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Structure of a dimeric fungal α-type carbonic anhydrase

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ABSTRACT

The crystal structure of *Aspergillus oryzae* carbonic anhydrase (*Ao*CA) was determined at 2.7 Å resolution and it revealed a dimer, which only has precedents in the α class in two membrane and cancer-associated enzymes. α carbonic anhydrases are underrepresented in fungi compared to the β class, this being the first structural representative. The overall fold and zinc binding site resemble other well studied carbonic anhydrases. A major difference is that the histidine, thought to be the major proton shuttle residue in most mammalian enzymes, is replaced by a phenylalanine in *Ao*CA. This finding poses intriguing questions as to the biological functions of fungal α carbonic anhydrases, which are promising candidates for biotechnological applications.

Structured summary: **AoCA** binds to **AoCA** by molecular sieving (View interaction)
AoCA binds to **AoCA** X-ray crystallography (View interaction)

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1. Introduction

Carbonic anhydrase (CA) catalyses the reversible conversion of CO_2 into bicarbonate ions and was first purified from bovine blood in 1933 [1] but has since been found in all domains of life [2]. Besides the biological (e.g., as essential enzyme for CO_2 fixation in plants [3]) and pharmaceutical importance of CAs (e.g., as drug targets for the treatment of glaucoma [4]), the enzyme has gained attention from an industrial perspective.

A promising approach for sustainable CO_2 capture from industrial gases (i.e. smoke gas) is the replacement of high-energy requiring absorption chemicals with a biocatalyst. Generally, these CO_2 capture techniques operate by bringing an absorber solution into contact with exhaust-CO_2 in presence of a soluble or immobilized CA. In previous work, bovine CA [5] and modified human CA II (hCAII) [6] were used as CO_2 capture catalysts.

CAs are classified in five classes, α, β, γ, δ and ε [2], which are unrelated in sequence and structure, but have all converged to a metal dependent mechanism for catalysis, where the metal is usually Zn. The α-class is predominantly mammalian, monomeric and by far the best mechanistically studied. α-CAs are also the only ones found in mammals. The β-class is found in plants, fungi and prokaryotes while γ-CAs have been so far only identified in archea. δ- [7] and ζ-CAs had until recently only been identified in marine phytoplankton.

No α-type fungal CA has been characterized in detail so far to the best of our knowledge. In fungi the intracellular β-class predominates [2] and the biological function of the putatively secreted α-CA enzymes is unknown. Only one structure representative for a fungal CA has been published, the structure of the β-CA from the pathogenic fungus *Cryptococcus neoformans* [8]. The structure of *A. oryzae* CA (*Ao*CA) presented here represents the first fungal α-CA. The structure reveals unusual characteristics, which may have implications for the biological function of the α-class in fungi.

2. Materials and methods

Cloning, expression and purification: Genomic DNA was isolated from *Ao* strain CBS 205.89 (Centraalbureau voor Schimmelcultures, Netherlands) according to a modified FastDNA SPIN protocol (MPBiomedical). Oligonucleotides F-Q2TWF5 (5′ACACAACTGG

Abbreviations: Ao, Aspergillus oryzae; *Ao*CA, *Aspergillus oryzae* carbonic anhydrase; beCA, bovine erythrocyte carbonic anhydrase; CA, carbonic anhydrase; hCAII, hCAIX and hCAXII, human carbonic anhydrases II, IX and XII, respectively; MR, molecular replacement; MS, mass spectrometry; *Ng*CA, *Neisseria ghonorroeae* carbonic anhydrase; NCS, non-crystallographic symmetry; *p*-NP, *para*-nitrophenol

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GGATCCACC**ATGAAGTTCGCCACTACTTTG**-3′) and R-Q2TWF5 (5′AG ATCTCGAGAAGCTTA**ACCGAATTGAGTTCAATTTCTG**-3′) were used to amplify the *Ao*CA gene from locus AO090010000582 of EMBL: AP007175. InFusion™ PCR (Clontech) was used to clone the PCR product into an *Aspergillus* expression vector (described in [9]). One PCR error free plasmid was transformed into *Ao* strain BECh2 using a similar procedure as in [10]. The derived transformants were re-isolated twice under selective conditions on Cove-N minimal media plates. To test expression and secretion of *Ao*CA, transformants were grown in YPM media (1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) maltose) for 3 days at 30 °C and fermentation samples analyzed by SDS-PAGE (NuPAGE, Invitrogen, Carlsbad, USA).

A high yielding transformant was grown in YPM media for 3 days at 26 °C and 150 rpm shaking on a New Brunswick Scientific orbital shaker. Culture broth was filtered through a Whatman GF/F 0.7 μm glass fiber filter (Maidstone, UK) and then through a 0.22 μm filter. $(NH_4)_2SO_4$ was added to this filtrate to 1.6 M final concentration prior to application on a Phenyl-sepharose FF (high sub) column (GE Healthcare) equilibrated in 20 mM HEPES/NaOH, 1.6 M $(NH_4)_2SO_4$, pH 7.0. *Ao*CA was eluted with a linear gradient between the equilibration buffer and 20 mM HEPES/NaOH, pH 7.0 over three column volumes. The *Ao*CA peak was transferred to 20 mM HEPES/NaOH, pH 7.0 using a G25 sephadex column (from GE Healthcare) and applied to a Q-sepharose FF column (from GE Healthcare) equilibrated in 20 mM HEPES/NaOH, pH 7.0. *Ao*CA was eluted with a linear 0–0.4 M NaCl gradient in the same buffer. Fractions judged as pure by SDS-PAGE were pooled and used for further characterization.

Characterization: Intact Mw was analysed with DataAnalysis version 3.3 and measured using a Bruker microTOF focus electrospray MS (Bruker Daltonik GmbH, Bremen, DE) calibrated with ES tuning MIX (Agilent). The protein was N-terminal sequenced using a Procise Protein Sequencer from Applied Biosystems after it was recovered by SDS-PAGE on a PVDF membrane. Analytical gel filtration was carried out on a Superdex 75 (10/300 GL) column using 20 mM HEPES pH 7.0, 0.1 M NaCl as elution buffer at 4 °C. 100 or 300 μg of *Ao*CA prepared as for crystallization were loaded. CA activity was measured essentially by the Wilbur–Anderson method [11] in a slightly modified form as described by Sigma–Aldrich (http://www.sigmaaldrich.com). 50 μl enzyme was diluted in a 20 mM Tris buffer at pH 8.3 and mixed with CO_2-saturated water (3:2 v/v). The time, in seconds, for the pH of the mixture to drop to 6.3, was measured using a pH meter and compared to a blank without enzyme. CA esterase activity was determined by using *p*-NP-acetate as substrate as described in [12]. As a reference for activity measurements, bovine erythrocytes CA (beCA) [1] from Worthington was used.

Crystallization: *Ao*CA was dialyzed against 100 mM sodium carbonate buffer pH 8.0 and concentrated using a Microcon YM-10 unit (MWCO 10 kDa) to 103 mg/ml as estimated by A_{280} assuming an extinction coefficient of 24,535 M^{-1} cm^{-1}. Screening was performed with the JCSG+, PACT and Index crystallization screens. The crystals used for structure determination resulted from optimization in Linbro plates. Rod-shaped crystals of approximate size 25 μm x 25 μm x > 100 μm grew in hanging drops within few days of setting up. The drop consisted of 3.0 μl protein solution and 1.0 μl of reservoir (2.2 M $(NH_4)_2SO_4$, 0.1 M Buffer system II pH 6.5 [13]).

Data collection: Crystals were mounted in nylon cryoloops in mother liquor containing 33% ethylene glycol. Data were collected at beamlines 911-2 and 911-3 of MAXLAB, Lund, Sweden, at 100 K. All data sets were processed with XDS [14]. A dataset suitable for structure determination was collected and the UCLA anisotropy correction server [15] was used to correct the significant anisotropy observed for the phasing step, but final refinement was performed against the unmodified dataset. A second lower resolution dataset was collected at the Zinc K-edge to obtain anomalous signal. The anomalous correlation from XDS was 17% overall and 32% for data up to 5.5 Å. Although this would not be sufficient for phasing, it could clearly identify the Zn^{2+} position after molecular replacement (MR) phasing. Data statistics are shown in Table 1.

Table 1
Data collection and refinement statistics (numbers in parenthesis are for the high resolution shell).

	Data used in refinement	Data used for anomalous map
Data collection wavelength	1.038 Å	1.283 Å
Space group	$P4_12_12$	$P4_12_12$
Unit cell	*a* = *b* = 80.24 Å, *c* = 247.48 Å	a = b = 80.2 Å, c = 248.3 Å
Resolution	20 – 2.7 Å (2.8 Å – 2.7 Å)	3.5 Å (3.7 Å-3.5 Å)
R_{int}	10.78% (40.37%)	11.2% (23.2%)
I/sigma	11.43 (2.70)	12.3 (6.7)
Completeness	98.6% (98.4%)	99.1% (98.5%)
R-factor	20.90%	–
R-free	25.32%	–
RMSD bonds	0.010 Å	–
RMSD angles	1.384°	–
PDB ID:	3Q31	–
Protein atoms	3656 with average B 43.9 Å^2	–
Zinc atoms	2 with average B 42.5 Å^2	–
Malate atoms	18 with average B 46.2 Å^2	–
Water	91 with average B 38.6 Å^2	–
Carbohydrate	28 with average B 55.9 Å^2	–

Structure determination: The structure (Fig. 1a) was determined by MR using an appropriately truncated structure of *N. gonorroeae* CA (*Ng*CA, [16]) as search model, which shares the highest sequence identity with *Ao*CA among available structures. MOLREP [17] found two molecules in the asymmetric unit. Anomalous difference at the Zn K-edge showed clear peaks where active site Zn^{2+} ions were expected (Fig. 1b). The structure was rebuilt in COOT [18], and refined in REFMAC [19] and PHENIX [20] including TLS refinement and NCS restraints. Final refinement statistics are shown in Table 1. Structure figures were made with PYMOL [21].

3. Results

Characterization: *Ao*CA was expressed in high yield and purified. The theoretical Mw (Uniprot sequence Q2TWF5) is 29728.5 Da while the apparent Mw on SDS-PAGE was 35 kDa with a broad band suggesting glycosylation. N-glycosylation was confirmed by MS analysis prior and after EndoH treatment indicating a mass difference of 1825 Da, corresponding to 11 glycosyl units. The dominant N-terminal sequence was AAGGLDD, corresponding to a start at position 27 in the Uniprot sequence. A minor sequence, about 20% of the sample, had three additional amino acids removed. The apparent Mw was 78 kDa and 80 kDa for two separate gel filtration runs (Fig. 2a), most consistent with a dimer as the predominant form in solution, thus confirming the dimer observed crystallographically (see below). CA activity was confirmed by two different assays. The specific activity of *Ao*CA in the Sigma–Aldrich CA assay was 220 U/mg, that is 7% of beCA (3115 U/mg) used as reference. Addition of 2.5 mM imidazole decreased beCA activity by 3% but increased *Ao*CA activity by 21%. The specific activity against pNP-acetate was 20 times lower for the *Ao*CA compared

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