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Cloning, expression, purification, crystallization and preliminary X-ray crystallographic analyses of threonyl-tRNA synthetase editing domain from *Aeropyrum pernix*

The proofreading function of aminoacyl-tRNA synthetases is crucial in maintaining the fidelity of protein synthesis. Most archaeal threonyl-tRNA synthetases (ThrRSs) possess a unique proofreading domain unrelated to their eukaryotic/bacterial counterpart. The crystal structure of this domain from the archaeon *Pyrococcus abyssi* in complex with its cognate and noncognate substrate analogues had given insights into its catalytic and discriminatory mechanisms. To probe further into the mechanistic and evolutionary aspects of this domain, work has been extended to another archaeon *Aeropyrum pernix*. The organism possesses two proteins corresponding to threonyl-tRNA synthetase, *i.e.* ThrRS1 and ThrRS2, encoded by two different genes, *thrS1* and *thrS2*, respectively. ThrRS1 is responsible for aminoacylation and ThrRS2 for proofreading activity. Here the purification, crystallization and preliminary X-ray crystallographic investigation of the N-terminal proofreading domain of ThrRS2 from *A. pernix* is reported. The crystals belong to either the $P4_12_12$ or $P4_32_12$ space group and consist of one monomer per asymmetric unit.

1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) are one of the major players that ensure fidelity during protein translation by specifically charging the tRNAs with their corresponding amino acids. However, mistakes are often made by these aaRSs while discriminating between closely resembling amino acids such as isoleucine/valine and threonine/serine. These errors result in the formation of misacylated-tRNAs which, if not corrected, can lead to erroneous incorporation of amino acids during protein synthesis. aaRSs which face such a tough substrate recognition problem possess an additional module called the proofreading domain which deacylates mischarged tRNAs (Jakubowski & Goldman, 1992). Compromise in this proofreading activity of aaRSs can have serious consequences including growth defects (Nangle *et al.*, 2006), genetic diseases (Bacher & Schimmel, 2006) and neurodegeneration (Lee *et al.*, 2006). Threonyl-tRNA synthetase (ThrRS) also possesses this proofreading activity to deacylate the near-cognate serine charged on tRNA^{Thr}. Two unrelated folds carry out this function in eukaryotes/bacteria and archaea (Beebe *et al.*, 2004). The eukaryote/bacteria-specific domain is homologous to alanyl-tRNA synthetase editing domain (Dock-Bregeon *et al.*, 2004), while the archaea-specific domain bears a striking structural similarity to D-aminoacyl-tRNA deacylase (DTD) from *Escherichia coli* (Dwivedi *et al.*, 2005), which deacylates D-amino acids mischarged on tRNAs.

The complex structure of *Pyrococcus abyssi* ThrRS editing domain (Pab-NTD) with substrate-mimicking analogues provided the mechanistic basis for deacylation, suggesting a substrate-assisted water-mediated catalytic mechanism (Hussain *et al.*, 2006). Interestingly, a subsequent study revealed that this domain designed for serine removal can even bind the cognate threonine with only a tenfold lower affinity (Hussain *et al.*, 2010). The extra methyl group in threonine does not lead to its steric rejection at the editing site as conceived by the classical double sieve model (Fersht, 1977). Based on the structural evidence, the key to cognate/noncognate discrimination in this domain was proposed to be functional positioning of

Table 1

Macromolecule production information.

Source organism	<i>Aeropyrum pernix</i>
DNA source	<i>Aeropyrum pernix</i> genomic DNA
Forward primer	GGAAATTCATATGAGGCTTCTATACCTCCACGC
Reverse primer	CCCCTCGAGCTCGGTGAAGCTCCTAGAGAG
Cloning vector	pET21b
Expression vector	pET21b
Expression host	BL21 (DE3) Codon Plus
Complete amino acid sequence of the construct	MRLLYLHADRFEYKTVKPKLNPPDPPGEASFG EALVVFTTVEDGDPQTVMYAASDIASHSSRL KVTVILYPYAHLSRLAKPMAAHKRLIELEG ALRTKFPGHVHRAPFGWYKSFISACKGHPLAE LSRSFTE

the substrate rather than steric exclusion (Hussain *et al.*, 2010). Thus, in order to test the universality of the discriminatory mechanism of Pab-NTD within archaea and to study the editing process in ThrRS from an evolutionary perspective, we initiated structural investigations in its orthologue from *Aeropyrum pernix*, which belongs to the phylum Crenarchaeota.

A. pernix has two genes for ThrRS in its genome, *thrS1* and *thrS2*, encoding proteins ThrRS1 and ThrRS2, respectively. ThrRS1 is responsible for the aminoacylation function while the editing activity resides in the N-terminal domain of ThrRS2 (Shimizu *et al.*, 2009). Here we report the cloning, purification, crystallization and preliminary crystallographic investigations of the first 136 amino acids from ThrRS2 which define the editing domain of ThrRS from *A. pernix*.

2. Materials and methods

2.1. Production of N-terminal domain from *A. pernix* ThrRS2

The gene corresponding to the N-terminal domain of ThrRS2 from *A. pernix*, comprising amino acids 1–136, was amplified by PCR from its genomic DNA (obtained from ATCC) using gene-specific primers (BioServe, India) and was cloned into the *Nde*I and *Xho*I sites of the pET21b (Novagen) expression vector (Table 1). The recombinant plasmid was transformed and overexpressed in *E. coli* BL21 (DE3) Codon Plus cells. The transformed cells were grown in Luria–Bertani medium with 100 µg ml^{−1} ampicillin and 36 µg ml^{−1} chloramphenicol at 310 K until the OD₆₀₀ reached 0.6 absorbance units. The cells were then induced with 0.5 mM IPTG and allowed to grow at 291 K for 16 h before being harvested by centrifugation at 6000 rev min^{−1} for 10 min at 277 K. The cell pellet was resuspended in ice-cold lysis buffer consisting of 50 mM Tris–HCl pH 7.0, 20 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed by sonication on ice. The crude cell extract was subjected to nucleic acid precipitation with 1% streptomycin sulfate for 30 min at 277 K with constant stirring and was then centrifuged at 16 000 rev min^{−1} for 45 min at 277 K. Three chromatographic steps were performed in order to purify the protein from the supernatant. The first step involved passing the supernatant through a Sulfopropyl-Sepharose column (Amersham Pharmacia) equilibrated with the lysis buffer and elution in a linear gradient from 20 to 500 mM NaCl. The eluate was treated with 30% ammonium sulfate with constant stirring at 277 K followed by centrifugation at 18 000 rev min^{−1} for 1 h. The supernatant was loaded onto a Phenyl-Sepharose (Amersham Pharmacia) column equilibrated with 30% ammonium sulfate and 50 mM Tris–HCl pH 8.0 and eluted in a linear gradient from 30 to 0% ammonium sulfate. The protein was further purified to homogeneity by gel-filtration chromatography using a Superdex 75 column. The final protein was

Table 2

Crystallization experiment details.

Method	Hanging-drop vapour diffusion
Plate type	Iwaki 24-well plate
Temperature (K)	277
Protein concentration	10 mg ml ^{−1}
Buffer composition of protein solution	50 mM Tris–HCl 8.0 and 150 mM NaCl
Composition of reservoir solution	0.1 M sodium citrate pH 5.6, 0.2 M ammonium acetate, 30% PEG 4000
Volume and ratio of drop	1 µl protein and 1 µl reservoir, 1:1
Volume of reservoir	500 µl

concentrated to 10 mg ml^{−1}. The concentration of protein was estimated from its absorbance at 280 nm determined using a NanoDrop 1000 Spectrophotometer from Thermo Scientific.

2.2. Crystallization

Initial crystallization trials were performed at 277 K using Crystal Screen HT from Hampton Research in sitting-drop format using 96-well MRC plates (Jena Bioscience). Crystallization drops were set with 1 µl of 10 mg ml^{−1} protein in 50 mM Tris–HCl pH 8.0 and 150 mM NaCl and 1 µl reservoir buffer using a Mosquito crystallization robot (TTP LabTech). Hits obtained in the screen were further optimized by slight variations in pH, ionic strength and precipitant concentration in a hanging-drop vapour-diffusion setup using 24-well plates from Iwaki.

2.3. Data collection and processing

Data collection was performed using an in-house Rigaku Micromax 007 HF rotating-anode generator which produces Cu K α X-rays of wavelength 1.5418 Å and a MAR345dtb image-plate detector from Mar Research. The crystal was mounted on a nylon loop and flash-cooled in a nitrogen-gas stream at 100 K using an Oxford Cryostream cooler (Oxford Cryosystems). A total of 140° of the data set was collected with an oscillation width of 0.5° and an exposure of 120 s for each diffraction image. Indexing was performed by AUTOMAR (MAR Research) and data processing was done using HKL-2000 (Otwinowski & Minor, 1997).

Figure 1

15% SDS-PAGE showing the purity of the final protein sample. Lane (1) marker; lane (2) protein sample. The molecular weight of the N-terminal editing domain of *A. pernix* ThrRS2 is 15.1 kDa.

Figure 2
Crystals of the N-terminal editing domain of *A. pernix* ThrRS2.

3. Results and discussion

The editing domain of ThrRS from *A. pernix* was overexpressed in a soluble form in *E. coli* BL21 (DE3) Codon Plus cells and purified to homogeneity by a three-step purification procedure consisting of cation-exchange chromatography, hydrophobic interaction chromatography and finally gel-filtration chromatography. Each litre of bacterial culture yielded approximately 0.5 mg of nearly 95% pure protein sample (Fig. 1). Hits obtained from initial crystallization screens were optimized in a hanging-drop vapour-diffusion setup. 1 µl of the 10 mg ml⁻¹ protein solution in 50 mM Tris-HCl pH 8.0 and 150 mM NaCl and 1 µl of reservoir buffer were used to set up each crystallization drop, and equilibrated against 500 µl of reservoir buffer (Table 2). Well diffracting crystals were obtained in 0.1 M sodium citrate pH 5.6, 0.2 M ammonium acetate, 30% PEG 4000 (Fig. 2). The crystals appeared within 24 h of setting up the drop and grew to a maximum size of ~200 × 150 × 150 µm. Diffraction data were collected at the in-house X-ray source and the crystal diffracted to a resolution of 1.66 Å (Fig. 3). The X-ray diffraction data indicated

Figure 3
X-ray diffraction image of crystals of the N-terminal editing domain of *A. pernix* ThrRS2. The resolution at the edge is 1.66 Å. (Inset: magnified image near the edge.)

Table 3

Data-collection and -processing statistics.

Values in parentheses are for the highest-resolution shell.

Diffraction source	Rigaku MicroMax 007 HF Cu
Wavelength (Å)	1.54
Temperature (K)	100
Detector	Mar345
Crystal-detector distance (mm)	120
Rotation range per image (°)	0.5
Total rotation range (°)	140
Exposure time per image (s)	120
Space group	<i>P</i> ₄ ₁ ₂ ₁ ₂ or <i>P</i> ₄ ₃ ₂ ₁ ₂
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 47.32, <i>c</i> = 113.62, α, β, γ = 90
Mosaicity (°)	0.69–0.76
Unit-cell volume (Å ³)	254363.2
Resolution range (Å)	25–1.66
Total no. of reflections	153075 (7758)
No. of unique reflections	15781 (1315)
Completeness (%)	98.4 (84.2)
Redundancy	9.7 (5.9)
<i>R</i> _{merge} [†] (%)	3.2 (22.3)
<i>I</i> /σ(<i>I</i>)	61.1 (5.1)
Overall <i>B</i> factor from Wilson plot (Å ²)	31.0
<i>V</i> _M (Å ³ Da ⁻¹)	2.18
Solvent content (%)	43.6
Molecules per asymmetric unit	1

[†] $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$.

that the crystals belonged to space group *P*₄₁₂₁₂ or *P*₄₃₂₁₂ with unit-cell parameters *a* = *b* = 47.32, *c* = 113.62 Å. The data-collection statistics are shown in Table 3. The Matthews coefficient (*V*_M = 2.18 Å³ Da⁻¹) indicated the presence of one monomer per asymmetric unit and a solvent content of about 43.6%. Since archaeal ThrRS editing domain forms a dimer, as shown in the case of Pab-NTD (Dwivedi *et al.*, 2005), it is likely that the dimeric twofold symmetry coincides with one of the crystallographic twofold symmetries. Structure solution by molecular replacement will be attempted using the editing domain of ThrRS from *P. abyssii* (PDB entry 1y2q; Dwivedi *et al.*, 2005) as the search model which shares 43% sequence identity with Ap-NTD (the N-terminal proofreading domain of ThrRS2 from *A. pernix*); the structure determination and refinement are in progress.

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