 30 min. The reaction was initiated by addition of 4 ml CO$_2$-saturated water to 6 ml reaction buffer (20 mM TRIS-HCl pH 8.5) in the presence or absence of purified protein. Activity was monitored by recording the pH change from 8.3 to 6.3 at 5 s intervals, and was calculated in Wilbur-Anderson units (WAU) using the following formula:

\[
WAU = \frac{(T_0 - T)}{T} \times 100
\]

where $T_0$ and $T$ refer to the time in seconds taken in the absence and presence of the enzyme, respectively (Wilbur and Anderson, 1948). For acetazolamide or anion inhibition, protein samples were mixed with varying concentrations of acetazolamide or anions (Sigma-Aldrich, USA) for 1 h on ice before the CO$_2$ hydration assay. Each assay was performed in triplicate using the same enzyme preparation.

**Measuring thermal and pH stability**

To measure thermal stability, proteins were pretreated at temperatures from 22°C to 100°C for 15 min. To measure pH stability, 20 μl protein was incubated at 4°C for 4 h in 80 μl of 50 mM buffer at different pH values between 4 and 10. Remaining enzymatic activity was measured by *in vitro* CO$_2$ hydration assay as described above, and was expressed as a percentage of the maximal activity at 22°C and pH 7.
Crystallization, X-ray diffraction data collection, and structure determination

Purified CafA was concentrated to 15-20 mg/ml for crystallization. Crystals were obtained at 22°C by the sitting-drop vapor diffusion method in 0.1 M MES (or TRIS-HCl) pH 6.0-7.5, 24%-30% (w/v) polyethylene glycol 2000 monomethyl ether (PEG2000MME), and 0.2-0.4 M sodium acetate. For co-crystallization, acetazolamide (Sigma-Aldrich) was added to a concentrated solution of protein at a 10:1 molar ratio and incubated on ice for 30 min. Crystals of CafA were cryo-protected in reservoir solution supplemented with ethylene glycerol and then flash-frozen in liquid nitrogen. X-ray diffraction data were collected at beamline 7A of the Pohang Accelerator Laboratory (PAL, Korea). Datasets were indexed, integrated, and scaled using the HKL2000 program (Otwinowski and Minor, 1997). The CafA structure was determined by molecular replacement using the structure of β-CA CAS2 from the fungus Sordaria macrospora (Protein Data Bank [PDB] code 4O1K) as the search model (Lehneck et al., 2014; McCoy et al., 2007). Models were built by several cycles of manual modeling in COOT (Emsley and Cowtan, 2004), and were refined by REFMAC5 and PHENIX (Adams et al., 2010; Murshudov et al., 1997). X-ray crystallographic data and refinement statistics are summarized in Table 1. All residues lie in the allowed region of the Ramachandran plot. All figures in the manuscript were prepared using the program PyMOL (www.pymol.org).

Accession codes

Coordinates and structure factors for CafA have been deposited in the PDB under accession codes 7COI (apo, crystal form 1) and 7COJ (acetazolamide-bound, crystal form 2).

### Table 1. X-ray data collection and refinement statistics

|                | CafA Form 1 (apo, PDB ID 7COI) | CafA Form 2 (acetazolamide-bound, PDB ID 7COJ) |
|----------------|--------------------------------|-----------------------------------------------|
| **Data collection** |                                |                                               |
| Space group     | P22,2,1 | P22,2,1                                           |
| Cell dimensions |          |                                                  |
| a, b, c (Å)     | 66.6, 88.7, 144.2 | 66.9, 87.9, 144.7                              |
| α, β, γ (°)      | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0                               |
| Resolution (Å)  | 50.0-1.8 (1.83-1.80) | 50.0-2.0 (2.07-2.00)                           |
| R_φm            | 0.03 (0.28) | 0.03 (0.12)                                      |
| I/σI            | 33.1 (4.7)   | 43.2 (10.5)                                     |
| CC1/2           | 0.9 (0.9)   | 0.9 (0.9)                                       |
| Completeness (%)| 99.9 (100.0) | 99.9 (100.0)                                    |
| Redundancy      | 7.2 (7.2)   | 7.1 (7.2)                                       |
| **Refinement**  |                                |                                               |
| Resolution (Å)  | 50.0-1.8 | 50.0-2.0                                        |
| No. of reflections (work/test) | 76,053/4,066 | 55,669/2,919                                  |
| R_work/R_free   | 17.2/20.4 | 16.3/20.4                                      |
| No. of atoms    |          |                                                 |
| Protein         | 6,400    | 6,436                                          |
| Zinc            | 4        | 4                                               |
| Acetazolamide   | -        | 52                                             |
| Acetate         | 16       | -                                              |
| Water           | 700      | 522                                            |
| B factors       |          |                                                 |
| Protein         | 25.7     | 31.3                                           |
| Zinc            | 18.5     | 22.0                                           |
| Acetazolamide   | -        | 28.0                                           |
| Acetate         | 28.6     | -                                              |
| Water           | 32.4     | 34.3                                           |
| R.m.s. deviations |        |                                                 |
| Bond lengths (Å) | 0.011   | 0.010                                          |
| Bond angles (°) | 1.625    | 1.682                                          |
| Ramachandran plot (%) |       |                                                 |
| Most favored    | 90.5     | 91.4                                           |
| Allowed         | 9.5      | 8.6                                            |
| Generously allowed | 0     | 0                                               |
| Disallowed      | 0        | 0                                               |

Numbers in parentheses were calculated with data in the highest resolution shell.
R.m.s. deviations, root-mean-square deviations.
RESULTS AND DISCUSSION

CafA is a tetramer in solution

To determine the catalytic activities of the purified enzyme, in vitro CO$_2$ hydration activities were measured by electrometric methods (Carter et al., 1969). CAs catalyze the efficient hydration of CO$_2$, and the hydrogen ions produced are released into the surrounding solvent, lowering its pH. Previously, we showed that CafA (20 WAU/mg) was active under our experimental conditions, with activity approximately 8-fold higher than that of CafC and CafD (2.4 WAU/mg), which are minor β-CAs in A. fumigatus (Kim et al., 2019a). However, CafA had much lower activity than the highly thermostable γ-CAs from Persephonella marina EX-H1 (4,960 WAU/mg) (Kanth et al., 2014), Thermovibrio ammonificans (5,236 WAU/mg) (James et al., 2014), and Sulfurihydrogenibium yellowstonense YO3AOP1 (7,254 WAU/mg) (Capasso et al., 2012a).

Next, we explored the thermal and pH stability of CafA. As shown in Fig. 2A, CafA retained > 70% of its initial activity after incubation at temperatures up to 40°C, although it retained only 30% activity after 50°C treatment, and activity was almost negligible after exposure to temperatures above 50°C. To test the effect of pH on enzyme activity, proteins were pre-incubated in solutions with different pH values (pH 4–10). CafA was stable over a wide pH range (pH 5–9), but was rapidly inactivated below pH 5 or above pH 10 (Fig. 2B). This is reminiscent of previous studies showing that A. fumigatus can survive in neutral or alkaline environments, but not under acidic growth conditions (Amich et al., 2010).

Acetazolamide is a potent sulfonamide-based CA inhibitor. Its inhibition of enzymatic activity of CAs has been studied extensively as CAs are essential for the survival of many cancer cells under acidic and hypoxic stress (Neri and Supuran, 2011). Recently anions were also shown to inhibit the catalytic activity of fungal β-CAs (Innocenti et al., 2008; 2009; Isik et al., 2008). To determine the effect of anions on the activities of CafA, various concentrations of nitrate (NO$_3^-$), nitrite (NO$_2^-$), and sulfate (SO$_4^{2-}$) ions were separately added, and mixtures were incubated on ice for 30 min before measuring

![Fig. 1. Analysis of purified proteins by SDS-PAGE and gel filtration chromatography.](image)

(A) SDS-PAGE analysis of full-length (1-287) and truncated (75-287 and 79-287) variants of CafA. Constructs used in this study are enclosed in a black box. (B) Elution profile of CafA obtained using a Superdex Increase 200 10/300 GL column. The molecular weight of CafA was estimated by comparison with gel filtration standards (black arrows) ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa).
CO₂ hydration. Acetazolamide was used as a positive control. As expected, CafA activity was reduced by increasing the acetazolamide concentration and was completely inhibited at 5 μM acetazolamide (Fig. 2C). Interestingly, CafA was also weakly inhibited by nitrate in the submillimolar range (Fig. 2D). By contrast, nitrogen dioxide and sulfate had no inhibitory effect over the concentration ranges tested. This result suggests that nitrate or nitrate-containing compounds may represent a promising class of antifungal agents for the treatment of invasive aspergillosis (Supuran, 2008).

Overall enzyme structure
To establish the structural basis for the catalytic activity of CafA, we crystallized the purified protein in the presence and absence of acetazolamide, and determined the structures by X-ray crystallography (Table 1). Residues of the N-terminus (75-76), the linker region (97-101 and 269-275), and the C-terminus (286-287) could not be fitted effectively into the electron density map and were therefore not included in the final model. In our structure, CafA forms a plant-type tetramer from a dimer of dimers in the asymmetric unit, consistent with the gel filtration data (Figs. 1B and 3A). We observed that tetramerization is driven by the association of pairs of relatively flat surfaces composed of α-helices orthogonal to the dimerization interfaces. CafA possess the typical structural features of other plant-type β-CAs (Figs. 3B and 4) (Cronk et al., 2006; Dostál et al., 2018; Lehneck et al., 2014; Schlicker et al., 2009). The overall structure of each protomer consists of three parts: an N-terminal α-helical extension, a conserved central core comprising 10 α-helices and 5 β-strands, and a C-terminal subdomain (Fig. 3B). The N-terminal α-helix, which includes two perpendicularly oriented helices, extends...
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Fig. 3. The molecular architecture of CafA. (A) Cartoon representation of the CafA tetramer. Zinc ions are shown as green spheres. (B) CafA protomer structure. Subdomains within the protomer are indicated by black dashed circles.

Fig. 4. Structural comparison of CafA and other plant-type β-CAs. Superimposition of one protomer of CafA onto one protomer of other β-CAs from (A) Escherichia coli (ECCA, PDB ID 2ESF), (B) Candida albicans (CaNce103p, PDB ID 6GWU), (C) Haemophilus influenza (HICA, PDB ID 2A8D), (D) Cryptococcus neoformans (Can2, PDB ID 2W3Q), and (E) Sordaria macrospora (CAS1, PDB ID 4O1J).
far out from the central core region, spanning the neighboring protomer and stabilizing dimer interface interactions. The core structures are composed of four parallel β-strands (β2-β1-β3-β4) accompanied by a fifth antiparallel β-strand (β5). The C-terminal subdomains flank the adjacent protomers, creating new contacts, thus further stabilizing tetramer formation. Structure-based sequence alignment showed that CafA shares a high degree of amino acid conservation with CafB, suggesting that they might share a similar protein fold (Fig. 5). Note that throughout this article, single apostrophes are used to differentiate residues from the other protomer of the dimer.

The CafA active site

CafA (form 1) has a typical Type-I or open conformation in which the substrate binds at the dimer interface (Fig. 6). The catalytic zinc ion is tetrahedrally coordinated by three conserved residues (C119, H175, C178) and an acetate anion presumably acquired from the crystallization solution that replaces zinc-bound water in the metal coordination sphere (Fig. 6). The carboxyl group of acetate is firmly positioned by strong hydrogen bonds with the side chains of D121 and Q110’ and the nitrogen atom of the acetyl amide group interacts with the backbone oxygen of G179. By contrast, the methyl group of acetate is oriented toward the hydrophobic side of the dimer interface composed of I143, F138’, and F160’. The highly conserved D121 also forms essential salt bridges with R123 (the Asp-Arg pair).

We also solved the crystal structure of CafA in complex with acetazolamide (form 2) (Fig. 7A). The binding mode of acetazolamide is similar to that reported previously for β-CAs (Huang et al., 2011). The sulfonamide group interacts with the backbone oxygen of G179 and hydroxyl groups of the sulfonamide moiety form hydrogen bonds with the side chains of D121 and Q110’, respectively, while the nitrogen atom of the acetyl amide group interacts with the backbone oxygen of G179. The thiadiazole ring is sandwiched between G179 and F160’. Comparison of the CafA structures with and without acetazolamide reveals minor differences in this sandwich region: in the presence of the inhibitor, the phenyl ring of F160’ undergoes a ~40° rotation, and G179 moves ~0.6 Å away from the thiadiazole ring to avoid steric clashes with the secondary structural features being shown above the sequence alignment; α-helices are indicated as cylinders and β-strands as arrows. Residues responsible for zinc ion coordination in CafA and CafB are highlighted in yellow.

![Fig. 6. The CafA active site.](image)

**Fig. 6. The CafA active site.** Close-up view of the zinc coordination sphere. Residues and the zinc-bound acetate (ACT) are shown in stick representation, while zinc ions are shown as green spheres. The Fo-Fc map superimposed on the refined acetate is contoured at the 3α level.
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bound acetazolamide (Fig. 7D). It is noteworthy that no local conformational changes are induced in α-CAs by binding of acetazolamide due to their large active sites, which are fully exposed to the solvent (Eriksson et al., 1988; James et al., 2014; Nair et al., 1995).

In summary, the structure of CafA reveals key features that may be responsible for its apparent in vitro CO₂ hydration activity. The effect of pH on the activity of CafA suggests that A. fumigatus may use different catalytic strategies to regulate enzyme reactions in a pH-dependent manner. Importantly, knowledge of the structure of CafA in complex with acetazolamide and its functional intolerance of nitrate ions could be exploited to develop a promising class of antifungal agents for the treatment of invasive aspergillosis.

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AUTHOR CONTRIBUTIONS
All authors contributed to the study design, performed experiments, and interpreted the data. S.K., J.Y., and J.S. purified and crystallized the protein. S.K. and J.Y. performed enzyme activity assays. S.K. determined the crystal structures. S.K. and J.Y. prepared the figures. S.K. and M.S.J. wrote the manuscript. All authors helped to improve the manuscript and approved the final version.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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