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Recombinant production, crystallization and X-ray crystallographic structure determination of the peptidyl-tRNA hydrolase of *Pseudomonas aeruginosa*

The peptidyl-tRNA hydrolase enzyme from the pathogenic bacterium *Pseudomonas aeruginosa* (Pth; EC 3.1.1.29) has been cloned, expressed in *Escherichia coli* and crystallized for X-ray structural analysis. Suitable crystals were grown using the sitting-drop vapour-diffusion method after one week of incubation against a reservoir solution consisting of 20% polyethylene glycol 4000, 100 mM Tris pH 7.5, 10% (v/v) isopropyl alcohol. The crystals were used to obtain the three-dimensional structure of the native protein at 1.77 Å resolution. The structure was determined by molecular replacement of the crystallographic data processed in space group $P6_122$ with unit-cell parameters $a = b = 63.62$, $c = 155.20$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The asymmetric unit of the crystallographic lattice was composed of a single copy of the enzyme molecule with a 43% solvent fraction, corresponding to a Matthews coefficient of 2.43 Å³ Da⁻¹. The crystallographic structure reported here will serve as the foundation for future structure-guided efforts towards the development of novel small-molecule inhibitors specific to bacterial Pths.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium commonly found in soil, water, plants, animals and humans. This bacterium is an opportunistic pathogen that causes pneumonia, septicemia and urinary-tract infections. It is primarily a nosocomial pathogen, accounting for about 10% of all hospital-acquired infections (Cook, 2000; Giannella *et al.*, 2012). It is the most common pathogen isolated from patients who have been hospitalized for longer than a week. *P. aeruginosa* is notorious for its antibiotic resistance, which is attributed to multidrug efflux pumps and genetically evolved antibiotic-resistance genes (Poole, 2004). Currently, only a few antibiotics are effective against *P. aeruginosa* infection (e.g. fluoroquinolones, gentamicin and imipenem), with limited degrees of effectiveness. Therefore, more efficacious antibacterial agents against *P. aeruginosa* and other multidrug-resistant bacteria are needed.

Bacterial peptidyl-tRNA hydrolases (Pths) are prime candidates for novel antibiotic development. They are essential enzymes that cleave the peptide moiety off peptidyl-tRNA that results from the premature termination of translation and minigene expression. Pths are a highly conserved class of enzymes that are structurally dissimilar to their eukaryotic functional analogs. Structures of bacterial Pths have been solved (Clarke *et al.*, 2011; Pulavarti *et al.*, 2008; Schmitt *et al.*, 1997; Selvaraj *et al.*, 2007). As predicted from the high degree of amino-acid sequence similarity, these enzymes have the same backbone fold. Pth family members are globular single-domain proteins that have a central mixed β -sheet surrounded by α -helices. Helix 4 occludes three residues, Asn10, His20 and Asp93 (as numbered in *Escherichia coli* Pth), that were identified by site-directed mutagenesis to be crucially involved in enzyme activity (Goodall *et al.*, 2004). The structure of *E. coli* Pth in complex with the T ψ C loop of tRNA has recently been reported and has provided invaluable structural insight into nucleotide binding (Ito *et al.*, 2011). Nevertheless, more structural information is required in order to clearly elucidate the mechanism of peptide cleavage.

Here, we report the cloning, expression, crystallization and X-ray crystallographic structure of the recombinant Pth from *P. aeruginosa* at 1.77 Å resolution. The obtained structural information will provide a foundation for future structure-guided efforts towards the development of novel small-molecule inhibitors specific to bacterial Pths.

2. Materials and methods

2.1. Cloning

The open reading frame of Pth was amplified by polymerase chain reaction (PCR) from the genomic DNA of *P. aeruginosa* using the forward and reverse primers 5'-GAGGAATTCATGCATCATCATCATCACACTGCCGTACAAGTATCGT-3' and 5'-CGAGAAGCTTTTATCAGGCCTTCTGGCTGTGC-3', respectively. Oligonucleotide primers were designed to contain the necessary *Hind*III/*Eco*RI restriction-enzyme sites as well as an N-terminal hexahistidine tag. The amplification reaction was performed using a 50 µl reaction volume containing 20 pmol of each primer, 40 ng genomic template digested with *Eco*RV and 25 µl EmeraldAmp PCR Mastermix (Takara/Clontech, Japan). PCR was performed using a thermal cycler program consisting of initial denaturation at 371 K for 45 s followed by 30 cycles of 10 s at 371 K, 30 s at 333 K and 45 s at 345 K and a final 90 s extension step at 345 K.

The amplified product was digested with FastDigest *Hind*III and *Eco*RI (Fermentas, USA) and ligated into a linear pKQV4 plasmid vector previously digested with the same enzymes. Vector ligation was accomplished by combining 4 µl of the PCR product, 750 ng linear plasmid and T4 DNA ligase (Fermentas, USA) and was allowed to take place at room temperature for 2 h. 5 µl of the ligation reaction was added to 100 µl chemically competent JM109 *E. coli* cells (Promega, USA) and the plasmid-containing cell suspension was incubated on ice for 45 min. The cells were subjected to heat-shock treatment at 315 K for 1 min followed by a 2 min incubation on ice. The transformed cells were added to 400 µl Luria-Bertani (LB) medium, incubated for 1 h at 310 K and spread on LB-agar plates containing 100 µg ml⁻¹ carbenicillin. Inoculated plates were incubated overnight at 310 K. A 3 ml overnight culture was grown from a single colony of transformed cells to propagate the recombinant plasmid harboring the Pth coding sequence. The plasmid was purified from the saturated cell culture using a commercially available plasmid miniprep kit (Fermentas, USA). The correct amino-acid sequence was confirmed by DNA sequencing.

2.2. Expression and purification

The sequence-validated plasmid encoding *P. aeruginosa* Pth was transformed into chemically competent *E. coli* BL21* pLysS cells. A single colony of cells was taken from the selective LB agar plate and used to prepare a 3 ml starter culture grown in LB medium at 310 K. When the cell density reached an OD₆₀₀ of 0.8, the cells were spun down, resuspended in 30 ml M9 minimal medium and grown overnight. In the morning, the cells were spun down and resuspended in 1 l M9 medium. When the cell density reached an OD₆₀₀ of 0.6, the culture was quickly cooled on ice to 291 K and protein expression was induced by the addition of 1 M isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The protein was allowed to express overnight in a shaking incubator at 291 K. The cells were harvested by centrifugation and stored at 193 K.

For purification, the frozen cell pellet was resuspended in 20 ml lysis buffer (50 mM Na₂HPO₄, 300 mM NaCl, 2 mM DTT pH 7.4, 20 µg ml⁻¹ lysozyme) and incubated at room temperature for 30 min. The cell solution was then sonicated at maximum power using a

Branson Sonifier 250 (VWR Scientific, USA) with 20 s pulses for 2 min using a 50% duty cycle. The crude cell lysate was centrifuged for 20 min at 20 000g and the supernatant was applied onto an Ni²⁺-chelating affinity column. The column was washed with 50 mM Na₂HPO₄, 300 mM NaCl, 2 mM DTT, 20 mM imidazole pH 7.4 prior to elution with an equivalent buffer containing 150 mM imidazole.

Fractions containing the purified protein were pooled and dialyzed against 1 l of a final buffer consisting of 20 mM bis-tris pH 6.6, 50 mM NaCl, 2 mM DTT. The buffer-exchanged protein was concentrated in a Centricon-10 ultrafiltration device (Millipore, USA) to reduce the sample volume to 3 ml. The concentrated solution was loaded onto a Superose 12 XK26-100 size-exclusion column (GE Healthcare) and eluted using the final buffer. Fractions containing Pth were pooled and concentrated to 18 mg ml⁻¹ for subsequent functional analysis and crystallization screening trials (Fig. 1).

2.3. Functional assay

The activity of *P. aeruginosa* Pth was determined qualitatively from the reaction of bulk peptidyl-tRNA with purified Pth. Briefly, 1 µl bulk peptidyl-tRNA (30 µg µl⁻¹) was incubated with 1 µl of the *P. aeruginosa* Pth (18 µg µl⁻¹) used for crystallization in an activity-assay buffer consisting of 10 mM Tris-acetate, 10 mM magnesium acetate, 20 mM ammonium acetate pH 8.0 at room temperature for 30 min. The samples of cleaved product and uncleaved peptidyl-tRNA were run side-by-side on an acid-urea gel and stained with methylene blue to confirm that the purified enzyme was functional.

2.4. Crystallization

Crystallization trials were performed at 293 K using the sitting-drop vapor-diffusion method in a 96-well Intelli-Plate (Art Robbins) with the commercial sparse-matrix screen Crystal Screen HT (Hampton Research; Jancarik *et al.*, 1991). The Crystal Screen HT reagents were used as reservoir solutions in the initial trial. Protein:precipitant ratios of 1:1 (2 µl:2 µl) and 2:1 (2 µl:1 µl) were used in the top and the bottom sample wells, respectively. The plate was allowed to equilibrate undisturbed for one week at 293 K. Small (~0.01 mm³) single crystals were observed in several conditions in which the protein was equilibrated against a reservoir solution that contained high-molecular-weight (*i.e.* 4000 or 8000) polyethylene glycol (PEG). Crystallization conditions were optimized in 24-well

Figure 1 Pth purification and functional assay. (a) 12% SDS-PAGE. Lane M, molecular-mass marker (labeled in kDa); lane 1, soluble fraction of Pth lysate; lanes 2 and 3, pooled fractions containing Pth from the affinity chromatography and the size-exclusion chromatography, respectively. (b) Functional assay in which bulk peptidyl-tRNA (lane 1) was cleaved by recombinant *P. aeruginosa* Pth (lane 2). Bulk peptidyl-tRNA (1 µl at 30 µg µl⁻¹) was incubated with the *P. aeruginosa* Pth (1 µl at 18 µg µl⁻¹) used for crystallization in an activity-assay buffer consisting of 10 mM Tris-acetate, 10 mM magnesium acetate, 20 mM ammonium acetate pH 8.0. The cleavage reaction took place at room temperature for 30 min.

sitting-drop vapor-diffusion plates (Hampton Research) to examine the size and the habit of the obtained crystals as a function of protein concentration, pH, PEG 4000 concentration and isopropyl alcohol concentration. The best conditions for routinely growing large single crystals ($>0.2 \text{ mm}^3$) of Pth were determined to exist when $6 \mu\text{l}$ of 5 mg ml^{-1} Pth was mixed with $3 \mu\text{l}$ of a reservoir solution consisting of 0.1 M Tris pH 8.2, 25% PEG 4000, 10%(v/v) isopropyl alcohol. Using these conditions, large single crystals could be grown in 3–4 days of undisturbed incubation at 293 K.

2.5. X-ray data collection and reduction

A single Pth crystal grown by sitting-drop vapor diffusion in a 24-well plate (as previously described) was used for synchrotron X-ray data collection at 100 K. The native crystal was mounted onto an appropriate-sized nylon loop, flash-cooled and stored in liquid nitrogen. X-ray data sets were collected using radiation of wavelength $0.97 \text{ \AA}$ on the BioCARS beamline (BM-14) at the Advanced Photon Source (Argonne National Laboratory, Chicago, Illinois, USA). A complete data set consisting of 180 diffraction images was collected. Each image was collected with 1° oscillation using a 400 mm CCD area detector, with 3 s recorded exposure per oscillation image. The resulting images were indexed and integrated using *DENZO* and scaled with the onsite *SCALEPACK* program packages included in the *HKL-2000* software suite. *phenix.xtriage*, which is included in the *PHENIX* software package (Adams *et al.*, 2002), was used to ensure the proper crystallographic space-group assignment. The resolution of the scaled intensities was limited to Bragg reflections with $I/\sigma(I) > 2$. The scaled data were further processed using the *TRUNCATE* program (Kumar *et al.*, 2012) to convert the intensities to structure factors and to identify the set of working reflections as well as the set of free reflections to be used for structure determination and subsequent model refinement.

2.6. Structure determination and refinement

The number of molecules expected in the asymmetric unit was determined by calculating the Matthews coefficient (Matthews, 1968) and it was predicted that the asymmetric unit was most likely to contain one copy of the Pth molecule. The structure was phased by molecular replacement using *Phaser* (McCoy *et al.*, 2007), as included in *PHENIX*, using *E. coli* Pth (PDB entry 2pth; Schmitt *et al.*, 1997) as the initial search model. The Pth structure was refined by performing iterative cycles of maximum-likelihood refinement with *phenix.refine* (Adams *et al.*, 2002) and manual model adjustments using the program *Coot* (Emsley & Cowtan, 2004). Structural models were adjusted and completed by manually fitting the calculated $F_o - F_c$ and $2F_o - F_c$ electron-density maps. In general, iterative rounds consisted of five cycles of maximum-likelihood restrained refinement and one cycle of water-molecule updating followed by manual adjustments in *Coot*. Iterations continued until the model was complete and a reasonable attempt had been made to account for all of the significant difference map peaks (above 3.5σ) in the $F_o - F_c$ density map. Atomic displacement parameters for nonsolvent atoms were refined isotropically. In the final round of refinement, the number of cycles was extended to 15 cycles of xyz, occupancy and B-factor refinement to ensure model convergence and to optimize the experimental weighting of X-ray terms and geometric restraints. During the final round, the asparagine and glutamine B factors were fixed to validate the correct side-chain orientation and to identify the most probable orientation of these residue side chains in case of ambiguity. The final structural validations were performed using *MolProbity* (Chen *et al.*, 2010) and clashes were eliminated with the assistance of the *probe* and *reduce Coot* add-on programs. The refined coordinates were deposited in the PDB as entry 4fyj.

3. Results and discussion

The recombinant Pth enzyme of *P. aeruginosa* has been cloned, expressed with a high yield and purified to homogeneity. 20 mg purified protein was routinely obtainable per 2 l of minimal growth

Figure 2

Structural comparison. (a) Overlay of *P. aeruginosa* Pth (blue) with *E. coli* Pth (gray). The r.m.s.d. between C^α atoms is $0.62 \text{ \AA}$. (b) Highlighting the C-terminus: Pro173 (shown in red) causes a kink in helix 5 of *P. aeruginosa* Pth.

medium. The apparent molecular mass of the protein was observed to be 20–22 kDa as estimated by SDS–PAGE (Fig. 1*a*) and was consistent with prediction based on the translated open reading frame cloned fragment amplified from *P. aeruginosa* genomic DNA. A functional peptidyl-tRNA cleavage assay confirmed that the purified protein was active (Fig. 1*b*).

Large ($\sim 0.1\text{ mm}^3$) crystals of Pth were grown within one week of room-temperature incubation. The crystals obtained were demonstrated to be useful for cryogenic X-ray diffraction analysis using synchrotron radiation. The Pth crystals were little affected by radiation damage after 180° of data collection and 6 min of direct exposure to an unattenuated X-ray beam. This allowed a complete high-quality diffraction data set to be obtained from a single crystal. The diffraction data were easily indexed and scaled as a result of the compact and well resolved reflection spots. One molecule was predicted to be present in the crystallographic asymmetric unit based on Matthews calculations ($2.43\text{ \AA}^3\text{ Da}^{-1}$, 43.10% solvent content; Matthews, 1968). The structure of *P. aeruginosa* Pth was obtained by molecular replacement of the processed data against the crystal structure of *E. coli* Pth (PDB entry 2pth) using Phaser (McCoy *et al.*,

2007). An initial examination of the solution displayed good crystal packing of two Pth molecules with no symmetry-induced clashes. The structure was refined to a final R_{work} of 16.8% and R_{free} of 22.0%. The data-collection and refinement statistics are summarized in Table 1.

The fold of *P. aeruginosa* Pth is the same as other bacterial Pths (Fig. 2), with His20 and Asp93 (numbered as in *E. coli* Pth) in a stereochemically favorable arrangement for the catalytic site (Fig. 3). Compared with the Pth crystal structures solved from mycobacteria (*Mycobacterium tuberculosis*, PDB entry 2z2i, Selvaraj *et al.*, 2007; *M. smegmatis*, PDB entry 3p2j, Kumar *et al.*, 2012), *P. aeruginosa* Pth more closely resembles the *E. coli* coordinates. For example, the reported variability in the binding cleft indicated by the distance between Asp98 O $^{\delta 2}$ and Gly113 C $^{\alpha}$ (Kumar *et al.*, 2012) is 6.1 Å for *P. aeruginosa* Pth, which is much closer to the 6.7 Å found in *E. coli* Pth than to the 4.5 Å found in *M. smegmatis* Pth and the 3.1 Å found in *M. tuberculosis* Pth. One notable difference is the presence of a proline residue at position 173 instead of the glutamate in the other species mentioned. Pro173 introduces a kink in helix 5 that may affect the stability in the C-terminal helix and that is clearly resolved. Also, a tryptophan residue replaces Leu181 of *M. smegmatis* Pth; Leu181

Figure 3

The active site of *P. aeruginosa* Pth. The active site of Pth is found in a cleft covered by helix 4. It contains the conserved residues Asn10, His20 and Asp93 (red) found in all bacterial Pths. Below is an enlarged view of the active site. Phe66, Met67, His113 and Ser142 (Lys in *E. coli* Pth), which are important residues for catalytic activity as determined by site-directed mutagenesis of *E. coli* Pth, are shown in purple. Residues lining the proposed peptide tunnel are shown in yellow. Residue numbers correspond to those of *E. coli* Pth.

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	BioCARS BM-14
Unit-cell parameters (Å, °)	$a = b = 63.6$, $c = 155.2$, $\gamma = 120$
Space group	$P6_122$
No. of observed reflections	380094 (10363)
No. of unique reflections	18783 (2590)
Resolution range (Å)	30.81–1.77 (1.87–1.77)
Multiplicity	20.2 (4.0)
$\langle I/\sigma(I) \rangle$	57.7 (11.2)
$R_{\text{merge}}^{\dagger}$ (%)	4.5 (25.8)
Data completeness (%)	99.21 (99.9)
$R_{\text{work}}^{\ddagger}$ (%)	19.1
$R_{\text{free}}^{\ddagger}$ (%)	23.5
R.m.s.d. bonds§ (Å)	0.007
R.m.s.d. angles§ (°)	0.673
Atoms (non-H)	1891
Solvent molecules	326
Ramachandran plot¶	
Outliers (%)	0.0
Favored (%)	100.0
Rotamer outliers (%)	0.59

$\dagger R_{\text{merge}} = 100 \times [\sum_{hkl} \sum_i |F^2(hkl)| - \langle F^2(hkl) \rangle_i] / \sum_{hkl} \sum_i F^2(hkl)$, where $F^2(hkl)$ is the intensity of reflection hkl and $\langle F^2(hkl) \rangle_i$ is the mean value of i multiple measurements of the n equivalent reflections. $\ddagger R = \sum_{hkl} [|F_{\text{obs}}| - |F_{\text{calc}}|] / \sum_{hkl} |F_{\text{obs}}|$; R_{free} is the R value computed using a 5% subset of data randomly excluded from structure determination (where F_{calc} includes all scale factors and corrections for bulk solvent). $\S$ Root-mean-square deviations of bond lengths and bond angles calculated using *phenix.refine* from the *PHENIX* program suite. $\P$ Ramachandran-plot quality assessment using *MolProbity*.

has been shown to make extensive hydrophobic interactions that have been suggested to stabilize the C-terminal helix (Kumar *et al.*, 2012). Similar hydrophobic interactions are observed supporting this assertion.

4. Conclusions

The X-ray crystallographic structure of *P. aeruginosa* Pth presented here provides an excellent foundation for future structure–function studies and structure-guided antibiotic development. High-resolution coordinates of the critical active-site residues are necessary for *in silico* inhibitor screening and design. Knowledge of the discrete differences between the active-site structure of *P. aeruginosa* Pth and other bacterial Pth enzymes with available high-resolution structures will facilitate the development of specific inhibitors. Future work based on theoretical modeling may help to identify novel lead

compounds to be assayed for their ability to specifically inhibit Pth activity. Successful candidates will be used in crystallization trials in efforts to obtain structures of the enzyme–inhibitor complex, which will be used to further optimize ligand affinity and specificity, thus advancing the development of potent new Pth inhibitors.

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