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Received 7 April 2014

Accepted 2 May 2014

PDB reference: peptidyl-tRNA hydrolase, 4p7b

Recombinant production, crystallization and X-ray crystallographic structure determination of peptidyl-tRNA hydrolase from *Salmonella typhimurium*

Peptidyl-tRNA hydrolase (Pth; EC 3.1.1.29) from the pathogenic bacterium *Salmonella typhimurium* has been cloned, expressed in *Escherichia coli* and crystallized for X-ray analysis. Crystals were grown using hanging-drop vapor diffusion against a reservoir solution consisting of 0.03 M citric acid, 0.05 M bis-tris propane, 1% glycerol, 3% sucrose, 25% PEG 6000 pH 7.6. Crystals were used to obtain the three-dimensional structure of the native protein at 1.6 Å resolution. The structure was determined by molecular replacement of the crystallographic data processed in space group $P2_12_12_1$ with unit-cell parameters $a = 62.1$, $b = 64.9$, $c = 110.5$ Å, $\alpha = \beta = \gamma = 90^\circ$. The asymmetric unit of the crystallographic lattice was composed of two copies of the enzyme molecule with a 51% solvent fraction, corresponding to a Matthews coefficient of 2.02 Å³ Da⁻¹. The structural coordinates reported serve as a foundation for computational and structure-guided efforts towards novel small-molecule Pth1 inhibitors and potential antibacterial development.

1. Introduction

Salmonella typhimurium is a Gram-negative, rod-shaped bacterium that can be found in the intestinal lumen. This pathogenic bacterium is a common cause of food poisoning, called typhoid fever, in humans and other animals. Symptoms include diarrhea, fever, vomiting and abdominal cramps. Drug-resistant *S. typhimurium* has been reported for decades and transmission from animals to humans has resulted in many human infections (Anderson, 1968). In addition, drug-resistant *S. typhimurium* has recently been reported to be the cause of invasive nontyphoidal disease in Africa (Kingsley *et al.*, 2009). With the continued heavy use of antibiotics in livestock, greater development of resistance and transmission of drug-resistant strains to humans are alarming. Therefore, there is a need for new antibiotic targets against *S. typhimurium*.

Bacterial peptidyl-tRNA hydrolases (Pth1s) are promising candidates for antibiotic development. They are essential enzymes that recycle peptidyl-tRNA resulting from premature termination of translation and expression of minigenes or short ORFs (Jørgensen & Kurland, 1990; Manley, 1978; Kurland & Ehrenberg, 1985; Cruz-Vera *et al.*, 2003; Hernández-Sánchez *et al.*, 1998; Tenson *et al.*, 1999) by hydrolyzing the ester bond between the peptide and nucleotide, returning free tRNAs for use in protein biosynthesis. Bacterial Pth1s are highly conserved, leading to the possibility of broad-spectrum inhibition. In contrast, eukaryotes have multiple Pth enzymes that are structurally and functionally unrelated. The bacterial Pth1 homologue in yeast cells has been shown to be non-essential for yeast growth, suggesting that eukaryotic cells have multiple mechanisms to deal with accumulation of peptidyl-tRNA (Menez *et al.*, 2002; Rosas-Sandoval *et al.*, 2002). To date, inhibition of bacterial Pth1s by natural products is encouraging (Harris *et al.*, 2011; McFeeters *et al.*, 2012; McFeeters, 2013).

Structures of bacterial Pth1s have been solved, showing similar backbone folds (Clarke *et al.*, 2011; Hughes *et al.*, 2012; Kumar *et al.*, 2012; Pulavarti *et al.*, 2008; Selvaraj *et al.*, 2007). They 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* Pth1), identified by site-directed mutagenesis to be crucially involved in enzyme activity (Goodall *et al.*, 2004). The structure of *E. coli* Pth1 in the complex of the T ψ C loop of tRNA has recently been reported and has provided invaluable structural insight into the nucleotide binding (Ito *et al.*, 2011). An overall shape of the complex furthers understanding (Hames *et al.*, 2013). Nevertheless, more structural information is needed to clearly elucidate the mechanism of peptide cleavage and small-molecule inhibition.

Here, we report the cloning, expression, crystallization and X-ray crystallographic structure of recombinant Pth1 from *S. typhimurium* at 1.6 Å resolution. The structural information presented provides a foundation for future computational and structure-guided efforts aimed at developing inhibitors and helping to establish the antibiotic potential of bacterial Pth1s.

2. Materials and methods

2.1. Cloning

The open reading frame of Pth1 was PCR (polymerase chain reaction) amplified from the genomic DNA of *S. typhimurium* (ATCC 700720) using the forward and reverse primers 5'-GAGGAATTCATGCATCATCATCATCACGCAATTAAATTGATTGTTGGTCTGGCG-3' and 5'-CGAGAAGCTTTCATTATTGCGCCTTAAAGGTATGTGCAAC-3', respectively. Oligonucleotide primers were designed to contain *Hind*III/*Eco*RI restriction-enzyme sites as well as an N-terminal hexahistidine tag. The PCR amplification reaction was performed using a 50 μ l reaction volume containing 20 pmol of each primer, 40 ng of genomic template digested with *Eco*RV and 25 μ l of EmeraldAmp PCR Mastermix (Takara/Clontech, Japan). The thermal cycler program consisted of a 45 s, 98°C initial denaturation, 20 cycles of 30 s at 98°C, 30 s at 50°C and 45 s at 72°C, followed by a final 2 min extension at 72°C.

The amplified product was digested with FastDigest *Hind*III and *Eco*RI (Fermentas, USA) and ligated into a pKQV4 plasmid vector

previously digested with the same enzymes. For ligation, 4 μ l of the PCR product was mixed with 750 ng of digested pKQV4. The ligation reaction was then initiated by supplying T4 DNA ligase (Fermentas, USA) and left to proceed for 2 h. 10 μ l of the ligation reaction was added to 100 μ l of chemically competent JM109 *E. coli* cells (Promega, USA) and incubated on ice. After 45 min incubation, the mixture was subjected to heat shock at 42°C for 1 min followed by a 2 min incubation on ice. The transformed cells were added to 400 μ l Luria–Bertani (LB) medium, incubated for 1 h at 37°C and spread on LB–agar plates containing 100 μ g ml⁻¹ carbenicillin. Inoculated plates were incubated overnight at 37°C. A 3 ml overnight culture was grown from a single colony of transformed cells to propagate the recombinant plasmid harboring the Pth1 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 *S. typhimurium* Pth1 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 30 ml starter overnight culture grown in LB–carbenicillin (100 μ g ml⁻¹) medium at 37°C. In the morning, the cells were spun down and used for inoculation of 1 l of LB–carbenicillin (100 μ g ml⁻¹) medium. Enough cells were resuspended in the large volume to reach a starting OD₆₀₀ of 0.1. The culture was kept at 37°C until an OD₆₀₀ of 0.6 when it was quickly cooled on ice to 30°C and protein expression induced by the addition of 1 M isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. *S. typhimurium* Pth1 was allowed to express for 6 h in a shaking incubator at 30°C. Cells were harvested by centrifugation and stored at -80°C.

For purification, the frozen cell pellet was resuspended in 20 ml lysis buffer (50 mM NaHPO₄, 300 mM NaCl, 2 mM DTT pH 7.4, 20 μ g 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 40 000g and the supernatant was applied onto a Ni²⁺-chelating affinity column. The column was washed with 50 mM NaHPO₄, 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 Amicon Ultracel (Millipore, USA) units to reduce the sample volume to 3 ml. The concentrated solution was loaded onto a Superose 12 (GE Healthcare, USA) XK26-100 size-exclusion column and eluted using the final buffer. Fractions containing Pth1 were pooled and concentrated to 120 mg ml⁻¹ for subsequent functional analysis and crystallization screening trials (Fig. 1).

2.3. Functional assay

The activity of *S. typhimurium* Pth1 was determined qualitatively from a reaction of bulk peptidyl-tRNA with purified Pth1 as previously reported (Harris *et al.*, 2011; McFeeters *et al.*, 2012). Briefly, 1 μ l of bulk peptidyl-tRNA (30 μ g μ l⁻¹) was incubated with 1 μ l of *S. typhimurium* Pth1 (30 μ g μ l⁻¹) used for crystallization in an activity assay buffer 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-

Figure 1

Pth1 purification and functional assay. (a) 12% SDS-PAGE gel. Lane MW, molecular-weight marker with sizes of the control proteins in kDa labeled. Lanes 1–6, Ni²⁺-affinity chromatography purification: lane 1, unbound fraction; lane 2, 25 mM imidazole wash; lanes 3–6, eluted fractions containing *S. typhimurium* Pth1; lane 7, total lysate; lane 8, soluble fraction of the lysate; lane 9, insoluble portion of the lysate; lane 10, *S. typhimurium* Pth1 after size-exclusion chromatography. (b) Functional assay for *S. typhimurium* Pth1 activity: bulk peptidyl-tRNA (lane 1) was cleaved by the purified recombinant enzyme (lane 2). The control band of 5S ribosomal rRNA in the samples is indicated.

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 18°C using the hanging-drop vapor-diffusion method in 24-well VDXm plates (Hampton Research, USA) with the commercial sparse-matrix screens Crystal Screen HT, PEG/Ion and PEG/Ion 2 (Hampton Research, USA; Jancarik *et al.*, 1991). The Hampton Research reagents were used as reservoir solutions in the initial trial. Protein and precipitant ratios of 1:1 (2 µl:2 µl) and 2:1 (2 µl:1 µl) were placed as two separate drops on the same siliconized well cover. The plates were allowed to equilibrate undisturbed for 3 d at 18°C. A small (~0.01 mm³) star-like crystal was observed in the condition consisting of 0.03 M citric acid, 0.07 M Bis-Tris propane pH 7.6, 20% PEG 3350. Crystallization conditions were optimized in 24-well hanging-drop and sitting-drop vapor-diffusion plates (Hampton Research, USA) to examine the size and habits of the obtained crystals as a function of protein concentration, pH, PEG size and concentration, and the presence of several different additives. The best conditions for routinely growing single crystals of *S. typhimurium* Pth1 were determined to exist when 3 µl 120 mg ml⁻¹ Pth1 solution was mixed with 3 µl of a reservoir solution consisting of 0.03 M citric acid, 0.05 M Bis-Tris propane, 1% glycerol, 3% sucrose, 25% PEG 6000 pH 7.6. Using these conditions, crystals suitable for X-ray diffraction could be grown in 2–3 d of undisturbed incubation at 18°C.

2.5. X-ray data collection and reduction

A single Pth1 crystal grown by hanging-drop vapor diffusion in a 24-well plate (previously described) was used for synchrotron X-ray data collection at -173°C. 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 0.98 Å wavelength radiation on the SBC-CAT beamline (19ID) at the Advanced Photon Source (Argonne National Laboratory, Chicago, Illinois, USA). A complete data set (180, 1° oscillation diffraction images) was collected using an ADSC Q315r 315 × 315 mm mosaic CCD detector.

Figure 2

Crystal of *S. typhimurium* Pth1 used for X-ray diffraction. The crystal dimensions were approximately 0.15 × 0.07 × 0.8 mm. Crystals were obtained within 3 d of 18°C incubation.

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

PDB code	4p7b
X-ray source	SBC-CAT 19ID
Unit-cell parameters (Å, °)	$a = 62.1, b = 64.9, c = 110.5,$ $\alpha = \beta = \gamma = 90$
Space group	$P2_12_12_1$
No. of observed reflections	843916
No. of unique reflections	59556
Resolution range (Å)	44.88–1.60 (1.66–1.60)
Multiplicity	14.2 (14.3)
$\langle I/\sigma(I) \rangle$	16.6 (5.9)
$R_{\text{merge}}^{\dagger}$ (%)	9.2 (39.4)
$CC_{1/2}^{\ddagger}$	0.998 (0.986)
Data completeness (%)	99.82 (99.6)
$R_{\text{work}}^{\S}$ (%)	17.4
$R_{\text{free}}^{\S}$ (%)	19.8
R.m.s.d., bonds ¶ (Å)	0.012
R.m.s.d., angles ¶ (°)	1.29
Atoms (non-H)	6464
Solvent molecules	611
Ramachandran plot ††	
Outliers (%)	0.0
Favored (%)	98.0
Rotamer outliers (%)	1.0

† $R_{\text{merge}} = 100 \times [\sum_{hkl} \sum_{i=1}^n |F^2(hkl)_i| - \langle F^2(hkl) \rangle] / [\sum_{hkl} \sum_{i=1}^n F^2(hkl)]$ where $F^2(hkl)$ is the intensity of the hkl reflection and $\langle F^2(hkl) \rangle_i$ is the mean value of i multiple measurements of the hkl equivalent reflections. ‡ $CC_{1/2}$ is the correlation coefficient of the mean intensities between two random half-sets of data (Evans, 2012; Karplus & Diederichs, 2012). § $R = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$; R_{free} is the R value computed using 5% randomly excluded from the structure-determination data subset (where F_{calc} includes all scale factors and corrections for bulk solvent). ¶ Root-mean-square deviations of bond lengths and bond angles calculated with *phenix.refine* in the *PHENIX* program suite. †† Ramachandran plot quality assessment using *MolProbity* (Chen *et al.*, 2010).

The resulting images were indexed and integrated using the *XDS* software package (Kabsch, 2010a,b). *phenix.xtriage* included in the *PHENIX* software package (Adams *et al.*, 2010) was used to ensure the proper crystallographic space-group assignment. The resolution of the scaled intensities was limited to Bragg reflections having $I/\sigma(I)$ greater than two. Scaled data were further processed using the *TRUNCATE* (French & Wilson, 1978) program 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 expected molecules in the asymmetric unit was determined by calculating the Matthews coefficient (Matthews, 1968) and it was predicted that the asymmetric unit is most likely to contain two copies of the Pth1 molecule. The structure was phased by molecular replacement using *Phaser* (McCoy *et al.*, 2007), included with *PHENIX*, using *Pseudomonas aeruginosa* Pth1 (PDB entry 4fyj; Hughes *et al.*, 2012) as the initial search model. The criteria used to determine the resolution cutoff for the reflection data used for refinement required an overall data completeness of >90% and an outermost resolution shell completeness of >50%. The Pth1 structure was refined by performing iterative cycles of maximum-likelihood refinement with *phenix.refine* (Adams *et al.*, 2010) and manual model adjustments using *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 update, followed by manual adjustments in *Coot*. Iterations continued until the model was complete and a reasonable attempt had been made to account for all

the significant difference map peaks (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, asparagine and glutamine B factors were fixed to validate the correct side-chain orientation and to identify the most probable orientation for these residue side chains in case of ambiguity. 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.

3. Results and discussion

The recombinant Pth1 enzyme of *S. typhimurium* has been cloned, expressed with a high yield and purified to homogeneity. 60 mg

purified protein were routinely obtainable per litre of minimal growth medium. The apparent molecular weight of the protein was observed to be 25 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 *S. typhimurium* genomic DNA. The enzyme was a monomer in solution as determined by size-exclusion chromatography. A functional peptidyl-tRNA cleavage assay confirmed that the purified protein was active (Fig. 1*b*).

S. typhimurium Pth1 was highly soluble and stable in the final buffer. It could be stored at concentrations of 120 mg ml⁻¹ for weeks without loss of activity or precipitation. During crystallization trials with lower concentrations of Pth1 (10 or 20 mg ml⁻¹), limited crystal formation was observed even after several weeks. At the high concentration (120 mg ml⁻¹) used for crystallization, a thick white precipitate formed immediately after mixing the protein solution with well solution. Nevertheless, in several hours the drop became completely clear and crystals of *S. typhimurium* Pth1 were observed

Figure 3

Structural comparison. (a), (b) The high-resolution structure of *S. typhimurium* Pth1 (PDB entry 4p7b). (c), (d) Overlay of the high-resolution *S. typhimurium* Pth1 structure (blue) with structures of other bacterial Pth1s (gray): *E. coli* Pth1 (PDB entry 2pth; Schmitt *et al.*, 1997), *P. aeruginosa* Pth1 (PDB entry 4fyj; Hughes *et al.*, 2012), *F. tularensis* Pth1 (PDB entry 3nea; Clarke *et al.*, 2011), *M. smegmatis* Pth1 (PDB entry 3p2j; Kumar *et al.*, 2012) and *Acinetobacter baumannii* Pth1 (PDB entry 3wh4; Kaushik *et al.*, 2013). The regions with high variations in tertiary structure between the enzymes are indicated with arrows. (e), (f) Surface representation of *S. typhimurium* Pth1 with residues colored depending on their conservation in bacterial Pth1 enzymes for which high-resolution structures have been solved: red, strictly conserved amino-acid residues; orange, highly homologous residues; yellow, homologous residues; gray, nonhomologous residues. The orientation of the molecule in the surface representation corresponds to the ribbon diagrams.

in 2–3 d of room-temperature incubation (Fig. 2). The obtained crystals were demonstrated to be useful for cryogenic X-ray diffraction analysis using synchrotron radiation. *S. typhimurium* Pth1 crystals were somewhat affected by radiation damage after 180° of data collection and 6 min of direct exposure to an unattenuated X-ray beam. However, a complete, high-quality diffraction data set was obtained from a single crystal. The diffraction data were indexed and scaled. Two molecules were predicted to be present in the crystallographic asymmetric unit based on Matthews-coefficient calculations ($2.02 \text{ \AA}^3 \text{ Da}^{-1}$, 51.43% solvent fraction; Matthews, 1968). The structure of *S. typhimurium* Pth1 (PDB entry 4p7b) was obtained by molecular replacement of the processed data against the crystal structure of *P. aeruginosa* Pth1 (PDB entry 4fyj; Hughes *et al.*, 2012) using *MOLREP* (Vagin & Teplyakov, 2010). An initial examination of the solution displayed good crystal packing of two Pth1 molecules with no symmetry-induced clashes. The structure was refined to a final R_{work} of 17.4% and R_{free} of 19.8%. The data-collection and refinement statistics are summarized in Table 1.

The fold of *S. typhimurium* Pth1 is the same as that of other bacterial Pth1s for which high-resolution structures have been solved (Fig. 3). From the set of bacterial Pth1 structures compared, *S. typhimurium* Pth1 most closely resembles the *E. coli* coordinates, with a backbone r.m.s.d. of 0.4 Å. Amino acids in the catalytic site of bacterial Pth1 enzymes are strictly conserved, as is apparent from the conservancy plot shown in Figs. 3(e) and 3(f). There are however several regions with larger variations in tertiary structure. Differences in these regions correlate well with the degree of conservancy of the amino-acid residues in the primary structures of the Pth1 enzymes compared (Fig. 4). Firstly, the amino-acid residues 48–63 (as numbered in *E. coli* Pth1) form two central β -sheets and a connecting loop and show most variability both in amino-acid composition and in spatial arrangement. Secondly, the short helix 4 with flanking loops that serves as a lid of the active site is comprised of residues 138–155.

NMR relaxation experiments have shown that this region is very mobile, exhibiting motion on both picosecond (loop residues) and microsecond to millisecond (helix residues) timescales (Pulavarti *et al.*, 2008). Mobility of this region in the crystal is apparent from the high B -factor values for all of the structures compared. In one of the molecules of *S. typhimurium* Pth1 in the asymmetric unit it was not even possible to detect electron density corresponding to residues 142–146. Thirdly, variations in the conformation of the long helix 5 result in different conformations of helix 6 in the C-terminal part of the molecule. The conformation of *S. typhimurium* Pth1 helix 5 most resembles that of *E. coli* Pth1 helix 5. The difference in conformation of helix 5 of *S. typhimurium* Pth1 compared with *P. aeruginosa* and *Mycobacterium smegmatis* Pth1 helix 5 is affected by the presence of a proline residue in positions 163 (*M. smegmatis*; Kumar *et al.*, 2012) and 181 (*P. aeruginosa*; Hughes *et al.*, 2012). On the other hand, in the available structure of *Francisella tularensis* Pth1 the C-terminus is highly flexible and helix 6 is not formed (Clarke *et al.*, 2011).

4. Conclusions

Bacterial Pth1 enzymes are promising new targets for antibiotic development. The X-ray crystallographic structure of *S. typhimurium* Pth1 presented here serves as a foundation for future computational, structure–function and structure-guided antibiotic development studies. Although their overall fold is well conserved, there are regions that differ. These differences may be exploited to develop narrow-spectrum or species-specific inhibitors, helping mitigate the development of resistance – an admirable goal for next-generation antibiotic development. The similarities in high-resolution structures of bacterial Pth1s in conjunction with bioinformatics studies may be used to explore the development of broad-range antibiotics as well as targeted specific inhibition of a particular species. With several

Figure 4

S. typhimurium Pth1 B factors. (a) *S. typhimurium* Pth1 structure with amino-acid residues labeled based on their B factors. The B -factor color scale is shown in (a). Regions discussed in the text are shown magnified: (b) helix 4 with flanking loops and (c) region of amino-acid residues 48–63.

potential Pth1 inhibitors available, future efforts will be directed to solving structures of enzyme–inhibitor complexes, identification of promising motifs in inhibitor molecules and the discovery, design and synthesis of new inhibitors.

The Office of Biological and Environmental Research supported research at the Oak Ridge National Laboratory Center for Structural Molecular Biology (CSMB) using facilities supported by the Scientific User Facilities Division, Office of Basic Energy Sciences, United States Department of Energy. Results shown in this study are derived from work performed at Argonne National Laboratory (ANL), Structural Biology Center at the Advanced Photon Source. ANL is operated by UChicago Argonne LLC for the United States Department of Energy, Office of Biological and Environmental Research under Contract DE-AC02-06CH11357.

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