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Purification, crystallization and preliminary crystallographic analysis of a 4-thiouridine synthetase–RNA complex

The sulfurtransferase 4-thiouridine synthetase (ThiI) is involved in the ATP-dependent modification of U8 in tRNA. ThiI from *Thermotoga maritima* was cloned, overexpressed and purified. A complex comprising ThiI and a truncated tRNA was prepared and crystallized, and X-ray diffraction data were collected to a resolution of 3.5 Å. The crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 102.9$, $b = 112.8$, $c = 132.8$ Å.

1. Introduction

More than 100 different modified nucleotides have been identified in RNA, most of them in tRNAs (Rozenki *et al.*, 1999; Machnicka *et al.*, 2013). The tRNA modifications affect different processes like the stability of the tRNA structure (Motorin & Helm, 2010), the binding of tRNAs to the ribosome (Ashraf *et al.*, 1999; Yarian *et al.*, 2002), or the fidelity and rate of translation (Krüger *et al.*, 1998; Björk *et al.*, 1999). Among the 16 different thio-nucleotides identified in RNAs, the 4-thiouridine (s^4U) is found at position 8 of eubacterial and archaeal tRNAs. s^4U8 stabilizes the fold of the tRNA (Romby *et al.*, 1987; Kumar & Davis, 1997) and, in addition, plays an important role as a sensor for UV radiation (Thomas & Favre, 1980). Upon exposure to near-UV light a covalent bond between s^4U8 and the cytidine at position 13 of the tRNA is formed (Favre *et al.*, 1971). This photocrosslink slows down the amino-acylation of the tRNA (Blondel & Favre, 1988), which provokes the stringent response and leads to growth delay of the bacteria (Ramabhadran & Jagger, 1976).

The biosynthesis of s^4U8 in tRNAs requires the enzyme 4-thiouridine synthetase ThiI (Mueller *et al.*, 1998). This enzyme activates the target uridine by adenylation and substitutes the oxygen linked to position 4 of the base by sulfur (Mueller *et al.*, 2001; You *et al.*, 2008). For 4-thiouridine synthesis in *Escherichia coli* it was shown that the sulfur is first transferred from the free amino acid cysteine to the enzyme IscS by formation of an internal persulfide bond and then further transferred to the rhodanese-like domain (RLD) of ThiI, also forming an internal persulfide bond, which finally attacks the adenylated U8 (Fig. 1) (Kambampati & Lauhon, 2000; Wright *et al.*, 2006).

The crystal structure of ThiI from *Bacillus anthracis* (ThiI_{Ba}) has previously been determined, revealing the spatial organization of three domains (Waterman *et al.*, 2006) and the crystal structure of a putative ThiI ortholog from the archaeon *Pyrococcus horikoshii* was also solved (Sugahara *et al.*, 2007). The N-terminal ferredoxin-like domain (NFLD) and the THUMP-domain are thought to be involved in tRNA binding, while the PPase domain contains the catalytic centre, which is defined by the bound AMP molecule in the ThiI_{Ba} crystal structure. However, the structure of a ThiI–RNA complex is yet unknown.

In order to unveil the structural basis for the specific recognition and modification of U8 in tRNA, we have prepared and crystallized the complex consisting of ThiI from *Thermotoga maritima* and a truncated tRNA^{Phe}. This truncated tRNA (TPHE39A) was previously shown to be a minimal substrate for ThiI (Lauhon *et al.*, 2004).

2. Materials and methods

2.1. Cloning, expression and purification of ThiI

The *thiI* gene from *T. maritima* (NCBI accession number AAD36761) was amplified from chromosomal DNA by PCR, cloned into the vector pET-28a (Novagen, USA) via the restriction sites *NcoI* and *HindIII* introduced by respective 5' and 3' PCR primers. Owing to the cloning procedure the lysine at position 2 (Lys2) was replaced by a glutamate residue (Lys2Glu). Full-length ThiI encoding Met1–Glu388 (including the Lys2Glu mutation, which will not be mentioned henceforth) was produced in *E. coli* BL21(DE3)pLysS cells (Invitrogen) at 310 K in LB medium containing the appropriate selective antibiotics (50 mg l⁻¹ kanamycin, 20 mg l⁻¹ chloramphenicol). Expression was induced at OD₆₀₀ = 0.5 by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Cells were harvested by centrifugation after 4 h of expression and cell pellets were stored at 193 K.

For protein preparation cells were lysed in buffer A (50 mM ammonium chloride, 2 mM DTT, 50 mM Tris–HCl pH 7.5) and the protease inhibitor cocktail (Roche) by incubation on ice for 10 min followed by sonification until the suspension appeared clear. The clarified lysate was incubated at 353 K for 10 min resulting in the denaturation of most innate *E. coli* proteins. After cooling on ice, precipitated proteins were removed by centrifugation (30 000g, 10 min, 277 K). Unless stated otherwise, all subsequent purification steps were carried out at room temperature. The heat denaturation step was followed by DEAE-sepharose chromatography. The supernatant was loaded onto a DEAE fast-flow sepharose column (GE Healthcare) equilibrated with buffer A and, after removal of unbound molecules, proteins were eluted by a linear gradient (7 column volumes) by adding buffer B (1 M ammonium sulfate, 50 mM ammonium chloride, 2 mM DTT, 50 mM Tris–HCl pH 7.5) to a final concentration of 100%. ThiI-containing fractions were pooled and concentrated at 289 K using Vivaspin 30 kDa concentrators (Sartorius) with a molecular-weight cut-off of 30 000 Da. Further

purification was achieved by a size-exclusion chromatography step [Superdex 75 (26/60), GE Healthcare] in a buffer consisting of 150 mM ammonium sulfate, 20 mM Tris–HCl pH 7.5. The pure protein was concentrated to 10 mg ml⁻¹ using Vivaspin 30 kDa.

2.2. ThiI–RNA complex formation

The synthetic RNA of 39 nucleotides corresponding to the previously described truncated tRNA^{Phe} (TPHE39A) from *E. coli* (Lauhon *et al.*, 2004) was purchased from IBA (Göttingen, Germany). For complex formation, ThiI and RNA were mixed in a 1:1.5 molar ratio and incubated for 1 h at 277 K. The complex was separated from unbound RNA by gel filtration using a Superdex S75 (10/30) column equilibrated in 150 mM ammonium sulfate, 20 mM Tris–HCl pH 7.5. In addition to a small shift in the elution volume of the ThiI peak, complex formation was confirmed by comparison of the OD₂₆₀/OD₂₈₀ ratio. Fractions were analyzed by SDS–PAGE on 17.5% acrylamide gels stained consecutively with STAINS ALL [0.5% (v/v) STAINS ALL, 50% (v/v) formamide] and Coomassie. The complex-containing fractions were pooled and concentrated to 10 mg ml⁻¹ (Vivaspin, 10 kDa cut-off).

2.3. Crystallization, X-ray data collection and crystallographic analysis

Crystallization was performed in Cryschem plates (Hampton Research) using sitting drops. Initial crystals were obtained in drops composed of 1 µl of complex solution (10 mg ml⁻¹ ThiI–RNA complex in a buffer consisting of 150 mM ammonium sulfate, 20 mM Tris–HCl pH 7.5, 2 mM ATP) mixed with 1 µl of freshly prepared reservoir solution (2.0 M sodium formate, 100 mM sodium citrate pH 4.6, 2 mM DTT). Many small crystals of the ThiI–RNA complex grew at 293 K within 24 h. Optimization of the ratio of a mixture between complex and reservoir solution to 1 µl:3 µl resulted in large single crystals (150 × 80 × 40 µm) after 7 d. For data collection crystals were transferred into cryobuffer [30% (v/v) glycerol, 2 M sodium

Figure 1

(a) The biosynthesis of 4-thiouridine only occurs for uridine at position 8 of bacterial and archaeal tRNAs. In *E. coli* the thiolation of U8 requires the action of both the 4-thiouridine synthetase ThiI and the cysteine desulfurase IscS (Lauhon, 2002). (b) The pyridoxal phosphate-dependent IscS catalyzes the sulfur transfer from free L-cysteine via persulfide to ThiI, which utilizes Mg–ATP to activate uridine at position 8 of tRNAs and transfers the sulfur to give the final product (Mueller *et al.*, 2001). In other organisms, like *B. subtilis*, IscS is replaced by the sulfurtransferase NifZ (Rajakovich *et al.*, 2012). (Abbreviations: PLP = pyridoxal phosphate, SSH = persulfide, s⁴U8 = 4-thiouridine at U8 of tRNA.)

Table 1

X-ray data-collection and processing statistics.

Values in parentheses are for the highest-resolution shell.

Space group	$P2_12_12_1$
Unit-cell dimensions (Å)	$a = 102.9, b = 112.8, c = 132.8$
Resolution (Å)	81.3–3.50 (3.83–3.50)
Wavelength (Å)	0.8430
$R_{\text{merge}}^{\dagger}$ (%)	10.6 (56.3)
Mean $I/\sigma(I)$	11.0 (2.8)
Completeness (%)	99.7 (100.0)
Reflections (observed)	72525 (17355)
Average redundancy	3.6 (3.7)
Wilson B value (Å ²)	93.0

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

formate, 100 mM sodium citrate pH 4.6] and flash-cooled at a temperature of 100 K. X-ray diffraction data of a single crystal were collected on a MAR345 image-plate detector at a wavelength of 0.8430 Å on EMBL beamline BW7A at DESY (Hamburg). The diffraction data were processed and scaled with *MOSFLM* and *AIMLESS* from the *CCP4* suite, respectively (Leslie & Powell, 2007; Evans, 2011; Winn *et al.*, 2011). A Patterson self-rotation function was calculated with *POLARRFN* as part of the *CCP4* program suite (Winn *et al.*, 2011) and molecular replacement (MR) was performed with *Phaser* (McCoy *et al.*, 2007). Data-collection and processing statistics are given in Table 1.

3. Results and discussion

The 44 kDa enzyme 4-thiouridine synthetase (ThiI) from *T. maritima* was overproduced in *E. coli*, purified and used for the preparation of the ThiI–RNA complex. The RNA corresponds to a truncated tRNA^{Phe}, which is recognized and modified by ThiI (Tanaka *et al.*, 2009; Lauhon *et al.*, 2004).

Crystals of the ThiI–RNA complex were obtained by vapor diffusion using sitting drops. After optimization of the crystallization condition, crystals grew to an average size of 150 × 80 × 40 µm (Fig. 2). In order to show that these crystals contain the ThiI–RNA complex, crystals were extensively washed in crystallization buffer and dissolved in water. The subsequent analysis by SDS–PAGE

Figure 2

A single crystal of the ThiI–RNA complex.

revealed that the crystals indeed consist of both the ThiI protein and the RNA (Fig. 3). Previous crystallization trials of ThiI–RNA complexes have yielded crystals only of tRNA^{Phe} or the truncated tRNA^{Phe} (TPHE39A) indicating the dissociation of the complex in the crystallization buffer. These co-crystallization experiments were performed with ThiI from *B. anthracis* (Byrne *et al.*, 2010) or with ThiI from *E. coli* (Tanaka *et al.*, 2009), while the complex crystals described here contain ThiI from the thermophilic organism *T. maritima*, which might explain the higher stability of the complex. Furthermore, ATP was added to the crystallization buffer, as ThiI catalyzes the formation of a covalent bond between the α-phosphate and the substrate U8, thereby releasing pyrophosphate (Fig. 1), which might also lead to increased stability of the ThiI–RNA complex. The structure of that reaction intermediate would be of particular interest.

A complete X-ray diffraction data set was obtained from one ThiI–RNA crystal providing data to 3.5 Å resolution (Table 1). Data processing revealed that the crystal belongs to the primitive orthorhombic space group $P2_12_12_1$ with unit-cell dimensions of $a = 102.9$, $b = 112.8$, $c = 132.8$ Å. The Matthews coefficient (Matthews, 1968) suggests the presence of either two or three monomers per asymmetric unit with a solvent content of 63.3% (3.4 Å³ Da^{−1}) or 45.0% (2.3 Å³ Da^{−1}), respectively. The higher solvent content corresponding to two ThiI–RNA complexes per asymmetric unit appears to be more likely as it is consistent with the results of a Patterson self-rotation analysis.

A structure determination by means of MR appeared feasible as the known structure of *B. anthracis* ThiI (ThiI_{Ba}; PDB entry 2c5s, Waterman *et al.*, 2006) shares 34% sequence identity with the ThiI from *T. maritima*. While the complete structure of ThiI_{Ba} as the search model did not provide an MR solution, a search using three separated domains (PPase, NFLD and THUMP) of ThiI_{Ba} led to partial reasonable solutions consisting of two PPase domains and one NFLD and one THUMP domain, which based on their relative position to each other and to one PPase domain have been assigned to form an entity with one PPase domain. Analysis of rotation peaks corresponding to two PPase domains revealed that this solution is consistent with the weak peak from the self-rotation function indicating the presence of the single non-crystallographic twofold axis.

Figure 3

SDS–PAGE analysis of ThiI–RNA complex crystals (lane M, molecular-weight marker proteins, labelled in kDa; lane C, crystals; lane R, RNA used for co-crystallization; lane P, ThiI protein used for co-crystallization).

Therefore the missing NFLD and THUMP domains of the second PPase domain have been obtained based on superposition between the two PPase domains followed by rigid-body refinement of individual domains in *Phaser*. This led to a reasonable solution indicating different relative positions of the NFLD and THUMP domains belonging to the two Thil monomers occupying the asymmetric unit, although the highest translation function *Z*-scores (TFZ) reported by the *Phaser* program for NFLD and THUMP domains were quite low (around 5). Locating the truncated tRNA molecules using the TPHE39A crystal structure (PDB entry 2zy6, Tanaka *et al.*, 2009) was not successful, despite using the two PPase domains or the two monomers of Thil as the partial structure. MR searches performed using shorter fragments of TPHE39A or tRNA (PDB entry 4tna, Hingerty *et al.*, 1978) yielded reasonable solutions differing in the directionality of tRNA fragments. Structure verification and model rebuilding are currently in progress.

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