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Cloning, purification and preliminary crystallographic analysis of the complex of *Helicobacter pylori* α -carbonic anhydrase with acetazolamide

Helicobacter pylori infection of the stomach can lead to severe gastroduodenal diseases such as gastritis, peptic ulcers and gastric cancers. Periplasmic *H. pylori* α -carbonic anhydrase (Hp α CA) is essential for the acclimatization of the bacterium to the acidity of the stomach. Through the action of urease and carbonic anhydrases, the *H. pylori* periplasmic pH is maintained at around 6 in an environment with a pH as low as 2, which in turn facilitates the maintenance of a cytoplasmic pH close to neutral, allowing growth in the gastric niche. Crystals of Hp α CA in complex with the inhibitor acetazolamide have been grown by the hanging-drop vapour-diffusion method using polyethylene glycol as a precipitating agent. The crystals have the symmetry of space group $P2_12_12_1$, with unit-cell parameters $a = 37.0$, $b = 82.4$, $c = 150.8$ Å. An X-ray diffraction data set was collected from a single crystal to 1.7 Å resolution. Calculation of the self-rotation function using this data and molecular replacement showed that the asymmetric unit contains an Hp α CA dimer.

1. Introduction

Helicobacter pylori is a Gram-negative, spiral-shaped, pathogenic bacterium that colonizes the stomach of approximately half of the human population (Dunn *et al.*, 2007). Much morbidity and mortality are associated with *H. pylori* infections, as they can develop into severe gastroduodenal diseases such as gastritis, peptic ulcers and gastric cancers (Kusters *et al.*, 2006; Ernst & Gold, 2000; Peek & Blaser, 2002). Eradication of *H. pylori* in patients with associated ulceration and gastritis greatly reduces the chance of disease re-occurrence and the risk of developing gastric cancer (Wroblewski *et al.*, 2010; Uemura *et al.*, 2001). Current *H. pylori* eradication therapies rely on the simultaneous use of two or more broad-spectrum antibiotics (commonly amoxicillin and clarithromycin; Graham & Fischbach, 2010) and a proton-pump inhibitor (Walsh & Peterson, 1995). However, owing to the increasing antibiotic resistance of *H. pylori* this form of treatment is becoming less effective (Graham, 1998; Graham & Fischbach, 2010). Therefore, there is a growing need to identify and develop new therapeutic targets.

Carbonic anhydrases (CAs; carbonate dehydratases; EC 4.2.1.1) are zinc metalloenzymes that reversibly catalyse the conversion of carbon dioxide to bicarbonate. CAs are ubiquitous and play important roles in many biological processes such as respiration, acid–base homeostasis and photosynthesis (Supuran, 2008; Hewett-Emmett & Tashian, 1996). These enzymes are classed into three evolutionarily distinct families, α -CAs, β -CAs and γ -CAs, based on amino-acid sequence, structure and oligomeric state (Supuran, 2008; Liljas & Laurberg, 2000; Hewett-Emmett & Tashian, 1996). Many bacterial species have been found to possess genes from multiple CA families (Smith & Ferry, 2000). *H. pylori* has CA genes from both the α and β families (Chirica *et al.*, 2002). Periplasmic *H. pylori* α CA (Hp α CA) shares 28% sequence identity with human carbonic anhydrase II (Chirica *et al.*, 2002) and is likely to follow the same reaction mechanism, in which CO₂ is converted into bicarbonate (HCO₃[−]) via a nucleophilic attack on CO₂ by the reactive zinc-bound hydroxide and the resultant bicarbonate is then displaced from the zinc by a water molecule (West *et al.*, 2012; Lindskog, 1997). Joint activities of α -CAs

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and β -CAs and urease are required to maintain the *H. pylori* periplasmic and cytoplasmic pH close to neutral in highly acidic media, allowing both survival and growth in the gastric niche. *H. pylori* buffers its periplasm by means of $\text{NH}_3/\text{NH}_4^+$ and $\text{CO}_2/\text{HCO}_3^-$ couples that are the products of the reactions catalysed by urease and α -CA and β -CA (Marcus *et al.*, 2005; Krulwich *et al.*, 2011).

As with α CAs from other species, Hp α CA is inhibited by sulfonamides, particularly by the anti-glaucoma drug acetazolamide (2-acetylamido-1,3,4-thiadiazole-5-sulfonamide; Chirica *et al.*, 2002; Nishimori *et al.*, 2006). Previous studies have shown that treating *H. pylori* with CA inhibitors drastically reduces the ability of the bacteria to survive within an acid environment, suggesting that CAs are essential for colonization of the stomach and duodenum (Sachs *et al.*, 2005; Bury-Moné *et al.*, 2008). These data suggest that CAs could potentially be a novel target for the treatment of *H. pylori*-associated gastroduodenal disease. In this paper, we report the crystallization and preliminary X-ray analysis of recombinant Hp α CA.

Figure 1 Crystallographic analysis of purified recombinant Hp α CA. (a) Reduced 15% SDS-PAGE Coomassie Blue-stained gel. Molecular-mass markers are labelled in kDa. (b) A crystal of the Hp α CA-acetazolamide complex. (c) A representative 0.5° oscillation image of the data collected using a MAR CCD ADSC Quantum 210r detector at station MX1 of the Australian Synchrotron, Victoria, Australia. (d) The self-rotation function for Hp α CA ($\kappa = 180^\circ$). The three crystallographic twofold axes are encircled by a bold line and the noncrystallographic twofold axis is encircled by a dashed line.

2. Materials and methods

2.1. Cloning and overexpression

The coding sequence for Hp α CA (247 amino-acid residues; UniProtKB ID K4NGD4_HELPY) lacking the N-terminal periplasmic targeting sequence was PCR-amplified from genomic DNA of strain 26695 of *H. pylori* using OneTaq Hot Start DNA Polymerase (New England Biolabs) and the primers CACCAATACCAATGGGATTATAAGAATA (forward) and TTAGCGGGTCTCAGCTGAG (reverse). The amplified fragment was cloned into the pET151/D-TOPO vector using the TOPO cloning kit (Invitrogen) to produce an expression vector that contains an N-terminal His₆ tag followed by a TEV protease cleavage site. The expression clone was confirmed by DNA sequencing. The recombinant protein used for crystallization comprised residues 20–247 of Hp α CA plus six additional residues from the TEV cleavage site (GIDPFT). The vector was transformed into *Escherichia coli* strain BL21(DE3) (Novagen). Cells were grown in LB medium containing 50 mg l^{-1} ampicillin at 310 K until an OD_{600} of 0.8 was reached, at which point overexpression of Hp α CA was induced by adding 0.5 mM IPTG and growth was continued for a further 3 h. The cells were then harvested by centrifugation at 6000g for 15 min at 277 K.

2.2. Purification and determination of the oligomeric state

Cells were resuspended in buffer A (20 mM sodium phosphate pH 7.4, 200 mM NaCl, 1 mM PMSF) and lysed by sonication. Cell debris was removed by centrifugation at 12 000g for 30 min at 277 K. The supernatant was collected and clarified by ultracentrifugation at 105 000g for 20 min at 277 K. NaCl and imidazole were then added to the supernatant to final concentrations of 500 and 10 mM, respectively, after which the supernatant was loaded onto a 5 ml HiTrap Chelating HP column (GE Healthcare) pre-washed with buffer A containing 500 mM NaCl. The column was washed with 20 column

Table 1

Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 37.0$, $b = 82.4$, $c = 150.8$, $\alpha = \beta = \gamma = 90$
V_M ($\text{\AA}^3 \text{Da}^{-1}$)	2.18
No. of crystals	1
Temperature (K)	100
No. of images	360
Rotation per image ($^\circ$)	0.5
Wavelength ($\text{\AA}$)	0.92
Resolution range ($\text{\AA}$)	32.9–1.7 (1.79–1.70)
Completeness (%)	83 (76)
Observed reflections	255286
Unique reflections	42998
Mean $I/\sigma(I)$	12.6 (4.0)
$R_{\text{merge}}^\dagger$	0.087 (0.363)
Mosaicity ($^\circ$)	0.8

 $\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of reflection hkl .

volumes of buffer *B* (20 mM sodium phosphate pH 7.4, 500 mM NaCl, 60 mM imidazole) and the protein was eluted with buffer *B* containing 500 mM imidazole. The N-terminal tag was cleaved off with His₆-TEV protease (Invitrogen) overnight at 277 K whilst dialysing the sample against buffer *C* [50 mM Tris–HCl pH 8.0, 2 mM DTT, 200 mM NaCl, 1% (v/v) glycerol]. NaCl and imidazole were then added to the sample to final concentrations of 500 and 15 mM, respectively, and the TEV protease and the uncleaved protein were removed on a HiTrap Chelating HP column. The flowthrough was concentrated to 2 ml in a VivaSpin 10 000 Da cutoff concentrator and passed through a Superdex 200 HiLoad 26/60 gel-filtration column (GE Healthcare) equilibrated with buffer *D* (50 mM Tris–HCl pH 8.0, 200 mM NaCl). Finally, the protein was buffer-exchanged into 30 mM Tris–HCl pH 8.0 by passing it through a HiPrep 26/10 desalting column (GE Healthcare). Protein concentration was determined using the Bradford assay (Bradford, 1976). The protein purity was estimated to be greater than 98% (Fig. 1a). The oligomeric state of HpαCA in solution was determined by calculating the molecular weight (MW) using a calibration plot of log MW versus the retention volume [$V_{\text{retention}}$ (ml) = 549.3 – 73.9 × log MW] available at the EMBL Protein Expression and Purification Core Facility website http://www.embl.de/pepcore/pepcore_services/protein_purification/chromatography/hiload26-60_superdex200/index.html.

2.3. Crystallization and preliminary X-ray analysis

Prior to crystallization, the protein was concentrated to 16 mg ml^{−1}, mixed with acetazolamide (final concentration of 1 mM) and centrifuged for 20 min at 13 000g to clarify the solution. Initial screening of crystallization conditions was carried out by the hanging-drop vapour-diffusion method using an automated Phoenix crystallization robot (Art Robbins Instruments) and Crystal Screen HT and PEG/Ion Screen HT (Hampton Research). The initial crystallization droplets comprised 100 nl protein solution mixed with 100 nl reservoir solution and were equilibrated against 50 µl reservoir solution in a 96-well Art Robbins Crystallization Intelli-Plate (Hampton Research). Crystals appeared after 1 d from condition No. 10 of Crystal Screen HT (Fig. 1b), which consisted of 20% (w/v) polyethylene glycol (PEG) 3350, 200 mM sodium iodide. This condition was subsequently refined to improve the crystal quality, yielding an optimal crystallization reservoir solution consisting of 20% (w/v) PEG 8000, 200 mM sodium iodide with protein and inhibitor concentra-

tions of 27 mg ml^{−1} and 2 mM, respectively. For data collection, the crystal was flash-cooled to 100 K after soaking in a cryoprotectant solution consisting of 25% (w/v) PEG 8000, 240 mM sodium iodide, 2 mM acetazolamide, 15% (v/v) glycerol. X-ray data were collected to 1.7 Å resolution on the MX1 beamline of the Australian Synchrotron (Fig. 1c). A total of 360 images were collected using a 0.5° oscillation. The statistics of data collection are summarized in Table 1.

2.4. Molecular replacement

A sequence-similarity search against proteins with known structure in the RCSB Protein Data Bank revealed that HpαCA shares 39% identity with αCA from *Sulfurihydrogenibium yellowstonense* YO3AOP1 (PDB entry 4g7a; Di Fiore *et al.*, 2013) in a 228-residue overlap and 38% identity with carbonic anhydrase from *Neisseria gonorrhoeae* (PDB code 1kop; Huang *et al.*, 1998) in a 231-residue overlap. Molecular replacement was performed with *Phaser* using data to 2.5 Å resolution (McCoy *et al.*, 2005). The search model was prepared using *CHAINS* (Stein, 2008) based on the coordinates of *S. yellowstonense* αCA.

3. Results and discussion

3.1. Overexpression, purification and biochemical characterization

Recombinant HpαCA was expressed from the pET151/D-TOPO plasmid in *E. coli* BL21(DE3) upon induction by T7 polymerase. The enzyme was purified to >98% electrophoretic homogeneity based on Coomassie Blue staining of SDS–PAGE gels (Fig. 1a). The protein migrates on SDS–PAGE with an apparent molecular weight of 25 kDa, which is close to the value calculated from the amino-acid sequence (29.6 kDa). When subjected to gel filtration, the protein eluted as a single peak with an apparent molecular weight of approximately 50 kDa, indicating that HpαCA forms a dimer in solution. This result is consistent with previous studies on αCAs from *S. yellowstonense*, *N. gonorrhoeae*, *Chlamydomonas reinhardtii* (Suzuki *et al.*, 2011), *Aspergillus oryzae* (Cuesta-Seijo *et al.*, 2011) and *Homo sapiens* (Pilka *et al.*, 2012; Alterio *et al.*, 2009; Whittington *et al.*, 2001), which suggested a dimeric state for this family of enzymes.

3.2. Crystallization and preliminary crystallographic analysis

Because of the high mosaicity (0.8°) of the crystals, as evident from the lack of space between the lunes in Fig. 1(c), the data collected over 180° had many spot overlaps and were only partially complete (83%). Analysis of the diffraction data and systematic absences using *iMOSFLM* (Battye *et al.*, 2011) and *POINTLESS* (Evans, 2006) suggested that the crystals have the symmetry of space group $P2_12_12_1$, with unit-cell parameters $a = 37.0$, $b = 82.4$, $c = 150.8$ Å. The self-rotation function computed using *POLARFN* (Winn *et al.*, 2011) with diffraction data between 10 and 1.9 Å resolution and an integration radius of 30 Å exhibited a twofold symmetry axis ($\kappa = 180^\circ$) with (θ, ϕ) values of (71, 90°) and with a height of 10.7σ (Fig. 1d). This suggested that the protein may form a dimer. The V_M value calculated under the assumption that there is one dimer of HpαCA in the asymmetric unit was 2.18 Å³ Da^{−1}, corresponding to a solvent content of approximately 44% (Matthews, 1968). These values fall within the range observed by Matthews for protein crystals.

A molecular-replacement search was performed using *Phaser* and a monomer of *S. yellowstonense* αCA as a model. Two copies of the search model were found in the asymmetric unit which were related by a noncrystallographic twofold axis and formed a dimer. One round of refinement of the coordinates and temperature factors using *CNS*

(Brunger, 2007) yielded values of 0.36 and 0.40 for R and R_{free} , respectively, which are consistent with a correct molecular-replacement solution. Ultimately, a complete solution of the Hp α CA–acetazolamide complex structure is expected to lead to a better understanding of the α CA family and reveal the *H. pylori*-specific structural features that are important for regulation of the α CA activity.

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