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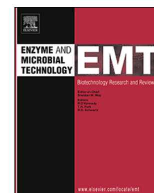

Structure of a hyperthermostable carbonic anhydrase identified from an active hydrothermal vent chimney

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ABSTRACT

Carbonic anhydrases (CAs) are extremely fast enzymes, which have attracted much interest in the past due to their medical relevance and their biotechnological potential. An α-type CA gene was isolated from DNA derived from an active hydrothermal vent chimney, in an effort to identify novel CAs with suitable properties for CO_2 capture. The gene product was recombinantly produced and characterized, revealing remarkable thermostability, also in the presence of high ionic strength alkaline conditions, which are used in some CO_2 capture applications. The T_m was above 90 °C under all tested conditions. The enzyme was crystallized and the structure determined by molecular replacement, revealing a typical bacterial α-type CA non-covalent dimer, but not the disulphide mediated tetramer observed for the hyperthermophilic homologue used for molecular replacement, from *Thermovibrio ammonificans*. Structural comparison suggests that an increased secondary structure content, increased content of charges on the surface and ionic interactions compared to mesophilic enzymes, may be main structural sources of thermostability, as previously suggested for the homologue from *Sulfurihydrogenibium yellowstonense*.

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are some of the fastest enzymes on earth, achieving k_{cat} of over $10^6\,s^{-1}$, which approach the diffusion limit. CAs catalyse the reversible hydration of CO_2 to produce bicarbonate ions (HCO_3^-) and protons. They are ubiquitous in all domains of life and are of significant biological, pharmaceutical and biotechnological importance [1]. In the last ten years, CAs, especially thermostable ones, have also attracted considerable attention for CO_2 capture applications [2,3]. Depending on the structure they are classified into several classes, the most prominent and best studied family being the α-class, which contains mammalian, fungal and bacterial members and are Zn-dependent. The active site Zn is coordinated by three histidine residues.

Although the α-CAs have traditionally been considered monomeric enzymes, some mammalian, fungal and bacterial members have been shown to be dimeric [1,4]. The dimerization interfaces are not conserved across all dimeric α-class CAs which have been structurally characterized. However, within the bacterial members of the class, conservation of the dimerization interface has been observed as first noted when comparing the α-CA from *Neisseria gonorrhoeae* [5] and the α-CA (SspCA) from *Sulfurihydrogenibium yellowstonense* YO3AOP1 [6]. SspCA is also the most thermostable CA known, able to retain activity at 100 °C for 3 h [6]. Another structurally characterized CA from *Thermovibrio ammonificans* (TaCA), shows a similar dimeric structure, and additionally a cysteine mediated tetramer [7]. Recently, another bacterial enzyme, SazCA from *Sulfurihydrogenibium azorense,* has been identified as the most efficient CA known to date [8] and its structure has been determined [9]. SazCA is more than 60% identical to SspCA and forms, as the other bacterial CAs characterized so far [7], non-

Abbreviations: CA, carbonic anhydrase; SspCA, carbonic anhydrase from *Sulfurihydrogenibium yellowstonense* YO3AOP1; TaCA, carbonic anhydrase from *Thermovibrio ammonificans*; SazCA, carbonic anhydrase from *Sulfurihydrogenibium azorense*; PMCA, carbonic anhydrase from *Persephonella marina* DSM 14350; LOGACA, carbonic anhydrase from the Logatchev hydrothermal field metagenome; DSC, differential scanning calorimetry; PDB, protein data bank; PEG, polyethylene glycol; PEGMME, polyethylene glycol monomethyl ether; TSA, thermal shift assay

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covalent dimers. Though SazCA is hyperthermostable, it is not so to the same extent as SspCA. Thus attempts have been made to transfer the properties of SazCA to SspCA by site directed mutagenesis, but have to date not succeeded [10]. Through a comparison of SspCA to human mesophilic counterparts, several structural determinants of thermostability were suggested by [6]: an increase in secondary structure elements and 'compactness'; an increase in charged residues contribution to surface area; increased long range ionic interactions and ionic networks. For TaCA [7], the main structural feature leading to thermostability was identified as the formation of tetramers through 4 ion pairs and 2 disulphide bridges. TaCA in its tetrameric form retains 90% activity upon incubation at 70 °C for 1 h.

In an effort to identify novel CAs with suitable properties for CO_2 capture, we have cloned, expressed, structurally and functionally characterized a CA from a metagenome derived from an active chimney structure emanating hot, reduced hydrothermal fluids.

2. Materials and methods

2.1. Sample collection and sample description

Parts of an active hydrothermal vent chimney were collected from the Logatchev hydrothermal field located on the Mid-Atlantic Ridge at 14°45′N and 44°58′W at a water depth of 3000 m. The sample was retrieved during a dive with the remotely operated vehicle Kiel ROV 6000 (GEOMAR, Kiel) during the cruise HYDROMAR VII (January/February 2009). On the inside of the chimney conditions can be characterized as extremely hot (temperatures of the emanating fluids at the chimney outlet measured 350 °C) and highly reduced [11], while on the outside of the chimney ambient seawater conditions prevail, i.e. oxygen is present and the temperature is 4 °C. Hence, a steep chemical and thermal gradient exists within the chimney (from inside to outside). The hydrothermal fluids are generally enriched in hydrogen and methane due to serpentinization reactions. After sample recovery the chimney piece was immediately frozen at −80 °C until further processing.

2.2. DNA extraction

A fragment of the frozen chimney sample was pestled to fine powder and the fine powder was suspended in 2 mL washing solution (800 mM NaCl, 100 mM EDTA pH 8.0) and incubated for 1 h on ice. Then 2 mL TE-sucrose buffer (10 mM Tris, 1 mM EDTA, 20% (w/v) sucrose, pH 8.0) were added and the sample was incubated in an ice bath for 15 min. After the addition of 2 mL cell lysis buffer (10 mM Tris, 1 mM EDTA, 10 mg/mL lysozyme, 1 mg/mL RNase A, pH 8.0) the sample was incubated at 37 °C for 1 h. Then 1 mL Proteinase K (1 mg/mL in 5% (w/v) sacrosyl) was added and the reaction was again incubated at 37 °C for 1 h. The DNA was extracted with phenol/chloroform (1:1). To precipitate the DNA from the upper phase, 2.5 vol ethanol (99%, vol/vol) and 0.1 vol 3 M sodium acetate (pH 5.5) were added. The tube was inverted and incubated at −20 °C for at least 1 h. Then the DNA was sedimented at 13,000 g and 4 °C for 20 min and the supernatant was decanted carefully. The DNA pellet was washed twice with 300 μL ice-cold ethanol (70%, vol/vol) each followed by a centrifugation of 2 min at 4 °C and 13,000 g. After discarding the supernatant, the pellet was air-dried. The DNA was then dissolved in an appropriate volume of sterile water. The DNA was subjected to Illumina sequencing (300 bp PE run). Read count was 12,141,468 with 2,888,817,382 bp. The N50 was 4941 and the raw coverage/x 52.5456. The assembled length was 54,977,361 bp on 17,000 contigs. The CA gene (GenBank entry MG547709) was discovered in the metagenome by homology searches. The metagenome CA protein sequence displayed 83% identity to bacterial CA from *Persephonella sp.* IF05-L8 (NCBI Reference Sequence WP_029521561) and 82% identity to CA from *Persephonella marina* EX-H1 or *Persephonella marina* DSM 14350 (PMCA, NCBI sequence reference WP_015898908 and sequence from patent WO 2012/025577 [12]).

2.3. Protein production

A synthetic CA (referred hereon as LOGACA) gene corresponding to the identified sequence was cloned into *Bacillus subtilis* in the same expression vector as previously used for PMCA [12]. The native CA signal peptide was exchanged with a Bacillus signal peptide for recombinant expression in *B. subtilis*, leaving the mature peptide of LOGACA after predicted cleavage with Signal Peptide peptidase with N-terminal residues 'GGVGHW'. The recombinant CA protein expression was performed in rich media supplemented with 6 mg/L chloramphenicol and successful expression and purification using ion-exchange chromatography (SP-Sepharose) was monitored by SDS-PAGE analysis. It was determined that there was very high CA activity in the culture broth solution, measured according to [13]. Purified LOGACA was stored in 50 mM MES pH 6, 100 mM NaCl at a protein concentration of 5.2 mg/mL.

2.4. Biochemical characterization

The thermostability of the recombinant LOGACA was determined by thermal shift assay (TSA) in a Roche Lightcycler 480 II machine running Roche LightCycler 480 software (release 1.5.0 SP4) as follows: TSA were run with enzyme samples diluted to 0.3 mg/mL in assay buffers: 0.1 M succinic acid, 0.1 M HEPES, 0.1 M CHES, 0.1 M CAPS, 0.15 M KCl, 1 mM $CaCl_2$, 0.01% Triton X100, pH adjusted to 5.0, 7.5 and 10.0 respectively. SYPRO Orange dye (Life Technologies S6650) was diluted 101× in deionized water. 10 μL diluted enzyme sample + plus 10 μL assay buffer + plus 10 μL dye were mixed in wells of TSA assay plates (Light¬Cycler 480 Multiwell Plate 96, white, Roche and covered with optic seal LightCycler 480 Sealing foil, Roche). Protein melting analysis was conducted at 25–99 °C at 200 °C/h. The LOGACA and a PMCA reference sample for comparison with [14] were analysed in duplicate and the data used to determine T_m, defined as the midpoint value of the protein melting curves.

The thermostability of LOGACA and PMCA was also determined by Differential Scanning Calorimetry (DSC) at high ionic strength, because certain high ionic strength alkaline solvents are used in CO_2 capture applications. Samples were diluted to approximately 1 mg/mL in 1.5 M Glycine buffer at pH 8, 9 or 10 and the thermal midpoint (T_m) determined by scanning from 20 to 120 °C at 200 °C per hour.

The specific activity of LOGACA determined according to [13] was compared to PMCA in order to assess industrial applicability. Esterase activity with *p*-nitrophenyl acetate (*p*-NPA) was additionally measured with a continuous assay in 96-well format. 15 μL dilution of the substrate in 2-propanol was added 285 μL enzyme in 0.1 M phosphate buffer pH 7.5 (total enzyme concentration 7.6 μM) at room temperature and the reaction monitored by following $A_{405.}$ Both LOGACA and PMCA followed Michaelis-Menten kinetics. K_m,V_{max} and k_{cat} could be calculated using a simple web-tool (http://www.ic50.tk/kmvmax.html). Activity at high pHs could not be measured accurately due to an extremely high background due to fast non-catalyzed reaction. An apparent ε_{405} of 14720 M^{-1} was used for pNP in the microtiter plate assay.

2.5. Crystallization and data collection

Initial crystallization attempts were carried out with an Oryx 8 liquid handling robot (Douglas Instruments Ltd) in MRC 2-well sitting drop trays, using 0.3 μL drops (protein:reservoir ratio of 1:1 and 3:1) and using the JCSG+ and Morpheus screens (Molecular Dimensions, Newman et al., 2005). Crystals were obtained in 20% w/v PEGMME 500, 10%w/v PEG 20000, 0.1 M MES/Imidazole pH 6.5, diffracting to around 4 Å in space group $P4_12_12$ (the crystals were thin needles with dimensions around $20 \times 20 \times 300\,\mu m^3$, length varying between

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