Mayte Conejero-Muriel,^a
Ana Isabel Martínez-Gómez,^b
Sergio Martínez-Rodríguez^{c*} and
Jose A. Gavira^{a*}

^aLaboratorio de Estudios Cristalográficos, IACT,
CSIC–Universidad de Granada, Avenida de las
Palmeras 4, 18100 Armilla, Spain,

^bDepartamento de Química–Física,
Universidad de Almería, 04120 Almería, Spain,
and ^cDepartamento de Química–Física,
Universidad de Granada, 18071 Granada, Spain

Correspondence e-mail: sergio@ugr.es,
gavi@lec.csic.es

Received 1 August 2014

Accepted 30 September 2014

Cloning, expression, purification, crystallization and preliminary X-ray characterization of allantoinase from *Bacillus licheniformis* ATCC 14580

Allantoinase, a member of the amidohydrolase superfamily, exists in a wide variety of organisms, including bacteria, fungi, plants and a few animals, such as fishes and amphibians. Allantoinase catalyzes the reversible hydrolysis of allantoin into allantoate by hydrolytic cleavage of the N¹–C² amide bond of the five-membered hydantoin ring. Allantoinase from *Bacillus licheniformis* (AllBali) presents an inverted enantioselectivity towards allantoin (*R*-enantioselective), which is a distinguishable feature that is not observed for other allantoinases. In this work, *B. licheniformis* ATCC 14580 allantoinase (AllBali) containing a C-terminal His₆ tag was overproduced in *Escherichia coli* and purified to homogeneity. Crystals of AllBali were obtained by the vapour-diffusion method using 0.1 M potassium thiocyanate, 20%(w/v) polyethylene glycol 3350 as a crystallization solution. X-ray diffraction data were collected to a resolution of 3.5 Å with an R_{merge} of 29.2% from a crystal belonging to space group *P*12₁1, with unit-cell parameters $a = 54.93$, $b = 164.74$, $c = 106.89$ Å, $\beta = 98.49^\circ$. There are four molecules in the asymmetric unit with a solvent content of 47% as estimated from the Matthews coefficient ($V_M = 2.34$ Å³ Da^{−1}).

1. Introduction

The amidohydrolase superfamily is comprised of a set of enzymes that catalyze the hydrolysis of a broad range of substrates with amide or ester functional groups at carbon and phosphorus centres (Holm & Sander, 1997; Syltatk *et al.*, 1999; Gerlt *et al.*, 2011; Gerlt & Babbitt, 2001; Seibert & Raushel, 2005). Among these enzymes, several amidohydrolases acting on cyclic amides (EC 3.5.2.–) have been shown to be involved in the metabolism of pyrimidines and purines; dihydroorotase (EC 3.5.2.3) participates in the *de novo* synthesis of pyrimidines (Holm & Sander, 1997), whereas dihydropyrimidinase (EC 3.5.2.2) and allantoinase (EC 3.5.2.5) are involved in the catabolism of pyrimidines and purines, respectively.

Allantoinase exists in a wide variety of organisms, including bacteria, fungi, plants and a few animals, such as fishes and amphibians (Kim *et al.*, 2000; Lee & Roush, 1964; Vogels & Van der Drift, 1966; Vogels *et al.*, 1966; Hayashi *et al.*, 1994; Masuda *et al.*, 2001; Gaines *et al.*, 2004). Allantoinase catalyzes the reversible hydrolysis of allantoin into allantoate through the hydrolytic cleavage of the five-membered hydantoin ring (Noguchi *et al.*, 1986; Vogels *et al.*, 1966). Allantoin and allantoate, collectively called ureides, play essential roles in nitrogen assimilation, storage and transport (Kim *et al.*, 2009). Biochemical studies using *Escherichia coli* allantoinase have proved that its activity depends on the presence and the type of metal in the active centre, analogous to what has been observed for other enzymes of this family (Kim *et al.*, 2000; Mulrooney & Hausinger, 2003). However, a metal-independent allantoinase (puuE) from *Pseudomonas fluorescens* has been reported (Ramazina *et al.*, 2008), allowing the organism to be functional even in the absence of the genes for the metal-dependent allantoinase.

Owing to the inverted enantioselectivity found for *Bacillus licheniformis* allantoinase when compared with the enzymes from other microorganisms (Martínez-Gómez *et al.*, 2014), we wanted to obtain further insights into the molecular determinants governing this feature. In this study, the allantoinase from *B. licheniformis* ATCC 14580 (AllBali) has been cloned, overexpressed in *E. coli* and purified

© 2014 International Union of Crystallography
All rights reserved

Table 1

Data collection and processing.

Values in parentheses are for the outer shell.

Beamline	ID23-1, ESRF
Space group	$P12_11$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 54.93$, $b = 164.74$, $c = 106.89$, $\beta = 98.49$
Wavelength ($\text{\AA}$)	0.9700
Resolution ($\text{\AA}$)	48.73–3.50 (3.78–3.50)
Total No. of reflections	160528 (32266)
Total No. of unique reflections	23737 (4880)
$R_{\text{merge}}^\dagger$ (%)	29.2 (68.5)
$\langle I/\sigma(I) \rangle$	7.5 (3.6)
Completeness (%)	99.9 (99.9)
Multiplicity	6.8 (6.6)
Protein molecules per asymmetric unit	4
Matthews coefficient ($\text{\AA}^3 \text{Da}^{-1}$)	2.34
Solvent content (%)	47
B factor ($\text{\AA}^2$)	67.2
Mosaicity ($^\circ$)	0.45

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

to homogeneity in a two-step procedure by metal-affinity and size-exclusion chromatography. Crystallization screening and initial X-ray characterization using a synchrotron-radiation source are also presented.

2. Materials and methods

2.1. Cloning

B. licheniformis ATCC 14580 genomic DNA was used as template to amplify the allantoinase gene by PCR (GenBank sequence accession No. CP000002.3; locus tag BL01094; 1365 bp). The forward primer (5'-ACACCATATGAATTTTGATTCAATTATCAAA-3') included an *NdeI* restriction site (bold) upstream of the initiation codon. The reverse primer (5'-AAAACCTCGAGGGATCCACGCGGAACAGAGGAATAAATCTTCTACTTT-3') included a thrombin recognition site (italics) followed by an *XhoI* restriction site (bold). The PCR fragment obtained (1397 bp) was purified from agarose gel using QIAquick (Qiagen) and digested at 310 K using *NdeI* and *XhoI* enzymes (Fermentas). The digested fragment was purified from agarose gel using QIAquick (Qiagen) and then ligated into pET-21b+ plasmid (Novagen) cut with the same enzymes. The resulting construction (pSMR1) allows the production of the recombinant AllBali fused to a C-terminal polyhistidine tag (His₆ tag), which can be removed by thrombin cleavage.

2.2. Protein expression

E. coli strain BL21 (DE3) containing pSMR1 was grown in LB medium supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin. A single colony was transferred into 10 ml LB medium with ampicillin at the above-mentioned concentration in a 50 ml flask. This culture was incubated overnight at 310 K with shaking. 1 l LB supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin was inoculated with 10 ml of the overnight culture in a 2 l flask. After 2 h of incubation at 310 K with vigorous shaking (OD_{600} 0.4–0.6), isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM and the culture was continued at 305 K for a further 4 h. The cells were collected by centrifugation (5000 rev min⁻¹, 20 min, 277 K) and stored at 253 K until use.

2.3. Purification

E. coli BL21 (DE3) pSMR1 cells were resuspended in 30 ml wash buffer (50 mM sodium phosphate, 300 mM NaCl pH 8.0). The cell walls were disrupted on ice by sonication using a Branson Digital Sonifier for six periods of 30 s with pulse mode 0.5 and sonic power 60%. The pellet was precipitated by centrifugation (13 000 rev min⁻¹, 15 min, 277 K) and discarded. The supernatant was applied onto a column packed with nickel metal-affinity resin (1 ml, GE Healthcare) and then washed with 10 ml wash buffer (50 mM sodium phosphate, 300 mM NaCl pH 8.0). The AllBali enzyme was eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole pH 8.0). The eluted enzyme was directly loaded onto a Tricorn Superdex 200 gel-filtration column (GE Healthcare) in an ÄKTA-prime FPLC system (GE Healthcare) using 20 mM Tris-HCl pH 8.0 as the running buffer. The fractions corresponding to AllBali were pooled and concentrated using a Vivaspin 20 ultrafiltration unit (Sartorius, 30 kDa cutoff), dialyzed against 20 mM Tris-HCl pH 8.0 and stored at 277 K. Protein purity was verified by SDS-PAGE. The His₆ tag was not removed for crystallization experiments, thus resulting in a theoretical molecular mass of the construction (residues 1–468) of 51 163 Da. Protein concentrations were determined spectrophotometrically at 280 nm using the theoretically calculated extinction coefficient of 37 860 $\text{M}^{-1} \text{cm}^{-1}$ (Gill & von Hippel, 1989).

2.4. Crystallization

Initial crystallization trials of purified His₆-tagged AllBali (residues 1–468) were carried out *via* the sitting-drop vapour-diffusion method with a STAR Plus liquid-handling and nanodroplet robotic system (Hamilton). A total of 288 conditions from the following commercially available crystallization screens were used for initial screening: Crystal Screen HT, PEG/Ion and PEG/Ion 2 (Hampton Research, California, USA) and The JCSG+ Suite (Qiagen, Germany) at 293 K. A drop consisting of 0.1 μl screening solution and 0.1 μl protein solution (14 mg ml⁻¹) in 20 mM Tris-HCl pH 8.0 was placed into each well of a CrystalQuick LBR, 3 $\times$ square well. All hit conditions were selected for size improvement by the hanging-drop vapour-diffusion method in 24-well Linbro plates (Crystalgen). Drops of 1 μl protein (14 mg ml⁻¹) in 20 mM Tris-HCl pH 8.0 and 1 μl crystal-

Figure 1

Size-exclusion chromatography profile of the purified AllBali carried out with a Tricorn Superdex 200 column. The inset represents the SDS-PAGE analysis of AllBali. The purity of AllBali was checked on a 12% SDS-PAGE gel before crystallization trials. The theoretical molecular mass of AllBali is 51 163 Da. Lane 1, purified AllBali; lane 2, molecular-mass marker.

Figure 2

Initial crystallization screening with AllBali (14 mg ml^{-1}) in 20 mM Tris-HCl pH 8.0. (a) $20\% (w/v)$ PEG 3350, 0.2 M sodium iodide. (b) $20\% (w/v)$ PEG 3350, 0.2 M potassium thiocyanate. (c) $20\% (w/v)$ PEG 3350, 0.2 M sodium bromide. (d) $20\% (w/v)$ PEG 6000, 0.1 M citrate pH 5.0. (e) Crystals that diffracted to 3.5 Å resolution obtained using the final crystallization condition ($20\% \text{ PEG 3350}$, 0.1 M potassium thiocyanate pH 6.5).

Figure 3

X-ray diffraction image of an AllBali crystal collected to 3.5 Å resolution. The inset is a magnification showing diffraction spots at 3.0 Å resolution.

lization condition were allowed to equilibrate against $500 \mu\text{l}$ reservoir solution. Further improvement was only performed with condition No. 14 ($20\% \text{ PEG 3350}$, 0.2 M potassium thiocyanate) of the PEG/Ion kit. A grid screen around the initial condition was performed by varying the concentration of PEG 3350 from 5 to 30% and that of potassium thiocyanate from 0.05 to 0.2 M at pH 6.5 in hanging-drop

experiments at 293 K . Although several conditions of the grid produced crystals, the largest crystals were obtained in $20\% \text{ PEG 3350}$, 0.1 M potassium thiocyanate pH 6.5.

2.5. X-ray diffraction data collection and preliminary analysis

Target crystals were identified under a microscope using polarized light. Crystals from the different conditions of the grid screening were transferred to a cryosolution consisting of the reservoir solution supplemented with 20% glycerol and flash-cooled in liquid nitrogen prior to data collection on ID23-1 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Portions of the larger crystals from the hanging-drop experiment ($20\% \text{ PEG 3350}$, 0.1 M potassium thiocyanate pH 6.5) were used for X-ray data collection. Reflections were recorded on a Pilatus detector at a wavelength of 0.9700 Å at 100 K . A total of 720 images were collected with an oscillation range of 0.5° and a crystal-to-detector distance of 525.36 mm . The diffraction data were indexed and integrated using *XDS* (Kabsch, 2010) and scaled with *SCALA* from the *CCP4* suite (Winn *et al.*, 2011). Details of data collection and processing are presented in Table 1.

3. Results and discussion

B. licheniformis ATCC 14580 allantoinase (AllBali), with a calculated molecular mass of $51\,163 \text{ Da}$ and a theoretical isoelectric point of 5.46 , was cloned containing a C-terminal His₆ tag, overproduced in *E. coli* BL21 (DE3) cells and purified to homogeneity in a two-step procedure by affinity and size-exclusion chromatography for crystallization (Fig. 1). The final purified sample was at least 95% pure as estimated by SDS-PAGE.

Initial screening using a STAR Plus liquid-handling and nanodroplet robotic system (Hamilton) produced crystals in four different conditions: Nos. 10 and 14 of PEG/Ion, No. 46 of PEG/Ion 2 and No.

21 of The JCSG+ Suite (Figs. 2*a*, 2*b*, 2*c* and 2*d*, respectively). Among them, condition No. 14 of PEG/Ion (20% PEG 3350, 0.2 *M* potassium thiocyanate) was selected for further optimization. Larger needle-shaped crystals ($50 \times 5 \times 5 \mu\text{m}$) were obtained after 3 d using 20% PEG 3350, 0.1 *M* potassium thiocyanate pH 6.5. Although the crystal external appearance did not improve (Fig. 2*e*), the larger size of the needles allowed us to cut the best looking portions of the crystal clusters to test their diffractive power.

A single data set was collected on ID23-1 at the ESRF and a representative diffraction pattern is shown in Fig. 3. The crystal belonged to space group $P12_11$, with unit-cell parameters $a = 54.93$, $b = 164.74$, $c = 106.89 \text{ \AA}$, $\beta = 98.49^\circ$. The diffraction data set was processed to 3.5 $\text{\AA}$ resolution with 99.9% completeness and an R_{merge} of 29.2%. Final data-collection statistics are given in Table 1.

Assuming the presence of four molecules per asymmetric unit, the Matthews coefficient (V_M) and solvent content were calculated to be $2.34 \text{ \AA}^3 \text{ Da}^{-1}$ and 47%, respectively, which are within the common range observed for protein crystals (Matthews, 1968). A BLAST search with the AllBali amino-acid sequence showed 54% and 40% identity to *E. coli* and *B. halodurans* allantoinases, the structures of which are available in the PDB [PDB entries 3e74 (Kim *et al.*, 2009) and 3hm7 (New York Structural Genomics Research Consortium, unpublished work), respectively]. As discussed previously, AllBali also presents a high sequence identity (approximately 40%) to other members of the amidohydrolase family, *i.e.* dihydropyrimidinases/hydantoinases or dihydroorotases (Syldatk *et al.*, 1999; Martínez-Gómez *et al.*, 2014). The crystallographic structural solution of AllBali was obtained using the three-dimensional model structure of the allantoinase from *E. coli* (PDB entry 3e74; Kim *et al.*, 2009; 54% sequence identity) as template for molecular replacement in Phaser (McCoy, 2007). Structure refinement of AllBali is currently in progress.

This research was funded by MICINN (Spain) projects BIO2010-16800 (JAG), 'Factoría Española de Cristalización' Consolider-Ingenio 2010 (JAG and MCM) and EDRF Funds (JAG). MCM

thanks CSIC for her JAE-Pre research contract. We are grateful to the staff at beamline ID23-1 of the European Synchrotron Radiation Facility (ESRF), Grenoble, France for providing assistance during data collection under Project MX-1541.

References

- Gaines, P. J., Tang, L. & Wisniewski, N. (2004). *Insect Biochem. Mol. Biol.* **34**, 203–214.
- Gerlt, J. A., Allen, K. N., Almo, S. C., Armstrong, R. N., Babbitt, P. C., Cronan, J. E., Dunaway-Mariano, D., Imker, H. J., Jacobson, M. P., Minor, W., Poulter, C. D., Raushel, F. M., Sali, A., Shoichet, B. K. & Sweedler, J. V. (2011). *Biochemistry*, **50**, 9950–9962.
- Gerlt, J. A. & Babbitt, P. C. (2001). *Annu. Rev. Biochem.* **70**, 209–246.
- Gill, S. C. & von Hippel, P. H. (1989). *Anal. Biochem.* **182**, 319–326.
- Hayashi, S., Jain, S., Chu, R., Alvares, K., Xu, B., Erfurth, F., Usuda, N., Rao, M. S., Reddy, S. K., Noguchi, T., Reddy, J. K. & Yeldandi, A. V. (1994). *J. Biol. Chem.* **269**, 12269–12276.
- Holm, L. & Sander, C. (1997). *Proteins*, **28**, 72–82.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Kim, K., Kim, M.-I., Chung, J., Ahn, J.-H. & Rhee, S. (2009). *J. Mol. Biol.* **387**, 1067–1074.
- Kim, G. J., Lee, D. E. & Kim, H.-S. (2000). *J. Bacteriol.* **182**, 7021–7028.
- Lee, K. W. & Roush, A. H. (1964). *Arch. Biochem. Biophys.* **108**, 460–467.
- Martínez-Gómez, A. I., Soriano-Maldonado, P., Andújar-Sánchez, M., Clemente-Jiménez, J. M., Rodríguez-Vico, F., Neira, J. L., Las Heras-Vázquez, F. J. & Martínez-Rodríguez, S. (2014). *Biochimie*, **99**, 178–188.
- Masuda, W., Fujiwara, S. & Noguchi, T. (2001). *Biosci. Biotechnol. Biochem.* **65**, 2558–2560.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J. (2007). *Acta Cryst.* **D63**, 32–41.
- Mulrooney, S. B. & Hausinger, R. P. (2003). *J. Bacteriol.* **185**, 126–134.
- Noguchi, T., Fujiwara, S. & Hayashi, S. (1986). *J. Biol. Chem.* **261**, 4221–4223.
- Ramazzina, I., Cendron, L., Folli, C., Berni, R., Monteverdi, D., Zanotti, G. & Percudani, R. (2008). *J. Biol. Chem.* **283**, 23295–23304.
- Seibert, C. M. & Raushel, F. M. (2005). *Biochemistry*, **44**, 6383–6391.
- Syldatk, C., May, O., Altenbuchner, J., Mattes, R. & Siemann, M. (1999). *Appl. Microbiol. Biotechnol.* **51**, 293–309.
- Vogels, G. D., Trijbels, F. & Uffink, A. (1966). *Biochim. Biophys. Acta*, **122**, 482–496.
- Vogels, G. D. & Van der Drift, C. (1966). *Biochim. Biophys. Acta*, **122**, 497–509.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.