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Cloning, expression, purification, crystallization and preliminary X-ray analysis of peptidyl-tRNA hydrolase from *Mycobacterium tuberculosis*

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Peptidyl-tRNA hydrolase catalyses the cleavage of the ester link between the peptide and the tRNA in peptidyl-tRNAs that, for various reasons, have dropped off the translating ribosomes. This enzyme from *Mycobacterium tuberculosis* has been crystallized in three related but distinct forms: $P2_12_12_1$, unit-cell parameters a = 36.30, b = 61.85, c = 73.97 Å, $P2_1$, a = 35.83, b = 73.79, c = 59.79 Å, β = 92.3°, and $P2_12_12_1$, a = 35.84, b = 57.06, c = 72.59 Å. X-ray data have been collected from all three forms.

1. Introduction

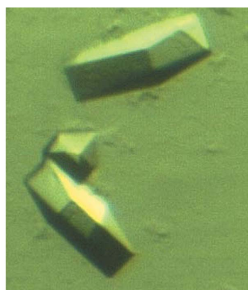
Occasionally, peptidyl-tRNAs drop off translating ribosomes. The frequency of peptidyl-tRNA drop-off is especially high during the translation of minigene ORFs (Heurgue-Hamard *et al.*, 2000). Accumulation of peptidyl-tRNA is toxic to the cell. Peptidyl-tRNA hydrolase (Pth) is an enzyme that salvages tRNA from peptidyl-tRNA by hydrolyzing the ester link between the peptide and the 2′- or 3′-OH of the sugar at the end of tRNA (Cuzin *et al.*, 1967; Kössel & RajBhandary, 1968; Das & Varshney, 2006). Pth is also capable of hydrolyzing the amide bond between the peptide and the 3′-amide group of the modified ribose at the end of tRNA in synthetic substrates (Jost & Bock, 1969). Genes coding Pth have been identified in organisms belonging to all three kingdoms of life. *Escherichia coli* and other eubacteria possess a single gene coding for Pth (also known as Pth1). A different class of Pth, known as Pth2, is found in archaea. An ensemble of proteins with Pth activity, including orthologues of the eubacterial and archaeal enzymes, is found in eukaryotes. Pth is an important potential drug target to control eubacterial growth.

The re-emergence of tuberculosis as a major disease, the evolution of multi-drug resistant strains of *Mycobacterium tuberculosis* and the long duration necessary for treatment using currently available drugs underscore the need to develop new drugs that target novel proteins. In this context, as part of a continuing effort in this laboratory to characterize the structure and interactions of mycobacterial proteins (Datta *et al.*, 2000; Roy *et al.*, 2004; Saikrishnan *et al.*, 2003, 2005; Das *et al.*, 2006; Krishna *et al.*, 2006), crystallization and preliminary X-ray studies of the 191 amino-acid residue Pth from *M. tuberculosis* (MW 20 455 Da) have been carried out.

2. Materials and methods

2.1. Cloning, expression and purification

The *M. tuberculosis pth* gene (Rv1014c) was PCR-amplified using Dynazyme EXT (Finnzymes) from *M. tuberculosis* H37Rv genomic DNA using 5′-TGCGCCATGGCCGAGCCGTTG-3′ and 5′-CACAAGCTTCGTTACCAGGCGTGGAC-3′ as forward and reverse primers, respectively. The PCR involved 30 cycles of initial denaturation at 367 K for 1 min, annealing at 328 K for 30 s and extension at 343 K for 1 min. The PCR product was then digested with *Nco*I and *Hin*dIII and cloned into the same sites of the pET11d vector. The clones were verified by sequencing. The *Nco*I–*Hin*dIII fragment of pET11d*Mtu*Pth was subcloned into the same sites of the pRSETB vector to obtain the N-terminally His-tagged *Mtu*Pth as



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pRSETB*Mtu*Pth. This construct appended a 41-amino-acid stretch to the N-terminus of *Mtu*Pth. To reduce the length of the tag, the pRSETB*Mtu*Pth was digested with *Nhe*I and *Nco*I, end-filled and religated. The construct thus obtained, pRSETB*Mtu*Pth(-*Nhe*I-*Nco*I) appending MRGSHHHHHHGMAS to the *Mtu*Pth N-terminus, was used for overproduction of the protein.

E. coli BL21 (DE3) transformed with pRSETB*Mtu*Pth(-*Nhe*I-*Nco*I) was grown in 2 l LB to an $A_{595\,\rm nm}$ of ∼0.6 and induced with 0.5 m*M* IPTG for 3 h. The cells were harvested at 5715*g* for 15 min (RA1500, Kubota), resuspended in buffer *A* [20 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl and 10%(*v*/*v*) glycerol] and lysed by sonication. The lysate was then centrifuged at 20 405*g* (AG508R, Kubota) for 30 min. The supernatant was loaded onto an Ni–NTA column (Pharmacia) equilibrated with buffer *A*, washed with the same buffer containing 20 m*M* imidazole and eluted with an imidazole gradient ranging from 10 to 500 m*M*. Fractions containing the desired protein were pooled and dialyzed against buffer *B* (20 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl and 2 m*M* β-mercaptoethanol). The purity of the protein was checked using SDS–PAGE and was found to be nearly homogeneous. The protein was then concentrated using Microsep (10 kDa cutoff, Pall Lifesciences) for crystallization experiments.

2.2. Crystallization

Initial crystallization attempts performed using Hampton Crystal Screens I and II (Jancarik & Kim, 1991) failed to give useful crystals. Therefore, a systematic search for crystallization conditions was performed by varying the molecular weight and the concentration of polyethylene glycol in the precipitant solution. In this search, using the microbatch method, 2 µl each of the protein solution and the precipitant solution were mixed. Microtestplate (Sigma) and Hampton paraffin oil and silicon oil in a 1:1 ratio were used in the search. The protein solution contained 6 mg ml^{-1} Pth, 20 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl and 2 m*M* β-mercaptoethanol. Clusters of thin needle-like crystals appeared when the precipitant solution contained 20%(*w*/*v*) PEG 8000 in 0.1 *M* HEPES pH 7.5 (Fig. 1*a*). These crystals diffracted to 7 Å resolution. Crystals diffracting to 3 Å resolution were obtained after one week by including 5%(*v*/*v*) 2-propanol or dioxane in the above solution (Fig. 1*b*). In order to improve the quality of the crystals further, the pH of the precipitant solution was varied between 4.5 and 8.5 using different buffers. Individual well separated crystals grew when the precipitant solution contained 25%(*w*/*v*) PEG 8000, 100 m*M* sodium cacodylate pH 6.6, 5%(*v*/*v*) 2-propanol (Fig. 1*c*). Crystals appeared after 5 d and grew to dimensions of 0.3 × 0.3 × 0.2 mm in two weeks. It turned out that the crystals belonged to two related but distinct forms (forms I and II). In the meantime yet another related form (form III) grew in a drop containing 0.1 *M* HEPES pH 7.5, 15%(*w*/*v*) PEG 8000 and 5%(*v*/*v*) 2-propanol.

Table 1
Crystal data and data-collection statistics.

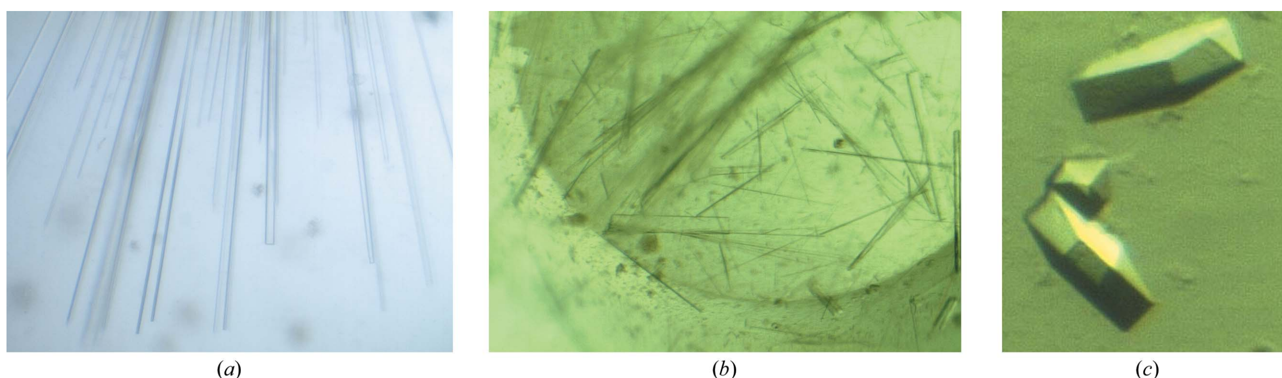
Values in parentheses are for the highest resolution shell.

	Form I	Form II	Form III
Temparature (K)	100	100	100
Space group	$P2_12_12_1$	$P2_1$	$P2_12_12_1$
Unit-cell parameters			
a (Å)	36.30	35.83	35.84
b (Å)	61.85	73.76	57.06
c (Å)	73.97	59.79	72.59
β (°)	—	92.3	—
Packing density ($V_{\rm M}$) (Å^3 Da^{-1})	2.1	2	1.9
Solvent content (%)	41	38	34
No. of molecules in ASU	1	2	1
Resolution range (Å)	30.0–1.97 (2.04–1.97)	30.0–2.35 (2.43–2.35)	30.0–2.49 (2.58–2.49)
Observed reflections	81818	37029	22020
No. of unique reflections	11461(1174)	12613 (1236)	5428 (522)
Completeness (%)	95.2 (99.8)	96.8 (95.4)	98.2 (95.0)
Multiplicity	7.1	2.9	4.1
$\langle I/\sigma(I)\rangle$	22.7 (3.7)	9.05 (2.7)	15.14 (3.6)
$R_{\rm sym}$† (%)	7.7 (48.7)	11.8 (41.1)	8.2 (38.4)

† $R_{\rm sym} = \sum_{\bf h}\sum_l |I_{{\bf h}l} - \langle I_{\bf h}\rangle| / \sum_{\bf h}\sum_l \langle I_{\bf h}\rangle$, where I_l is the *l*th observation of reflection **h** and $\langle I_{\bf h}\rangle$ is the weighted average intensity for all observations *l* of reflection **h**.

2.3. X-ray data collection and processing

Crystal form I of Pth picked up from a microbatch droplet using a mounted nylon loop (Hampton Research, Laguna Niguel, CA, USA) was placed directly into a cold nitrogen-gas stream at 100 K. In the case of forms II and III, crystals were dipped in mother liquor containing 15%(*v*/*v*) PEG 400 prior to mounting. X-ray diffraction data were collected from a single crystal using a MAR Research image-plate system (diameter 345 mm) with Osmic mirrors and a Rigaku RU-200 rotating-anode X-ray generator with a 300 µm focal cup. The crystal-to-detector distance was set to 150 mm. All frames were collected at 100 K using a 1.0° oscillation angle, with an exposure time of 1800 s per frame. The data revealed significant diffraction to 1.97 Å resolution in the case of form I. In all cases the data were processed using *DENZO* and scaled using *SCALEPACK* from the *HKL* program package (Otwinowski & Minor, 1997).



(*a*) (*b*) (*c*)

Figure 1
Crystals grown from solutions containing (*a*) 20%(*w*/*v*) PEG 8000, 0.1 *M* HEPES pH 7.5, (*b*) 20%(*w*/*v*) PEG 8000, 0.1 *M* HEPES pH 7.5, 5%(*v*/*v*) 2-propanol and (*c*) 25%(*w*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.6 and 5%(*v*/*v*) 2-propanol.

3. Results and discussion

The three crystal forms, two orthorhombic and one monoclinic, are closely related to one another. Preliminary X-ray data are given in the Table 1. The two orthorhombic forms have somewhat similar unit-cell parameters and are broadly isomorphic. On the basis of the solvent content (Matthews, 1968), the orthorhombic forms contain one molecule in the asymmetric unit, while the monoclinic form contains two. Structure analysis is in progress.

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