Crystal structure of a putrescine aminotransferase from *Pseudomonas* sp. strain AAC

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The putrescine aminotransferase KES24511 from *Pseudomonas* sp. strain AAC was previously identified as an industrially relevant enzyme based on the discovery that it is able to promiscuously catalyse the transamination of 12-aminododecanoic acid. Here, the cloning, heterologous expression, purification and successful crystallization of the KES24511 protein are reported, which ultimately generated crystals adopting space group *I*2. The crystals diffracted X-rays to 2.07 Å resolution and data were collected using the microfocus beamline of the Australian Synchrotron. The structure was solved using molecular replacement, with a monomer from PDB entry 4a6t as the search model.

1. Introduction

A putrescine aminotransferase, KES24511, was identified in 2015 as one of 14 aminotransferases in a 12-aminododecanoic acid-metabolizing strain of *Pseudomonas* sp. strain AAC (amino alkanoate catabolism; Wilding *et al.*, 2015). Of the 14 proteins, three were identified that could catabolize 12-aminododecanoic acid; two of the three enzymes, KES23458 (a β -alanine:pyruvate transaminase) and KES23360 (a 4-aminobutyrate:pyruvate transaminase), have been studied in more depth, but significant details of the third, KES24511, have not been reported until now.

There are very few known examples of enzymes that can catalyse the conversion of 12-aminododecanoic acid into dodecanoic acid semialdehyde, which is perhaps unsurprising since the substrate is relatively large and does not naturally occur (Wilding *et al.*, 2016; Schrewe *et al.*, 2013; Song *et al.*, 2014). The paucity of enzymes that work on 12-aminododecanoic acid suggests that it might be unusual to find three proteins in the same organism that all share this activity. Whilst it may simply be a promiscuous activity with no physiological relevance in all three cases, the reaction itself is of interest (Fig. 1). The generation of 12-aminododecanoic acid is potentially valuable in industrial biocatalysis for use in polyamide production, with Nylon 12 having significant applications, particularly in the automotive industry. Since aminotransferases can be utilized to run reactions in either direction, these enzymes can therefore be developed for the generation of 12-aminododecanoic acid and potentially a range of other, similar materials. Other than a prediction of the physiological role of KES24511, and confirmation of its enzymatic activity with 12-aminododecanoic acid, little is known about this protein. Here, we report the purification, crystallization and structure of KES24511.

2. Materials and methods

2.1. Macromolecule production

The gene encoding the KES24511 protein was amplified by PCR and cloned into a pETcc2 vector as described previously (Wilding *et al.*, 2015). Transformation of the vector into *Escherichia coli* BL21 (DE3) produced large quantities of insoluble protein, but soluble protein expression was achieved after transformation into the Rosetta 2 (DE3) cell line. Cells were grown in LB medium supplemented with ampicillin ($100 \mu\text{g ml}^{-1}$) at 37°C . When the optical density at 600 nm (OD_{600}) reached 0.6–1.0, the culture was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG; 1 mM final concentration). The cells were further incubated with shaking overnight at 15°C and harvested by centrifugation in a Beckman Avanti J-E centrifuge (4000g; 20 min), and the supernatant was discarded. The pellet was resuspended in imidazole–sodium chloride–potassium phosphate buffer (5 mM imidazole, 250 mM NaCl, 10 mM phosphate; pH 7.5) and cell lysis was achieved by homogenization at 137 900 kPa using a Microfluidics M-110P Microfluidizer. Cellular debris was precipitated by centrifugation (40 000g; 45 min) and the supernatant was passed over a HiTrap Chelating HP column (GE Healthcare) on an ÄKTA fast protein liquid-chromatography (FPLC) system (GE Healthcare). The protein was eluted with an increasing concentration of imidazole (5–500 mM), and the fractions containing protein were transferred into 10 mM potassium phosphate buffer pH 7.5, concentrated by centrifugation (10 000 molecular weight cutoff; GE Healthcare) and further purified by gel filtration (Superdex 200; GE Healthcare) in the same phosphate buffer. The protein purity was estimated to be >95% by SDS–PAGE. The protein eluted as a single peak that was concentrated to 8.7 mg ml^{-1} in a 10 kDa spin concentrator (GE Healthcare).

2.2. Assays

The activity of KES24511 was determined using an alanine dehydrogenase enzyme-coupled assay as described previously (Wilding *et al.*, 2015). The transaminase reaction co-product (L-alanine) was converted *in situ* by the dehydrogenase in an

Table 1

Macromolecule-production information.

The NdeI site and BamHI site used for cloning are underlined in the forward and reverse primers, respectively.

Source organism	<i>Pseudomonas</i> sp. strain AAC
DNA source	gDNA
Forward primer	TTTTTTCATATGACGAAACAGACCAGCGCCAG- CCCAAC
Reverse primer	AAAAAAGGATCCTCAAGACAGCGCTACGCGGCG- GTTTG
Cloning vector	pETcc2
Expression vector	pETcc2
Expression host	<i>E. coli</i> Rosetta 2 (DE3)
Complete amino-acid sequence of the construct produced	MGSSHHHHH SSGLVPRGSH MTKQTSAQTK HWQALSREHH LAPFTDYKQL NEKGARIITK AEGVYLWDE GNKILDGMAG LWCNVGYGR KELAEVAYKQ MLELPYYNLF FQTAHPPALE LAKAIADIAP EGMNHVFTG SGSESDNTVL RMVRHYWSIK GKPQKVVIG RWNGYHGSTV AGVSLGGMKA LHSQGDLPPI GIVHIAQPYW YGEAGDMSAE EFGVWAAEQL EKKILEVGEE NVAAFIAEPI QGAGGVIVPP DTYWPKIREI LAKYEILFIA DEVICGFGRT GEWFGSQYYG NAPDLMPIAK GLTSGYIPMG GVIVRDEIVD TLNEGGEFYH GFTYSGHPVA AAVALENIRI LREEKIVETV KAETAPYLQK RWQELADHPL VGEARGVGMV GALELVKNKK TRERFENGVG MLCREHCFRN GLIMRAVGDT MIISPLLVIT KPEIDELITL ARKCLDQTAA VALS

NAD⁺-dependent oxidation (Fig. 1), and nicotinamide conversion was monitored by the change in UV absorbance at 340 nm in a SpectraMax M2 spectrophotometer (Molecular Devices, Australia). A typical assay comprised 6.25 mM putrescine, co-substrate (0.5 mM pyruvate), 1.25 mM NAD⁺, dehydrogenase (0.035 U alanine dehydrogenase; ADH), 0.025–2 μM transaminase and 100 mM potassium phosphate pH 9. Macromolecule-production information is summarized in Table 1.

2.3. Crystallization

384 initial crystallization trials were set up with protein in 10 mM potassium phosphate buffer at two concentrations: $\sim 9 \text{ mg ml}^{-1}$ from the original purification and at a 2:1 dilution (4.5 mg ml^{-1}). The screens used were Shotgun (Fazio *et al.*,

Figure 1
The activity of KES24511.

Table 2
Crystallization.

Method	Vapour diffusion
Plate type	Swissci SD-2 sitting-drop plates ('MRC' plates)
Temperature (°C)	20
Protein concentration (mg ml ⁻¹)	10.5
Buffer composition of protein solution	TBS (25 mM Tris chloride, 137 mM NaCl, 3 mM KCl pH 8)
Composition of reservoir solution	0.066 M calcium acetate, 18%(w/v) PEG MME 5K, 0.1 M Tris chloride pH 7.6
Volume and ratio of drop	300 nl total, 1:1 ratio
Volume of reservoir (µl)	50

2014) at both 8 and 20°C as well as PACT (Newman *et al.*, 2005) and an in-house developed salt/pH screen, PS gradient, at 20°C. Details of the conditions can be found at <http://c6.csiro.au> (Newman *et al.*, 2010). Initial crystals were found both in Shotgun condition H3 [0.2 M ammonium iodide, 20%(w/v) PEG 3350] and PACT condition D11 [0.2 M CaCl₂, 20%(w/v) PEG 6K, 0.1 M Tris chloride pH 8] at both protein concentrations. All crystallization trials were set up with droplets consisting of 150 nl protein + 150 nl reservoir solution against a reservoir of 50 µl in SD-2 sitting-drop plates (Molecular Dimensions, UK). Optimization around Shotgun condition H3 led to hexagonal rods that showed absolutely no diffraction at the MX1 beamline at the Australian Synchrotron. Optimization around PACT condition D11 led to

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	MX2 beamline, Australian Synchrotron
Wavelength (Å)	0.9537
Temperature (K)	100
Detector	ADSC Quantum 315
Crystal-to-detector distance (mm)	270
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	<i>I</i> 2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	76.4, 95.4, 114.4
α , β , γ (°)	90, 100.2, 90
Mosaicity (°)	0.27
Resolution range (Å)	47.7–2.07 (2.13–2.07)
Total No. of reflections	356994 (27812)
No. of unique reflections	49266 (3794)
Completeness (%)	100 (100)
CC _{1/2}	0.997 (0.790)
Multiplicity	7.2 (7.3)
$\langle I/\sigma(I) \rangle$	11.2 (2.6)
$R_{\text{p.i.m.}}$	0.046 (0.327)
Overall <i>B</i> factor from Wilson plot (Å ²)	31.0

irregular rectangular prisms that diffracted to 2.4 Å resolution on the same beamline. After running a thermal melt experiment (Fig. 2) to look for the effects of the formulation on protein stability (Seabrook & Newman, 2013), the protein was

Figure 2

Summary of the thermal melt analysis (Rosa *et al.*, 2015) for formulations of the KES24511 protein. The red dashed line is the T_m of the protein in the initial 10 mM potassium phosphate buffer ($56.9 \pm 0.2^\circ\text{C}$). Orange symbols represent the T_m of formulations containing 200 mM NaCl; blue symbols represent the T_m of formulations containing 50 mM NaCl. Each buffer is used at 50 mM. The T_m of KES24511 in 50 mM Tris chloride pH 8, 200 mM NaCl was $62.3 \pm 0.1^\circ\text{C}$. Circle symbols are used when all three replicate curves were used in the automated T_m analysis and diamond symbols are used when at least one of the three replicate curves was discarded before the analysis; thus, results shown with diamonds are less reliable.

Table 4
Structure solution and refinement.

Resolution range (Å)	47.7–2.07
Completeness (%)	100
σ Cutoff	—
No. of reflections, working set	46794
No. of reflections, test set	2468
Final R_{cryst} (%)	18.5
Final R_{free} (%)	23.8
No. of non-H atoms	
Protein	6018
Ion	2
Water	153
Total	6173
R.m.s. deviations from ideal geometry	
Bonds (Å)	0.017
Angles (°)	1.741
Average B factors (Å ²)	
Protein	38.8
Ion	34.0
Water	36.8
Ramachandran plot	
Most favoured (%)	94.5
Allowed (%)	99.6

exchanged into TBS (see Table 2) and concentrated to 10 mg ml⁻¹; the crystals grown from protein in this formulation (Fig. 3) diffracted to approximately 2.1 Å resolution. During the optimization, seeding and supplementing the protein with additional saturated aqueous pyridoxal 5'-phosphate (PLP; Sigma catalogue No. P3657, 1% final concentration) cofactor was trialled, but made little difference. Crystals were also grown in the presence of gabaculine, a known transaminase suicide inhibitor (Sigma catalogue No. A3539, 1 mM final concentration). Crystallization information is summarized in Table 2.

2.4. Data collection and processing

Crystals were cryoprotected using a cryoprotectant created by the addition of glycerol to the reservoir solution to give a 20%(v/v) final concentration and were cryocooled in liquid nitrogen before being placed into pucks for transport. Data were collected on the MX2 beamline at the Australian Synchrotron with X-rays at 13 000 eV. 360 1° oscillation images were obtained to give a complete data set with approximately sevenfold multiplicity in the monoclinic space group *I*2. The data were indexed and integrated with *XDS* (Kabsch, 2010) and were scaled with *AIMLESS* (Evans & Murshudov, 2013). Data-collection and processing statistics are summarized in Table 3.

2.5. Structure solution and refinement

The structure was solved using *Phaser* (McCoy *et al.*, 2007) with a monomer from PDB entry 4a6t (Humble *et al.*, 2012) as the molecular-replacement model. The model was modified using *CHAINS*AW (Stein, 2008) prior to molecular replacement. Two monomers were found in the asymmetric unit, with an RFZ of 38.0 and a TFZ of 29.2, to give a clear solution. Much of the structure was rebuilt using *Buccaneer* (Cowtan, 2006) prior to manual rebuilding using *Coot* (Emsley *et al.*, 2010) and isotropic refinement was then performed using

automatically generated NCS restraints with the addition of jelly-body refinement (sigma set to 0.2) in *REFMAC* (Murshudov *et al.*, 2011). Refinement statistics are summarized in Table 4.

3. Results and discussion

We previously reported the overproduction and purification of KES24511 from *Pseudomonas* sp. strain AAC in *E. coli* Rosetta 2 (DE3) (Wilding *et al.*, 2015). Using an alanine dehydrogenase-coupled assay, the purified protein exhibited activity with two substrates, putrescine and 12-aminododecanoic acid, with pyruvate as the co-substrate for transamination. Putrescine has a potential application in the production of heteronylon (Schaffer & Haas, 2014), whilst 12-aminododecanoic acid is an interesting substrate with potential applications in the synthesis of polyamides. Based on these observed activities, and the proximity of a number of genes

Figure 3
(a) KES24511 crystal grown from an optimization based on well H3 of Shotgun [0.219 M ammonium iodide, 16.9% (w/v) PEG 3350]; the crystal is 80 μm in the longest dimension. (b) Crystal grown in PACT condition D11 [0.2 M calcium acetate, 20% (w/v) PEG 6000, 0.1 M Tris chloride pH 8]; the largest crystal is 50 μm in the longest dimension. The scale bar represents 0.1 mm.

within the genome that have homology with polyamine metabolism, KES24511 is believed to be a putrescine aminotransferase.

The purified KES24511 protein was also used for biophysical characterization and crystallization trials. Stability studies using dye-based thermal melts showed (Fig. 2) that the protein was stable, with a T_m of over 60°C in many neutral buffers, particularly in the presence of a modest amount of salt. Crystals grown (Fig. 3) from protein formulated in TBS against a crystallant containing calcium acetate diffracted to

2.07 Å resolution on the MX2 beamline at the Australian Synchrotron.

The structure was solved using *Phaser* with PDB entry 4a6t (the holo form of the enzyme; Humble *et al.*, 2012) as the molecular-replacement model (approximately 58% identity to the KES24511 amino-acid sequence). Two protomers were observed in the asymmetric unit (Fig. 4) and the calcium used to obtain crystals was clearly seen to bring the proteins together at a crystallographic interface. The calcium was coordinated by the side chain of Asn114 and the backbone

Figure 4

(a) and (b) (approximate 90° rotation) show the secondary structure of KES24511 with β -strands represented as yellow arrows, α -helices in red and loop structures in green. In (b) the glutamic acid residue (Glu111) is shown as a stick model and the Ca atom is shown as a green sphere. (c) and (d) show the dimeric structure of KES24511, with (d) being rotated by approximately 90° from (c).

carbonyl of Ala106 of one protomer, and the side chain of Glu111 of a crystallographically related protomer, along with several waters. This seems to be a hot spot for ion binding as the PDB entry 4a6u (apo form; Humble *et al.*, 2012) structure has a sodium ion bound by homologous residues. Two protomers form a tight dimer with a buried surface area of about 4900 Å² (Figs. 4c and 4d). *PISA* (Krissinel & Henrick, 2007) predicts that the structure in solution is a tetramer formed by two dimers coming together using the coordination of two Ca atoms, with roughly double the buried surface area of the dimer alone (9800 *versus* 10 600 Å²); little additional surface area is sequestered by the 'tetramer' interface (about 800 Å²). Four protomers are also seen in the 4a6t structure (two tight dimers), and as such the tetramer predicted by *PISA* for the KES24511 structure roughly mirrors the asymmetric unit of the 4a6t structure. Gel filtration of KES24511 (Supplementary Fig. S1) suggests that it runs as a dimer in solution, which in turn suggests that the tetramer interface is potentially quite weak and not particularly relevant in solution, at least at moderate concentrations.

In the active site of aminotransferases, the PLP cofactor is typically covalently bound to a lysine residue in an internal aldimine conformation (Christen & Metzler, 1985; Eliot & Kirsch, 2004). In KES24511, Lys290 assumes this role (based on structural overlay with Lys288 in PDB entry 4a6t), and although there is good density for Lys290 (and surrounding residues), there is no sign of the PLP cofactor in a free or covalently bound state. Even in crystals grown in the presence

of additional PLP, no cofactor was observed in any X-ray data set and although a few positive difference density peaks in the map were observed (which have not been modelled), these were not in the area where PLP was expected to bind (in proximity to Lys290). Four structures were deposited by Humble and coworkers (PDB entries 4a6t, 4a6u, 4a6r and 4a72) which show the *Chromobacterium violaceum* ω -transaminase in different states. Humble and coworkers also had trouble obtaining crystals with the PLP cofactor bound to the protein in the crystalline state. Superposition of a monomer from either of the two apo structures (PDB entry 4a6r or 4a6u) gives an r.m.s.d. of 1.1 Å between 367 aligned C α atoms (out of 387) with the same N-terminal and 'roof' residues missing as in the 4a6u structure (see below). There is a shift in how the two protomers are bound in the dimer such that the r.m.s.d. doubles (to $\sim$ 2 Å) if one tries to superpose a dimer instead of a monomer, and it is clear that this difference becomes greater the further one moves away from the interface to the far side of the molecule (a pivot of one protomer relative to the other from the interface outwards).

The KES24511 structure is more similar to the 4a6u apo structure than the 4a6t holo structure (Fig. 5) of Humble and coworkers. It is missing the first 35 amino acids, which are mostly present in the related 4a6t structure (5–35), but these residues are also missing in the 4a6u and 4a6r apo structures. Another region, from 155 to 177 in KES24511, is also missing in the 4a6u apo structure. This forms one face of the PLP cofactor-binding pocket in the 4a6t structure (the 'roof'), and

Figure 5

Superposition of the KES24511 structure (green) with the apo structure (PDB entry 4a6u; cyan) and the holo structure (PDB entry 4a6t; magenta) from Humble *et al.* (2012). In (a) the residues in the holo structure (right side; N-terminal helical bundle) are highlighted as not present in the apo structures. (b) shows a close-up of the active site showing the PLP cofactor and roof seen in the holo enzyme (PDB entry 4a6t, magenta) which are not seen in the apo enzymes. Lys290 of the KES24511 structure is in green and shown to overlay extremely well with Lys288 in the holo structure, whereas Lys288 of the apo structure (PDB entry 4a6u; cyan) from Humble *et al.* (2012) has moved away and is pointing away from the PLP pocket.

disorder in this region may explain why no density is seen for the cofactor in KES24511. There is an additional missing loop from 315 to 322 which should connect two α -helices and comes close to the phosphate moiety of the PLP cofactor in the 4a6t structure (also missing in the 4a6u apo structure of Humble and coworkers). Otherwise, most of the model is revealed to have good density. Two of the three outliers found in the Ramachandran plot for each protomer are Ala289 and Lys290, which is not unusual in the transaminase family of structures (Newman *et al.*, 2013; Wilding *et al.*, 2016; Humble *et al.*, 2012; unpublished data).

In conclusion, we report here the crystal structure of a putative putrescine aminotransferase at 2.07 Å resolution. In the case of KES24511, unlike the *C. violaceum* apo structure, PDB entry 4a6u, it is clear that the PLP is not driving Lys290 into an 'active' conformation: there is no phosphate or sulfate molecule pulling together the disordered loops around the active site, as seen in the holo structure 4a6t, and yet the Lys290 side chain is poised to accept the PLP and form the aldimine in the 'active' conformation (Fig. 5*b*). Coordinates have been deposited with the Protein Data Bank (Berman *et al.*, 2003) and have been given the accession code 5ti8.

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