Neutron diffraction analysis of *Pseudomonas aeruginosa* peptidyl-tRNA hydrolase 1

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Perdeuterated peptidyl-tRNA hydrolase 1 from *Pseudomonas aeruginosa* was crystallized for structural analysis using neutron diffraction. Crystals of perdeuterated protein were grown to 0.15 mm³ in size using batch crystallization in 22.5% polyethylene glycol 4000, 100 mM Tris pH 7.5, 10% (v/v) isopropyl alcohol with a 20-molar excess of trylisine as an additive. Neutron diffraction data were collected from a crystal at room temperature using the MaNDi single-crystal diffractometer at Oak Ridge National Laboratory.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative pathogenic bacterium that causes pneumonia, septicemia and urinary-tract infections. It is one of the leading organisms associated with nosocomial infections, accounting for 10% of all hospital-acquired infections (Cook, 2000; Giannella *et al.*, 2012). While several antibiotics against *P. aeruginosa* infection are available, they have limited degrees of effectiveness. Therefore, more efficacious agents against *P. aeruginosa*, as well as other multidrug-resistant bacteria, are needed. Increasing numbers of multidrug-resistant *P. aeruginosa* strains are concerning, as efficacious antimicrobial options are limited. Notorious for its antibiotic resistance, *P. aeruginosa* is a particularly dangerous and dreaded pathogen.

Bacterial peptidyl-tRNA hydrolase 1 (Pth1) is a promising candidate for novel antibiotic development. Pth1 enzymes are responsible for recycling peptidyl-tRNA that results from the premature termination of the translation and expression of minigenes and short ORFs (Jørgensen & Kurland, 1990; Manley, 1978; Kurland & Ehrenberg, 1985; Cruz-Vera *et al.*, 2003; Hernández-Sánchez *et al.*, 1998; Tenson *et al.*, 1999). In most bacteria, Pth1 is essential. The structurally unrelated Pth2 has been found in a few select strains (Powers *et al.*, 2005), but functional redundancy has not been established. In eukaryotes there are multiple Pth enzymes and Pth1-domain-containing proteins, and Pth1 knockouts do not affect viability (Menez *et al.*, 2002; Rosas-Sandoval *et al.*, 2002). Thus, Pth1 is not essential in eukaryotes. Targeting protein biosynthesis is a proven antibiotic strategy. With 100-fold fewer Pth1 enzymes in a typical bacterial cell than ribosomes (Cruz-Vera *et al.*, 2000; Dutka *et al.*, 1993), there is a tremendous stoichiometric advantage to targeting Pth1.

While the function of Pth1 is understood, the exact mechanism of enzymatic peptidyl-tRNA hydrolysis remains unknown. Generally, hydrolysis reactions depend on the activation of a water molecule (to either H⁺ or OH[−] ions). In order to obtain further insight into the Pth1 hydrolysis

mechanism, neutron diffraction data have been collected that will allow the examination of the protonation states of amino-acid residues in the enzyme active site and the presence of conserved water molecules in its proximity. In turn, these data will provide a foundation for improvement of current small-molecule natural product inhibitors of Pth1 (Harris *et al.*, 2011; McFeeters *et al.*, 2012; McFeeters, 2013) as well as the design and synthesis of new antibiotics.

Structure determination of *P. aeruginosa* Pth1 (PaPth1) by X-ray diffraction has recently been reported by our group (Hughes *et al.*, 2012). From initial crystal growth, the propensity for the growth of large crystals suitable for neutron diffraction was apparent. Here, we report the preparation of large PaPth1 crystals and room-temperature neutron data collection. This is one of the very first data sets to be collected using the MaNDi single-crystal diffractometer at Oak Ridge National Laboratory.

2. Materials and methods

2.1. Expression and purification of perdeuterated PaPth1

PaPth1 with an N-terminal hexahistidine tag was cloned into a pKQV4 vector as described previously (Hughes *et al.*, 2012) and transformed into chemically competent *Escherichia coli* BL21(DE3) cells. To generate fully deuterated (perdeuterated) recombinant PaPth1, a frozen cell stock was first revived in 3 ml Luria broth (LB) and subcultured into 20 ml H₂O minimal medium (Törnkvist *et al.*, 1996) using 0.5% (w/v) unlabeled glycerol as a carbon source. Selection with carbenicillin (100 mg l⁻¹) was used in this and all other media. After approximately 12 h of incubation at 310 K, the cells were harvested by centrifugation and resuspended in 20 ml minimal medium in 25% D₂O. The cells were successively adapted to increasing concentrations of D₂O (50, 75 and 100%) minimal medium before centrifugation and resuspension in approximately 100 ml perdeuterated minimal medium that included 0.5% (w/v) deuterated glycerol (d₈, 99%) and salts that had been subjected to H/D exchange *via* D₂O dissolution and rotary evaporation (Meilleur *et al.*, 2009). This final D₂O-

adapted preculture was grown to an OD₆₀₀ of 2 before it was used to inoculate 1 l perdeuterated medium in a 1.6 l stirred BioFlo 3000 bench-top fermentor system (New Brunswick Scientific, Edison, New Jersey, USA) equipped with sensors to maintain the pD (>7.3) and dissolved oxygen (>30%). The temperature for the growth phase was controlled at 308 K, foaming was curtailed by manual addition of polypropylene glycol 2000 and the pD was controlled with 10% (w/w) sodium deuterioxide. Once the initial deuterated glycerol (d₈, 99%) had been depleted, the culture was fed with a solution consisting of 10% (w/w) deuterated glycerol (d₈, 99%), 0.2% (w/v) MgSO₄. At an OD₆₀₀ of 8.4, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 1 mM and the culture temperature was reduced to 291 K. SDS-PAGE analysis comparing pre-induction and post-induction samples was used to confirm protein overexpression. Following 18 h of induction, the final wet weight of cell paste was 28.8 g from 1 l of medium. Cells were harvested by centrifugation at 7000g for 30 min and stored at 193 K until further use. The purification procedure of PaPth1 has been described in detail elsewhere (Hughes *et al.*, 2012) and the perdeuterated protein was purified in the same manner before concentrating to 15 mg ml⁻¹ for crystallization.

2.2. Crystallization

For batch crystallization of PaPth1 (Rayment, 2002), the protein at a concentration of 15 mg ml⁻¹ was mixed with trilycine (Sigma-Aldrich, St Louis, Missouri, USA) in a 1:20 molar ratio in a 1.5 ml centrifuge tube. 150 µl of the mixture was continuously vortexed at a low speed. While vortexing, 150 µl of the precipitant [22.5% polyethylene glycol 4000, 100 mM Tris pH 7.5, 10% (v/v) isopropyl alcohol] was carefully added to the mixture. Following the addition of the precipitant, the mixture was vortexed for an additional 5 s. The tubes were then sealed and incubated at 291 K for several months. Crystals appeared within two weeks and continued to grow in size for another six weeks. The largest crystals, with volumes of approximately 0.15 mm³, were then mounted in fused quartz capillary tubes for neutron data collection (Fig. 1).

2.3. Data collection

Time-of-flight (TOF) neutron diffraction data were initially recorded from a 0.15 mm³ perdeuterated PaPth1 crystal at 293 K to 2.77 Å resolution using the MaNDi instrument (Coates *et al.*, 2010, 2015) at the Spallation Neutron Source (SNS) onsite at Oak Ridge National Laboratory (ORNL). A single ω angle was chosen for data collection and each image was separated by a 10° φ rotation. These six images (Fig. 2) were processed and integrated using the *MANTID* package (Arnold *et al.*, 2014) and the *LAUENORM* program from the *LAUEGEN* package (Campbell *et al.*, 1998). *LAUENORM* was used for wavelength normalization of the Laue data and for scaling between Laue diffraction images. X-ray diffraction data were collected at 293 K on an in-house Rigaku MicroMax-007 HF generator equipped with an R-Axis IV⁺⁺

Figure 1

A perdeuterated crystal of PaPth1 mounted within a capillary for neutron data collection. The volume of the crystal is approximately 0.15 mm³.

Table 1
Data-collection statistics.

	X-ray	Neutron
Diffraction source	Rigaku MicroMax-007 HF	MaNDi
Wavelength (Å)	1.54	2–4
Temperature (K)	293	293
Detector(s)	R-Axis IV ⁺⁺	18 SNS Anger cameras
Crystal-to-detector distance (mm)	150	450
Rotation range per image (°)	0.5	Fixed
No. of images collected	100	6
Total rotation range (°)	50	60
Exposure time per image	30 s	12 h
Space group	<i>P</i> 6 ₁ 22	<i>P</i> 6 ₁ 22
<i>a</i> , <i>b</i> , <i>c</i> (Å)	64.93, 64.93, 156.52	64.93, 64.93, 156.52
α , β , γ (°)	90, 90, 120	90, 90, 120
Mosaicity (°)	0.07	0.07
Resolution range (Å)	19.68–1.95 (2.00–1.95)	11.97–2.77 (2.87–2.77)
Total No. of reflections	80451 (3479)	8376 (483)
No. of unique reflections	14671 (832)	3756 (302)
Completeness (%)	98.2 (82.0)	70.0 (58.4)
Multiplicity	5.5 (4.2)	2.3 (1.6)
$\langle I/\sigma(I) \rangle$	27.6 (6.0)	9.4 (2.7)
<i>R</i> _{p.i.m.}	0.022 (0.135)	0.139 (0.167)
<i>B</i> factor from Wilson plot (Å ²)	22	31

detector and were processed using the *XDS* package (Kabsch, 2010). Data-collection statistics are given in Table 1.

3. Results and discussion

In order to obtain crystals large enough for neutron diffraction, optimization of crystal growth was conducted prior to setting up the crystallization of the perdeuterated protein. Starting with the conditions used previously to obtain crystals for X-ray diffraction, the protein concentration, the PEG concentration and the pH of the precipitant solution were

Figure 2
A selected time-of-flight slice corresponding to neutrons with wavelengths between 2.4 and 2.6 Å from the PaPth1 diffraction pattern. For clarity, the three-dimensional detector orientations of the Anger cameras have been mapped onto a two-dimensional plane and 11 detectors are shown.

varied. Subsequently, the effect of additives on the size and the quality of crystals was probed. The best conditions for the growth of large crystals were determined to be 100 mM Tris pH 7.5, 22.5% PEG 4000, 10%(v/v) isopropyl alcohol, 15 mg ml^{−1} protein and a 1:20 ratio of protein to trylisine. The presence of trylisine significantly improved the crystal size and shape. Additionally, several crystallization techniques were screened, including vapor diffusion and batch crystallization. Batch crystallization yielded the highest quality crystals, followed

by hanging-drop vapor diffusion. The quality of crystals from sitting-drop vapor diffusion varied greatly and depended on the presence of trylisine. Although the crystals obtained using the sitting-drop technique were large, most of them were inferior in diffracting X-rays compared with the crystals obtained by the batch method. The crystals that were grown in the absence of trylisine often showed imperfections. Thus, the crystals that were obtained by the batch-crystallization technique were chosen for neutron diffraction experiments. The crystallization and preliminary neutron diffraction studies make the solution of the structure of *P. aeruginosa* Pth1 possible. Joint X-ray and neutron refinement (Afonine *et al.*, 2010) will indicate the protonation sites within the active site of the enzyme and possibly provide insight into the mechanism of this important essential bacterial enzyme. In addition to providing mechanistic information about this uncharacterized family of hydrolases, these studies will provide a foundation for future Pth1 inhibitor studies.

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