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# Expression, purification, crystallization and preliminary diffraction studies of the tRNA pseudouridine synthase TruD from *Escherichia coli*

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Pseudouridine, the 5-ribosyl isomer of uridine, is the most common modification of structural RNA. The recently identified pseudouridine synthase TruD belongs to a widespread class of pseudouridine synthases without significant sequence homology to previously known families. TruD from *Escherichia coli* was overexpressed, purified and crystallized. The crystals diffract to a minimum Bragg spacing of 2.4 Å and belong to space group $P2_12_12_1$, with unit-cell parameters $a$ = 63.4, $b$ = 108.6, $c$ = 111.7 Å.

## 1. Introduction

All organisms have enzymes responsible for RNA modification; the most common RNA modification in the biosphere is pseudouridine ($\psi$), the 5-ribosyl isomer of uridine (U). Pseudouridine is found in all three kingdoms of life including eukaryotic organelles such as mitochondria and chloroplasts. The synthesis of $\psi$ is performed by pseudouridine synthases, which are able to recognize and convert specific uridine residues in rRNA, tRNA and snRNA and snoRNA to $\psi$. The $\psi$ synthases are a large and ancient class of enzymes with four distinct families: TruA, TruB, RsuA and RluA (Gustafsson *et al.*, 1996; Koonin, 1996). These share a short sequence motif (Koonin, 1996) containing an aspartate residue that is essential for catalytic activity (Huang *et al.*, 1998). Representatives of all families have been studied structurally (Foster *et al.*, 2000; Hoang & Ferré-D'Amaré, 2001; Sivaraman *et al.*, 2002, 2004; Del Campo *et al.*, 2004) and their catalytic domains display the same overall fold, suggesting a common ancestry (Hoang & Ferré-D'Amaré, 2001; Mueller, 2002; Sivaraman *et al.*, 2002).

Recently, the synthase responsible for the $\psi$13 modification of *Escherichia coli* $tRNA^{Glu}$ was discovered by classic biochemical methods (Kaya & Ofengand, 2003). Given that no redundant pseudouridine synthase exists, this was the only remaining additional tRNA pseudouridine synthase expected to be found in *E. coli* (Kaya & Ofengand, 2003). The open reading frame, Ygbo, encoding the protein, does not show any significant sequence homology to previously known pseudouridine synthase subfamilies, although it has orthologues in all kingdoms of life. To reflect the fact that Ygbo is the fourth tRNA-specific $\psi$ synthase, it was renamed TruD (Kaya & Ofengand, 2003). Interestingly, the yeast orthologue Pus7, identified as catalysing $\psi$35 formation in U2 snRNA (Ma *et al.*, 2003), has recently been shown to have multisite multisubstrate specificity in that it also modifies the position U13 of various tRNAs in *Saccharomyces cerevisiae* (Behm-Ansmant *et al.*, 2003). Here, we describe the cloning, overexpression, crystallization and preliminary crystallographic characterization of TruD from *E. coli*.

## 2. Methods and results

### 2.1. Cloning, expression and purification

The *E. coli* TruD gene was amplified by PCR using chromosomal DNA from strain K12 as a template. The amplified DNA was subcloned into the *E. coli* expression vector PT73.3 (Tobbell *et al.*, 2002) using the Gateway system (Invitrogen, Carlsbad, CA, USA). The resulting vector, PT73.3-TRUD, encodes a polypeptide with the TruD gene and an N-terminal tail (N-MHHHHHHGSTSLYKKAGFEGDRT) encompassing an attB site for the Gateway recombination event, a silent ribosome-binding site and a hexahistidine sequence to simplify purification. A C-terminal hexahistidine tail (STHHHHHH-C) was also used to monitor full-length expression in a multi-well filtration dot-blot expression screening procedure (Knaust & Nordlund, 2001; Andersson *et al.*, in preparation). The expression plasmid was transformed into BL21(DE3) and the cells were grown in Luria–Bertani (Miller) medium supplied with tetracycline at 310 K. Protein overproduction was induced at an $OD_{600}$ of 0.6 by the addition of 0.25 m$M$ isopropyl-$\beta$-D-thiogalactopyranoside and was continued for 4 h at 298 K. Harvested cells were resuspended and sonicated in lysis buffer [0.5 $M$ NaCl, 20 m$M$ imidazole, 10 m$M$ $\beta$-mercaptoethanol, 10%($v$/$v$) glycerol, 20 m$M$ sodium phosphate buffer pH 7.4, 0.1 mg ml$^{-1}$ lysozyme, 0.5 mg ml$^{-1}$ DNAse I and Complete EDTA-free Protease Inhibitor mixture (Roche

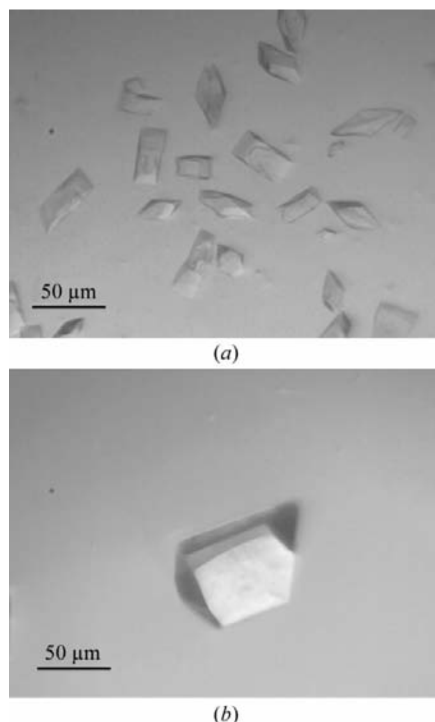


**Figure 1**
Crystals of TruD. (*a*) From condition No. 79 of the Index screen. (*b*) From optimised conditions.

Biosciences, Stockholm, Sweden)]. The cell lysate obtained by centrifugation at 50 000*g* for 20 min was loaded onto an $Ni^{2+}$-loaded Hi-Trap Chelating column (Amersham Biosciences, Uppsala, Sweden) equilibrated in wash buffer (lysis buffer without lysozyme and DNAse I). His-tagged TruD was eluted with elution buffer (wash buffer containing 200 m*M* imidazole) and the fractions containing TruD were pooled and applied onto a HiPrep 26/60 Superdex 200 gel-filtration column (Amersham Biosciences, Uppsala, Sweden) equilibrated in GF buffer [20 m*M* HEPES pH 7.4, 150 m*M* NaCl, 10 m*M* $\beta$-mercaptoethanol, 10%(*v*/*v*) glycerol, 2 m*M* EDTA]. The apparent molecular weight on the gel-filtration column was 43 kDa, suggesting that TruD is a functional monomer. Fractions containing TruD were concentrated to approximately 10 mg ml$^{-1}$ using an Amicon Ultra device (Millipore, Bedford, MA, USA). The 23-residue N-terminal and eight-residue C-terminal tag were not cleaved off prior to crystallization trials. The purified TruD exhibited a single band on SDS–PAGE analysis, but electrospray ionization mass-spectrometry (ESI–MS) analysis revealed two minor degradation products resulting from proteolytic cleavage between the TruD protein and the N-terminal tail, and in the attB site in the N-terminal tail (data not shown).

**Table 1**
Data-collection statistics.

Values in parentheses are for the last shell.

| | |
|---|---|
| Wavelength (Å) | 1.089 |
| Resolution (Å) | 20–2.4 (2.5–2.4) |
| Unique/observed reflections | 30800/222989 |
| Completeness (%) | 99.7 (99.9) |
| $\langle I/\sigma(I)\rangle$ | 13.35 (4.24) |
| $R_{sym}$† (%) | 11.3 (46.5) |

† $R_{sym} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of the reflection $I(hkl)$ and $\langle I(hkl)\rangle$ is the mean intensity of the reflection.

### 2.2. Crystallization

Initial crystals of TruD were obtained at 293 K by the sitting-drop vapour-diffusion method in Intelli-plates (Art Robbins Enterprises, Mountainview, CA, USA) using the Index screen from Hampton Research (Laguna Niguel, CA, USA). Condition No. 79 [0.2 *M* ammonium acetate, 0.1 *M* bis-Tris pH 6.5, 25%(*w*/*v*) PEG 3350] yielded small crystals with growth defects (Fig. 1*a*). By optimizing both the concentration and the type of PEG in the pH range 6–7, we could obtain relatively large single crystals of dimensions 0.1 × 0.1 × 0.2 mm which grew in 24 h (Fig. 1*b*) by the hanging-drop vapour-diffusion method. The best crystals were obtained by using 2 µl protein sample (10 mg ml$^{-1}$) in GF buffer mixed with 2 µl reservoir solution consisting of 10%(*w*/*v*) monomethylether PEG 5000 and 100 m*M* MES pH 6.0 (Fig. 1*b*). A thick skin of presumably oxygen-denatured protein developed within a few hours on the surface of the hanging drop. Inspired by the success of reducing the oxygen content in the crystallization well of another oxygen-sensitive pseudouridine synthase, TruB (Hoang & Ferré-D'Amaré, 2001), we placed a small volume of glucose mixed with glucose oxidase in a Microbridge (Hampton Research, Laguna Niguel, CA, USA) under the hanging drop (Benesch & Benesch, 1953). Although this was successful in eliminating the skin, it also inhibited crystal growth, giving rise to a heavy colourless protein precipitate in the drop.

### 2.3. Data collection and analysis

Crystals were mounted in nylon loops and swept through a cryosolution [10%(*w*/*v*) PEG 4000, 100 m*M* MES pH 6.0 and 25% glycerol] before being plunged into liquid nitrogen. Diffraction data were collected from the native crystals at beamline I711 (MAX-Lab II, Lund, Sweden) to a resolution of 2.4 Å (Table 1). The TruD pseudouridine synthase from *E. coli* crystallized in space group $P2_12_12_1$, with unit-cell parameters $a$ = 63.4, $b$ = 108.6, $c$ = 111.7 Å, suggesting the presence of two protein molecules in the asymmetric unit, giving a $V_M$ value of 2.3 Å$^3$ Da$^{-1}$ (Matthews, 1968), which corresponds to a solvent content of 45%. Data integration, scaling and merging were performed with the *XDS* program package (Kabsch, 1993). Since TruD displays no sequence homology to any protein of known structure, currently we are expressing selenomethionine-substituted TruD in *E. coli* with the aim of determining the structure by SAD or MAD phasing (Hendrickson, 1985).

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