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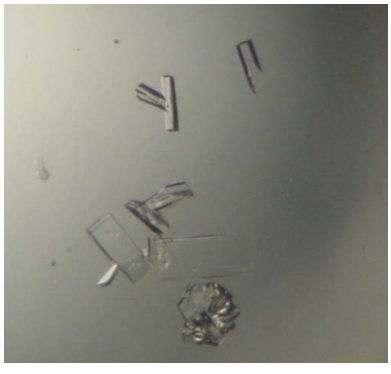
Crystallization and preliminary X-ray crystallographic characterization of TrmFO, a folate-dependent tRNA methyltransferase from *Thermotoga maritima*

TrmFO, previously classified as GID, is a methyltransferase that catalyzes the formation of 5-methyluridine or ribothymidine (T) at position 54 in tRNA in some Gram-positive bacteria. To date, TrmFO is the only characterized tRNA methyltransferase that does not use *S*-adenosylmethionine as the methyl-group donor. Instead, the donor of the methyl group is N^5,N^{10}-methylenetetrahydrofolate. The crystallization and preliminary X-ray crystallographic studies of TrmFO are reported here. The recombinant protein, cloned from *Thermotoga maritima* genomic DNA, was overproduced in *Esherichia coli* and crystallized in 25%(*v*/*v*) PEG 4000, 100 m*M* NaCl and sodium citrate buffer pH 5.0 at 291 K using the hanging-drop vapor-diffusion method. The plate-shaped crystals diffracted to 2.6 Å and belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 79.94, b = 92.46, c = 127.20 Å.

1. Introduction

Transfer RNA (tRNA) contains various modified nucleotides (Sprinzl *et al.*, 1998). To date, 107 modified nucleosides have been described, most of which occur in tRNA (Limbach *et al.*, 1994). From a chemical perspective, the extent of these modifications ranges from very simple to very complex. Certain modifications are invariably found at the same positions in tRNAs across different species (Björk, 1986). In fact, various structural features of tRNA were named according to the modifications found in that specific part of the tRNA molecule. For example, the 'TΨC' loop is named after ribothymidine, pseudouridine and cytidine, which are present at positions 54, 55 and 56, respectively. Even though the 'T' in 'TΨC' is structurally identical to thymidine, it is referred to as 5-methyluridine or ribothymidine, mainly because it is present in RNA rather than DNA. This modification occurs at position 54 in 60% of all sequenced tRNAs and has been shown to contribute to the overall stability of the tRNA molecule by interacting with the D-loop (Nobles *et al.*, 2002). Until recently, TrmA (previously named RUMT) was the only known enzyme responsible for the methylation of uridine in position 54 of tRNA. This methyltransferase, which was first discovered in *Escherichia coli*, carries out the reaction using *S*-adenosylmethionine as the methyl-group donor. Substrate recognition and the detailed chemical mechanism were proposed two decades ago (Björk *et al.*, 1987; Kealey *et al.*, 1991). However, it has been known for over 30 y that some Gram-positive bacteria use other sources of the methyl group for the methylation of tRNA. For example, when *Enterococcus faecalis* was grown in folate-depleted media, 5-methyluridine could not be detected in position 54 of any tRNAs (Samuel & Rabinowitz, 1974). It was later discovered that the donor of the methyl group is N^5,N^{10}-methylenetetrahydrofolate (Delk *et al.*, 1980). Recent bioinformatics searches revealed a novel gene encoding TrmFO, an enzyme that uses N^5,N^{10}-methylenetetrahydofolate as a methyl-group donor to catalyze the formation of 5-methyluridine at position 54 (Urbonavičius *et al.*, 2005). TrmFO is a folate-dependent tRNA methyltransferase from *Bacillus subtilis* that was originally classified as glucose-inhibited division protein (GID). It has been observed that Gram-negative bacteria, and probably all eukarya, utilize an *S*-adenosylmethionine-dependent methylation pathway, while most



Gram-positive bacteria use a folate/FAD-dependent pathway (Urbonavičius *et al.*, 2007). TrmFO shares sequence homology with two other classes of enzymes: GidA and GidA-small. GidA is involved in the biosynthesis of 5-methylaminomethyl-2-thiouridine in the wobble position of bacterial tRNAs (Björk *et al.*, 1999) and binds FAD (White *et al.*, 2001), while the function of GidA-small is still unknown (Iwasaki *et al.*, 2004).

2. Methods and results

2.1. Gene cloning

The *trmFO* gene was PCR-amplified from *Thermotoga maritima* genomic DNA using the forward primer 5′-TAGGGCGAGCTCAAGGAGATATACATATGATAGTGAATGTCATCGGAGCAGGA-3′ and the reverse primer 5′-TAGGCAAAGCTTTCACCAGGGATTCTCTTCCAGGAAC-3′, which contained a *Sac*I and a *Hin*dIII restriction site, respectively. The amplified gene was then digested with *Sac*I and *Hin*dIII and inserted into the pLM-1 expression vector (MacFerrin *et al.*, 1993). Recombinant plasmids were isolated and confirmed by agarose-gel electrophoresis and sequencing.

2.2. Protein expression and purification

The untagged recombinant protein was overexpressed in *E. coli* BL21 (DE3) strain. The cells were grown in LB medium at 310 K until the OD_{600} was ∼0.45, induced with 1 m*M* IPTG and harvested 12 h after induction. The cells were lysed using a French press. The lysis buffer contained 100 m*M* Tris–HCl pH 8.5, 20 m*M* NaCl, 1 m*M* DTT (DEAE-*A* buffer). The cell lysate was incubated at 333 K for 20 min, which resulted in the denaturation of most *E. coli* proteins. The cell lysate was then centrifuged at 13 500 rev min^{-1} in a table-top centrifuge. After centrifugation, the supernatant was purified using a three-step purification protocol. Firstly, the cell lysate suspended in DEAE-*A* buffer was loaded onto a low-resolution ion-exchange DEAE column (GE Healthcare Inc.). The protein was eluted off the column with DEAE-*B* buffer (100 m*M* Tris–HCl pH 8.5, 1 *M* NaCl, 1 m*M* DTT). The elution profile showed two major peaks: a TrmFO peak and a nucleic acid peak. SDS–PAGE was used to determine which fractions contained the protein. Fractions containing TrmFO were buffer-exchanged to MonoS-*A* buffer (100 m*M* sodium citrate pH 5.0, 20 m*M* NaCl, 1 m*M* DTT) and loaded onto a high-resolution ion-exchange MonoS column (GE Healthcare Inc.). After elution with MonoS-*B* buffer (100 m*M* sodium citrate pH 5.0, 1 *M* NaCl, 1 m*M* DTT), fractions containing TrmFO were concentrated using an Amicon centrifugal filter device (Millipore, USA) and further purified on a Superdex 200 size-exclusion column (GE Healthcare Inc.). The elution volume on Superdex 200 corresponds to a protein of approximately 42 kDa in size, indicating that TrmFO (MW = 49.7 kDa) is a monomer in solution (data not shown). This three-step liquid-chromatography protocol yielded TrmFO protein with >95% purity. Crystallization trials were performed immediately after the purification steps. The rest of the protein was stored at 253 K in 50%(*v*/*v*) glycerol.

2.3. Crystallization

Purified TrmFO at a concentration of 8 mg ml^{-1} was subjected to a grid crystallization screen using the vapor-diffusion method. Hanging drops of 1.6 µl in volume were set up in a 24-well plate (Hampton Research, USA). Initial small plate-shaped crystals grew in 7 d in 25% PEG 4000, 100 m*M* NaCl and 100 m*M* sodium citrate buffer pH 5.0. These plate-shaped crystals underwent considerable stacking, rendering them unsuitable for data collection. Further refinement of crystallization conditions using the standard Hampton Research Additive Screen kit (Hampton Research, USA), as well as the addition of FAD/$FADH_2$, did not improve crystal growth. However, lowering the concentration of the protein significantly improved the quality of the crystals. TrmFO crystallized at a concentration of 2 mg ml^{-1} yielded larger plate-shaped crystals that were suitable for data collection (Fig. 1). Before mounting, these crystals were flash-frozen after a brief soaking in a cryoprotectant composed of 20%(*v*/*v*) PEG 4000 and 20%(*v*/*v*) glycerol.

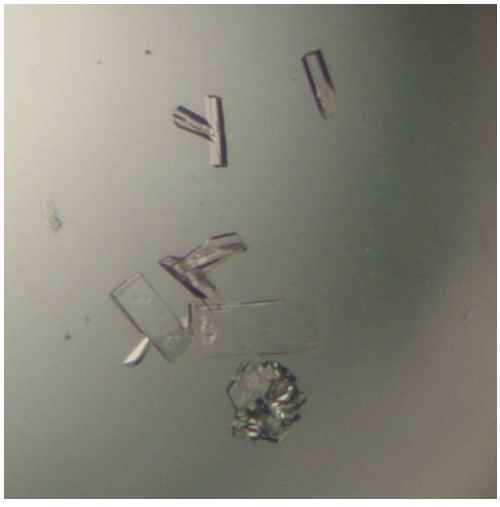


Figure 1
Crystals of TrmFO grown from a hanging drop in 25%(*v*/*v*) PEG 4000, 100 m*M* NaCl and 100 m*M* sodium citrate buffer pH 5.0. The approximate dimensions of the largest plate crystal are 0.1 × 0.3 × 0.001 mm.

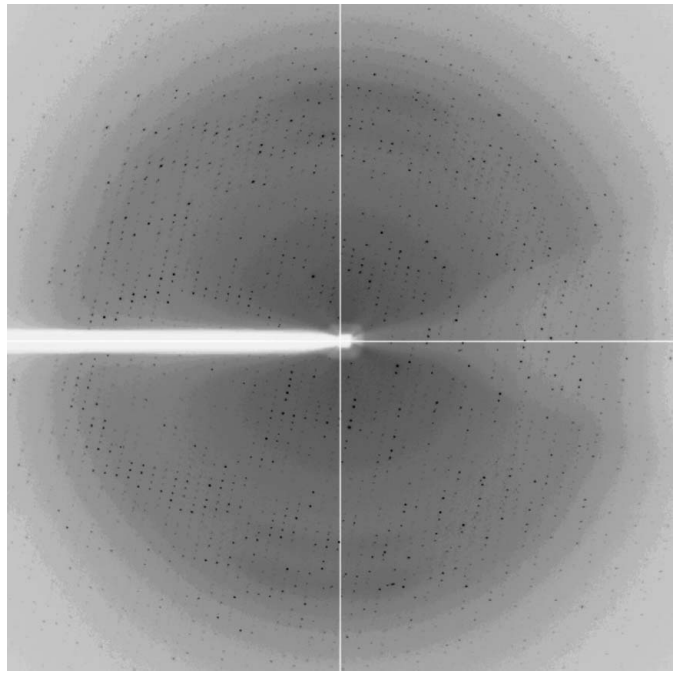


Figure 2
A 1° oscillation X-ray image of TrmFO taken at the 17-ID beamline at the Advanced Photon Source (APS), Argonne, Illinois, USA. The resolution at the edge of the detector is 1.8 Å. The image was taken from the crystal that showed the best diffraction. The data set was collected from a different crystal.

Table 1
X-ray data-collection parameters.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 79.94, b = 92.46, c = 127.20
Resolution range	50.0–2.6
No. of measured reflections	127262
No. of unique reflections	29068
Completeness (%)	97.6
$R_{\rm merge}$ (%)	7.0 (20)
$\langle I/\sigma(I)\rangle$	9.1 (6.7)

2.4. Crystallographic data collection and processing

The crystals were screened for diffraction quality using a Rigaku X-ray source (Rigaku, Japan). A 2.6 Å data set was collected from a thin plate-shaped crystal at the 17-ID beamline at the Advanced Photon Source (APS), Argonne, Illinois, USA. Data collection was performed with an oscillation angle of 1° for each diffraction image and a total oscillation range of 180° (Fig. 2). The exposure time for each image was 5 s. Processing and scaling were accomplished using *HKL*-2000 (Otwinowski & Minor, 1997). The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 79.94, b = 92.46, c = 127.20 Å (Table 1). If two protein molecules are assumed to be present in the asymmetric unit, the Matthews coefficient is 2.35 Å^3 Da^{-1}. This value corresponds to a solvent content of 47.7% (Matthews, 1968). Selenomethionine-labeled protein was also prepared using the method described by Van Duyne *et al.* (1993). It was overexpressed and purified as described above and crystallized under the same conditions as the native crystals. These crystals are awaiting data collection at Argonne National Laboratory.

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