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Preliminary X-ray crystallographic analysis of tRNA pseudouridine 55 synthase from the thermophilic eubacterium *Thermotoga maritima*

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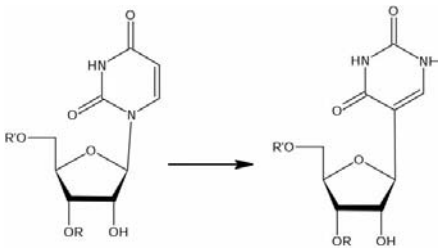
Thermotoga maritima TruB, an enzyme responsible for the formation of pseudouridine in tRNA, has been purified and crystallized by the hanging-drop vapour-diffusion method in 100 m*M* citrate pH 3.5, 200 m*M* Li_2SO_4, 20% glycerol, 13% PEG 8000. Crystals display orthorhombic symmetry, with unit-cell parameters $a = 47.39$, $b = 83.88$, $c = 98.72$ Å, and diffract to 2.0 Å resolution using synchrotron radiation. A solution was obtained by molecular replacement using part of the recently published crystal structure of *Escherichia coli* TruB bound to a synthetic RNA.

1. Introduction

Pseudouridine (5-β-D-ribofuranosyluracil; Ψ) is the C5–C1′ isomer of uridine. It is the most abundant modified nucleoside found in RNA. Pseudouridine synthases are responsible for the formation of Ψ in transfer RNA (tRNA), ribosomal RNA (rRNA) and small nuclear RNA (snRNA). These enzymes require no cofactor. The reaction involves breakage of the N-glycosidic bond of the target uridine, a 180° rotation of the detached base with respect to the ribose and formation of a new C1′—C5 carbon–carbon bond.



Possible mechanisms of pseudouridine synthases have recently been discussed in detail (Huang *et al.*, 1998; Mueller, 2002).

The formation of Ψ55 in the T-loop of tRNA is catalyzed by tRNA Ψ55 synthase, the product of the bacterial *truB* gene (*PUS4* in yeast; Nurse *et al.*, 1995; Becker *et al.*, 1997). Virtually all mature tRNAs sequenced so far contain Ψ55 (Sprinzl *et al.*, 1998). Thus, tRNA Ψ55 synthase is an interesting model for the study of protein–RNA recognition because its specificity contrasts with that of other enzymes such as aminoacyl tRNA synthetases which only recognize cognate tRNAs among all tRNA species.

Recently, the crystal structure of *Escherichia coli* TruB bound to a synthetic RNA corresponding to a T-stem and loop (TSL) has been determined (Hoang & Ferré-D'Amaré, 2001). This 1.85 Å resolution structure of TruB (PDB code 1k8w) shows that the protein adopts a mixed α/β fold with distinct N- and C-terminal domains.

In this TruB–TSL complex, the bases of nucleotides 55, 56 and 57 are everted from their position in the helical stack of isolated tRNA. The co-crystal structure showed that TruB gains access to its substrate by flipping out uridine 55 of the tRNA.

The structure of TruA, another *E. coli* pseudouridine synthase responsible for the formation of Ψ38, 39 and 40 in the anticodon arm of tRNA, has been determined without bound RNA (Foster *et al.*, 2000). The three-dimensional structures of the cores of TruA and TruB align closely. However, the RNA-binding segments of TruB, in particular the C-terminal domain, are not present in the structure of TruA.

Recently, a third structure of a pseudouridine synthase, that of Rsua, has been reported (Sivaraman *et al.*, 2002). Comparison of all three structures shows that the N-terminal domain of TruB is the conserved structural core of all pseudouridine synthases.

In order to determine whether the binding of TruB to its tRNA substrate induces conformational changes in TruB itself, we have undertaken the X-ray crystallographic analysis of the unliganded TruB protein. Here, we report the overexpression, crystallization and preliminary X-ray crystallographic analysis of *Thermotoga maritima* TruB.

2. Material and methods

2.1. Cloning and overexpression

The *T. maritima truB* gene was amplified by the polymerase chain reaction (PCR) using genomic DNA as template. The *T. maritima*

MSB8 strain (Hüber *et al.*, 1986) was cultured at 353 K as described previously (Van de Casteele *et al.*, 1990) and genomic DNA was prepared as described by Murray & Thompson (1980). The forward (5′-CATATGAAGCACGGAATCCTTGTAGCTTACAAGCC-3′) and reverse (5′-GCGGCCGCCCTCGTGTTGAAGACCTTTCTGAGGG-3′) oligonucleotide primers were designed to amplify the *truB* gene (GenBank AE000512). PCR amplification was achieved using the GC-rich PCR system (Roche). The amplified fragment was cloned into pCR2.1 vector using the TA Cloning kit (Invitrogen). After digestion with *Nde*I and *Not*I enzymes, the fragment corresponding to the *truB* gene was then ligated into the pET30b vector (Novagen), giving the pVD1 plasmid. The resulting protein, identified by DNA sequencing as TruB carrying a C-terminal histidine tag (AAALEHHHHHH), was overexpressed in *E. coli* BL21(DE3)pLysS cells (Novagen). Expression of the recombinant enzyme was induced for 5 h at 310 K using 0.5 m*M* IPTG. The cells were resuspended in 100 m*M* phosphate buffer pH 7.4 and disrupted by sonication for 10 min using a Vibra Cell 75041 sonicator. Debris was removed by centrifugation at 15 000*g* for 10 min. The supernatant was incubated at 343 K for 15 min, centrifugated at 15 000*g* for 10 min and dialyzed overnight against 50 m*M* Tris–HCl buffer pH 7.4.

2.2. Enzymatic assay

In vitro transcribed *T. thermophilus* HB27 $tRNA^{Phe}$ was used as a substrate to test the TruB enzymatic activity. The gene encoding *T. thermophilus* $tRNA^{Phe}$ was amplified by PCR from genomic DNA as template, using forward (TTPHE-1) and reverse (TTPHE-2) primers (5′-TATTAATACGACTCACTATAGCCGAGGTAGCTCAGTTGGTAGAG-3′ and 5′-TATCCTGGTGCCGAGGAGCGGAATCGAACCG-3′, respectively). The primers were designed using the published sequence of *T. thermophilus* $tRNA^{Phe}$ (Grawunder *et al.*, 1992). The TTPHE-1 primer contains the sequence of a T7 polymerase promoter and the TTPHE-2 primer contains an *Mva*I restriction site. PCR amplification was performed using *Pwo* DNA polymerase (Roche). The amplified fragment was cloned into the *Sma*I restriction site of pUC18, giving the pML2 plasmid. The template for *in vitro* transcription was obtained by *Mva*I restriction of this plasmid.

The TruB enzymatic activity was tested at 333 K in 50 m*M* Tris–HCl pH 8.0, 10 m*M* $MgCl_2$ using 1–2 fmol of (^{32}P-UTP)-radiolabelled T7-runoff transcript of *T. thermophilus* $tRNA^{Phe}$ as substrate. The reaction was terminated by phenol extraction and ethanol precipitation. The tRNA was hydrolyzed by nuclease P1 and the resulting nucleotides were separated by two-dimensional thin-layer chromatography on cellulose plates according to Becker *et al.* (1997). The formation of pseudouridine was revealed by autoradiography of the plates.

2.3. Purification

Purification was achieved using nickel-affinity chromatography with resin (Chelating Sepharose supplied by Amersham Pharmacia) pre-equilibrated with 50 m*M* Tris–HCl pH 7.4. Bound protein was eluted with a linear gradient of 0–0.5 *M* imidazole in 50 m*M* Tris–HCl pH 7.4. Active fractions were pooled, dialyzed against 50 m*M* Tris–HCl pH 7.4, 10 m*M* β-mercaptoethanol, 2 m*M* EDTA, 100 m*M* KCl and analyzed by SDS–PAGE under reducing conditions (Fig. 1). The purified enzyme sample was concentrated to about 10 mg ml^{-1}, as estimated by UV absorption (Kalckar & Shafran, 1947), snap-frozen in liquid nitrogen and stored at 193 K. The yield was 40 mg pure protein per litre of culture.

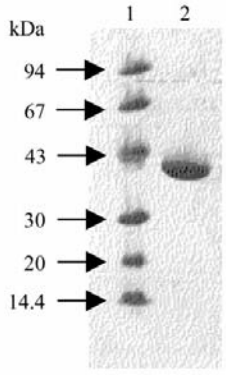


Figure 1
Polyacrylamide gel electrophoresis under denaturing conditions of the purified *T. maritima* TruB. Lane 1, molecular-weight markers (Amersham Pharmacia). Lane 2, purified enzyme.

2.4. Crystallization

Crystallization trials were performed by the hanging-drop vapour-diffusion method (McPherson, 1982) using 24-well tissue-culture VDX plates (Hampton Research) at 293 K. Each drop was prepared by mixing 2 µl of protein solution with the same volume of reservoir solution. The drops were suspended over 0.6 ml of reservoir solution. Preliminary crystallization conditions were tested using the Crystal Screen Cryo kit (Hampton Research). Crystals appeared in the presence of PEG 8000 and PEG 5000. Crystallization conditions were refined by systematic variations of PEG 8000 concentrations at different pH values. The best crystals were obtained after 5 d in 100 m*M* citrate pH 3.5, 200 m*M* Li_2SO_4, 20% glycerol, 13% PEG 8000. Crystals (Fig. 2) always grew as isolated aggregates of single crystals from which fragments had to be cut.

3. Data collection and analysis

Preliminary diffraction data were collected on a MAR345 imaging-plate system from MAR Research equipped with Osmic optics and running on an FR591 rotating-anode generator (Cu $K\alpha$). The crystals display orthorhombic symmetry, with unit-cell parameters a = 47.39, b = 83.88, c = 98.72 Å.

A first complete data set was collected from a flash-frozen crystal to a resolution of 2.7 Å. This same crystal was defrosted and placed in a drop containing mother-liquor solution (100 m*M* citrate pH 3.5, 200 m*M* Li_2SO_4, 20% glycerol, 13% PEG 8000) plus 0.5 *M* KI. After 2 min of soaking, the crystal was flash-frozen a second time in order to record diffraction data from the iodine derivative. Although the resolution of the images was reduced to about 3.0 Å, as a consequence of either the freezing–defrosting process or owing to damage induced by soaking, data were collected with a view to SAD phasing (Evans & Bricogne, 2002; Dauter *et al.*, 2000).

A complete diffraction data set was collected on beamline BM30 at ESRF (Grenoble) on a flash-frozen crystal using a MAR CCD detector at a wavelength of 0.97985 Å. The crystal diffracted to 2.0 Å. The distance between the crystal and the

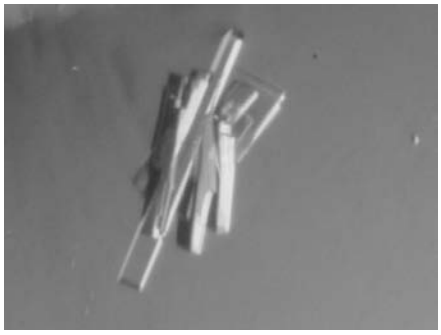


Figure 2
Typical plate crystals of *T. maritima* TruB obtained by equilibration against a solution of 100 m*M* citrate pH 3.5, 200 m*M* Li_2SO_4, 20% glycerol, 13% PEG 5000. The maximum dimension of the crystals is about 0.250 mm.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.97985
Resolution (Å)	99–2.07 (2.10–2.07)
No. measured reflections	77641
No. unique reflections	24675
Multiplicity	4
$I/\sigma(I)$	11.7 (3.9)
Completeness (%)	96.5 (98.6)
$R_{\rm merge}$ (%)	8.1 (27.4)

detector was set to 150 mm. Data were processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and the results are summarized in Table 1. The overall completeness is 96.5% in the resolution range 99.0–2.07 Å, with an overall $R_{\rm sym}$ value of 8.1%.

4. Structure analysis

In the *T. maritima* TruB crystal, the solvent content is calculated to be 49.7% assuming one molecule in the asymmetric unit and a density of 1.30 g ml^{-1} and based on the unit-cell size and space group $P2_12_12_1$. The Matthews coefficient (Matthews, 1968) is 2.47 Å^3 Da^{-1} based on a molecular weight of 35 970 Da per subunit for *T. maritima* TruB.

Sequence identity between *E. coli* and *T. maritima* TruB is about 30%. The 1.85 Å resolution structure of *E. coli* TruB bound to RNA (PDB code 1k8w) was used as search model for molecular replacement using the program *AMoRe* (Navaza, 1994). Using the two domains (N- and C-terminal domains) of *E. coli* TruB did not lead to a reasonable solution. Dividing the structure into two distinct search domains (residues 9–250 and 250–311) led to a possible solution which after fitting refines to an *R* factor of 53.7 and a correlation coefficient of 19.0 in the 30–4 Å resolution range. Packing is reasonable. In comparison with the *E. coli* structure, the relative positions of the N-terminal and C-terminal domains in *T. maritima* differ. Preliminary density maps suggest that a large amount of manual refinement will be necessary to obtain the structure of unbound *T. maritima* TruB and that it will be significantly different from the structure of the *E. coli* enzyme bound to RNA.

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