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Crystallization and preliminary X-ray crystallographic analysis of dihydrouridine synthase from *Thermus thermophilus* and its complex with tRNA

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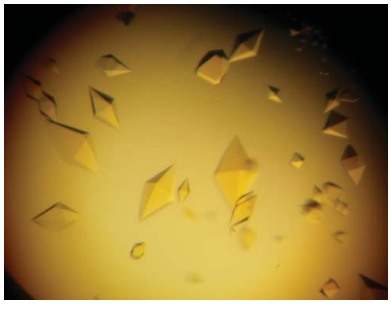
Dihydrouridine synthase (Dus) is responsible for catalyzing dihydrouridine formation in RNA by the reduction of uridine. To elucidate its RNA-recognition mechanism, Dus from *Thermus thermophilus* (*Tth*Dus) and its complex with tRNA were crystallized. Diffraction data sets were collected from crystals of native and selenomethionine-substituted *Tth*Dus to resolutions of 1.70 and 2.30 Å, respectively. These crystals belonged to space group $P1$. Preliminary X-ray crystallographic analysis showed that two molecules of *Tth*Dus were contained in an asymmetric unit. In addition, diffraction data were collected to 3.51 Å resolution from a crystal of selenomethionine-substituted *Tth*Dus in complex with tRNA, which belonged to space group $P4_12_12$. Preliminary structural analysis showed that the asymmetric unit contained two *Tth*Dus–tRNA complexes.

1. Introduction

Post-transcriptional modification is a critical step in the maturation of transfer RNAs (tRNAs). Dihydrouridine modification is found in almost all tRNAs from a wide variety of organisms (Sprinzl *et al.*, 1998). However, little is known about its biochemical roles. It has been proposed that dihydrouridine modification enhances the conformational flexibility of tRNA (Dalluge *et al.*, 1996) and the amount of dihydrouridine in the tRNAs of thermophilic organisms is usually low (Dalluge *et al.*, 1997).

Members of the dihydrouridine synthase (Dus) family of enzymes, which are responsible for catalyzing the reduction of uridine in tRNA, have been identified in *Saccharomyces cerevisiae* (Xing *et al.*, 2004) and *Escherichia coli* (Bishop *et al.*, 2002). The four Dus enzymes from *S. cerevisiae* introduce dihydrouridine modifications at different positions (Xing *et al.*, 2004). Likewise, site-specificity and nonredundant catalytic functions have also been confirmed for the three Dus enzymes from *E. coli* (Bishop *et al.*, 2002).

The crystal structure of Dus from *Thermotoga maritima* (Park *et al.*, 2004) has been reported and important residues for activity (Savage *et al.*, 2006) have been determined for the Dus homologue from *E. coli* by mutational analysis. Kinetic analysis of Dus2 from *S. cerevisiae* showed that other tRNA modifications are required for rapid uracil reduction and that a cysteine residue located at the active site is likely to protonate the uracil ring (Rider *et al.*, 2009). However, the tRNA-recognition and catalytic mechanisms of Dus have not been determined. To clarify them from a structural viewpoint, we attempted to determine the structures of *Tth*Dus and of *Tth*Dus in complex with tRNA. Here, we report the crystallization and preliminary X-ray diffraction analysis of *Tth*Dus and of *Tth*Dus in complex with tRNA.



2. Materials and methods

2.1. Preparation of *Tth*Dus and selenomethionine-substituted *Tth*Dus

A DNA fragment encoding *Tth*Dus with a His_6 tag attached at the C-terminus (350 residues; molecular weight 40 kDa) was cloned into

the *Nde*I and *Kpn*I sites of the CDFDuet-1 vector (Merck, Darmstadt, Germany). The recombinant plasmid was introduced into *E. coli* strain B834 (DE3) by electroporation. The transformed cells were incubated on an LB–agar plate containing 50 mg l^{-1} streptomycin overnight at 310 K. A single colony was inoculated into 100 ml LB preculture containing 50 mg l^{-1} streptomycin and incubated overnight at 310 K with shaking at 150 rev min^{-1}. A 100 ml aliquot of the preculture was transferred into 1 l LB culture containing 50 mg l^{-1} streptomycin and incubated at 310 K until an A_{600} of 0.7 was reached. After adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 m*M*, the culture was incubated at 298 K for an additional 18 h. The cells were collected by centrifugation at 4500*g* for 20 min and resuspended in a sonication buffer consisting of 50 m*M* HEPES pH 7.6, 1 m*M* $MgCl_2$, 500 m*M* KCl, 7 m*M* β-mercaptoethanol, 10% glycerol and 0.1% Triton X-100. The cells were sonicated for 20 min on ice and the cell debris was removed by centrifugation at 40 000*g* for 30 min at 283 K. The supernatant was heat-treated at 343 K for 20 min, followed by centrifugation at 40 000*g* for 1 h. The supernatant was loaded onto an Ni Sepharose 6 Fast Flow column (GE Healthcare, Waukesha, Wisconsin, USA) pre-equilibrated with sonication buffer. After washing with sonication buffer, adsorbed protein was eluted with a 0–0.5 *M* gradient of imidazole in purification buffer (20 m*M* HEPES pH 7.6, 1 m*M* $MgCl_2$, 200 m*M* KCl, 7 m*M* β-mercaptoethanol, 10% glycerol). Fractions containing *Tth*Dus were dialyzed against purification buffer containing 50 m*M* imidazole and then loaded onto a HiTrap Heparin HP column (GE Healthcare). The adsorbed protein was eluted with a 200–1000 m*M* gradient of KCl in purification buffer. The collected fractions were further purified using a HiLoad 26/60 Superdex 200 column (GE Healthcare) pre-equilibrated with purification buffer. The protein concentration was determined using the absorption at a wavelength of 280 nm with a molar extinction coefficient of 24 750 M^{-1} using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Purified native *Tth*Dus was concentrated to 13 mg ml^{-1} (the yield was 1.4 mg per litre of LB culture).

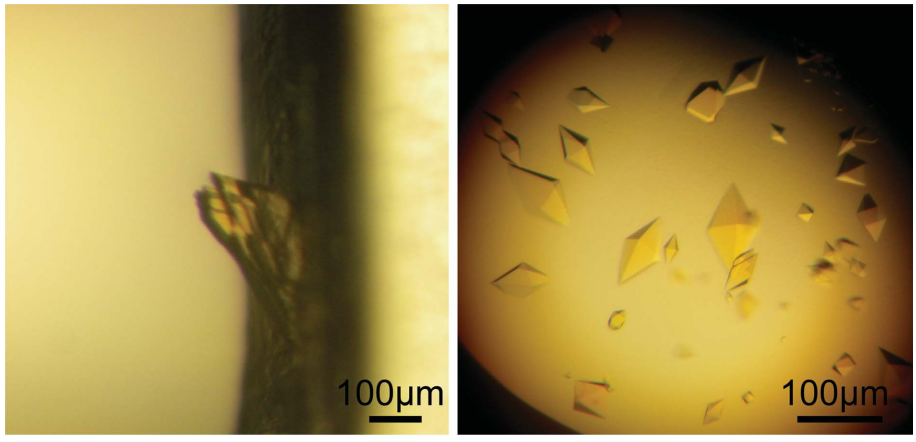
A DNA fragment encoding *Tth*Dus was cloned into the *Nde*I and *Bam*HI sites of the pET28a vector (Merck), in which a His_6 tag was attached at the N-terminus, for expression of selenomethionine-substituted *Tth*Dus (SeMet-*Tth*Dus; 362 residues; molecular weight 41 kDa) in M9 medium supplemented with 1 m*M* selenomethionine by *E. coli* strain B834 (DE3). SeMet-*Tth*Dus was purified by heat-treatment and Ni Sepharose 6 Fast Flow column chromatography (GE Healthcare) followed by dialysis against purification buffer containing 50 m*M* imidazole. The His_6 tag attached at the N-terminus was digested with thrombin (GE Healthcare) overnight at 310 K and was then removed using an Ni Sepharose 6 Fast Flow column. The collected fractions were further purified using a HiTrap Heparin HP column and a HiLoad 26/60 Superdex 200 column. Finally, purified SeMet-*Tth*Dus was concentrated to 15 mg ml^{-1}.

2.2. Preparation of *Tth*Dus in complex with tRNA

The DNA fragment encoding *Tth*Dus was cloned into the *Bam*HI and *Eco*RI sites of the CDFDuet-1 vector (Merck), in which a His_6 tag was attached at the N-terminus (363 residues; molecular weight 41 kDa). The DNA fragment encoding $tRNA^{Phe}$ from *T. thermophilus* (*Tth*-$tRNA^{Phe}$; 76 nucleotides; molecular weight 24 kDa) was first cloned into the *Xba*I and *Bpu*1102I sites of the pET22b vector (Merck). A DNA fragment between the T7 promoter and the T7 terminator of the vector obtained was amplified using KOD-Plus DNA polymerase (Toyobo, Japan). The PCR products were inserted into the *Eco*RI and *Hin*dIII sites of pUC19 (New England Biolabs, Ipswich, Massachusetts, USA). The plasmids for the expression of *Tth*Dus and *Tth*-$tRNA^{Phe}$ were introduced into *E. coli* strain B834 (DE3) by electroporation. The transformed cells were grown in LB medium containing 100 mg l^{-1} ampicillin and 50 mg l^{-1} streptomycin at 310 K until an A_{600} of 0.7 was reached. To induce expression of *Tth*Dus and *Tth*-$tRNA^{Phe}$, IPTG was added to a final concentration of 0.5 m*M* and the culture was incubated at 298 K for an additional 18 h. The purification methods were the same as those used for native *Tth*Dus, except that fractions containing the *Tth*Dus–tRNA complex were collected after purification by Ni Sepharose 6 Fast Flow column chromatography (GE Healthcare). The concentration of *Tth*Dus in the purified *Tth*Dus–tRNA complex was determined with a Quant-iT Protein Assay Kit using a Qubit fluorometer (Invitrogen, Eugene, Oregon, USA). The purified *Tth*Dus–tRNA complex was concentrated to 9.3 mg ml^{-1} in the purification buffer mentioned above. The final yield of the native complex was about 2.5 mg from 1 l LB culture. SeMet-*Tth*Dus in complex with tRNA was prepared by the same method as described above but using M9 medium supplemented with 1 m*M* SeMet.

2.3. Crystallization

Initial screening for crystallization conditions of native *Tth*Dus, SeMet-*Tth*Dus and the native *Tth*Dus–tRNA complex was per-



(*a*) (*b*)

Figure 1
(*a*) Native crystals of *Tth*Dus. (*b*) SeMet-substituted crystals of the *Tth*Dus–tRNA complex.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	SeMet-*Tth*Dus	Native *Tth*Dus	SeMet complex
X-ray source	SPring-8 BL41XU	PF NE3A	PF BL-5A
Wavelength (Å)	0.9792	1.0000	0.9788
Space group	*P*1	*P*1	$P4_12_12$
Unit-cell parameters (Å, °)	a = 43.6, b = 60.3, c = 67.3, α = 75.7, β = 87.7, γ = 70.5	a = 42.0, b = 60.2, c = 67.0, α = 76.2, β = 88.7, γ = 70.7	$a = b$ = 118.9, c = 319.6
Resolution (Å)	50.00–2.30 (2.38–2.30)	19.77–1.70 (1.79–1.70)	48.60–3.51 (3.70–3.51)
Unique reflections	26878 (2590)	63586 (8907)	29454 (4077)
Multiplicity	7.6 (6.5)	3.9 (3.7)	13.2 (11.8)
Completeness (%)	97.3 (94.0)	95.6 (91.7)	99.5 (97.1)
$\langle I/\sigma(I)\rangle$	16.4 (2.3)	17.9 (2.8)	17.1 (4.6)
$R_{\rm merge}$	0.098 (0.403)	0.046 (0.457)	0.116 (0.531)
No. of molecules in asymmetric unit	2	2	2
$V_{\rm M}$ (Å^3 Da^{-1})	2.1	2.0	4.3
Solvent content (%)	41.4	37.3	71.7

formed using NeXtal Classics Suite, NeXtal JCSG$^+$ Suite, NeXtal AmSO4 Suite, NeXtal Nucleix Suite, NeXtal ProComplex Suite and NeXtal JCSG Core Suite I–IV (Qiagen, Hilden, Germany) by the sitting-drop vapour-diffusion method, in which 1 µl sample and 1 µl reservoir solution were mixed and equilibrated against 100 µl reservoir solution in 96-well CrystalQuick plates (Greiner Bio-One, Kremsmuenster, Austria) at 293 K. After initial crystals had been obtained, the conditions were optimized by changing the pH value of the reservoir solution and the concentration of the precipitant (except for native *Tth*Dus). Well diffracting crystals of native *Tth*Dus were obtained from reservoir solution consisting of 0.1 *M* HEPES pH 7.0, 0.1 *M* $MgCl_2$ and 15% PEG 4000 (Fig. 1*a*), while crystals of SeMet-*Tth*Dus were obtained from reservoir solution consisting of 0.1 *M* trisodium citrate pH 5.6, 16% PEG 4000 and 12.5% 2-propanol. The crystallization conditions of the SeMet-*Tth*Dus–tRNA complex were optimized based on those of the native *Tth*Dus–tRNA complex. Finally, a crystal of the SeMet-*Tth*Dus–tRNA complex suitable for data collection was obtained from reservoir solution consisting of 0.1 *M* Tris–HCl pH 7.0–8.0, 2.4 *M* ammonium sulfate and 20% glycerol (Fig. 1*b*).

2.4. X-ray data collection

All data collections were carried out at 100 K after soaking the crystals in crystallization buffer containing 20% glycerol. Single-wavelength anomalous diffraction (SAD) data were collected from SeMet crystals to a resolution of 2.3 Å on beamline BL41XU of SPring-8 (Harima, Japan). The wavelength of 0.9792 Å for data collection was determined based on the fluorescence spectrum of the Se *K* absorption edge (Rice *et al.*, 2000). The data were indexed, integrated and scaled with *HKL*-2000 (Otwinowski & Minor, 1997). The crystal of SeMet-*Tth*Dus belonged to space group *P*1, with unit-cell parameters a = 43.6, b = 60.3, c = 67.3 Å, α = 75.7, β = 87.7, γ = 70.5°. A data set was collected from a native crystal to a resolution of 1.7 Å on beamline NE3A of the Photon Factory (PF; Tsukuba, Japan). The data were indexed and integrated with *XDS* (Kabsch, 2010). The crystal of native *Tth*Dus belonged to space group *P*1, with unit-cell parameters a = 42.0, b = 60.2, c = 67.0 Å, α = 76.2, β = 88.7, γ = 70.7°.

A SAD data set was collected from a crystal of the SeMet-*Tth*Dus–tRNA complex to 3.51 Å resolution on beamline BL-5A of PF. The wavelength of 0.9788 Å for data collection was determined based on the fluorescence spectrum of the Se *K* absorption edge (Rice *et al.*, 2000). The diffraction data were integrated with *XDS* (Kabsch, 2010) and scaled with *SCALA* (Evans, 1997). The crystal of the SeMet-*Tth*Dus–tRNA complex belonged to space group $P4_12_12$, with unit-cell parameters $a = b$ = 118.9, c = 319.6 Å. Data-collection statistics are summarized in Table 1.

3. Results and discussion

The purified *Tth*Dus had absorption peaks at around 370 and 450 nm which are charactersitic of oxidized flavin mononucleotide (FMN), suggesting that *Tth*Dus possesses an FMN cofactor. Although we attempted to determine the structure by the molecular-replacement method using the structure of Dus from *Thermotoga maritima* (PDB entry 1vhn; Park *et al.*, 2004) as a search model, no obvious solution was obtained. Therefore, we prepared crystals of SeMet-*Tth*Dus and determined the structure by the SAD method. Ten Se sites were determined with *SHELXD* (Sheldrick, 2008, 2010). The sites were refined and primary phases with an overall figure of merit (FOM) of 0.259 were calculated with *SOLVE* (Terwilliger & Berendzen, 1999). Phase improvement and automatic model building were performed using *RESOLVE* (Terwilliger, 2000), which increased the overall FOM to 0.586. Structural refinement using native data at 1.7 Å resolution is currently in progress.

To obtain a stable complex of *Tth*Dus and tRNA, we co-expressed these molecules in *E. coli*. $tRNA^{Phe}$ from *T. thermophilus* was chosen as the targeted tRNA for complexation with *Tth*Dus since its modifications and three-dimensional structures of $tRNA^{Phe}$ from other organisms have been reported. *Tth*Dus purified as a complex with tRNA migrated significantly more slowly than *Tth*Dus on SDS–PAGE (lanes 1 and 2 in Fig. 2*a*), in which several bands were confirmed. The band of the complex treated with RNase A moved to a position similar to that of *Tth*Dus (lane 3 of Fig. 2*a*), indicating that the purified *Tth*Dus formed a complex with RNA. The molecular weight of the uppermost band of the complex was calculated to be approximately 67 kDa from a comparison with molecular markers. This was almost consistent with the molecular weight of 65 kDa calculated from the sequence of the complex: *i.e.* 41 kDa for *Tth*Dus and 24 kDa for *Tth*-$tRNA^{Phe}$. These results suggest that the purified complex was the *Tth*Dus–tRNA complex and that it is so outstandingly stable that it cannot be dissociated even on SDS–PAGE. The structure of the complex will be necessary in order to address its stable character. The purified complex was further confirmed by native PAGE (Fig. 2*b*). Although there were several bands on SDS–PAGE, only one band was confirmed on native PAGE. The multiple bands of the complex on SDS–PAGE suggest the existence of partially denatured *Tth*Dus–tRNA complex in the presence of SDS.

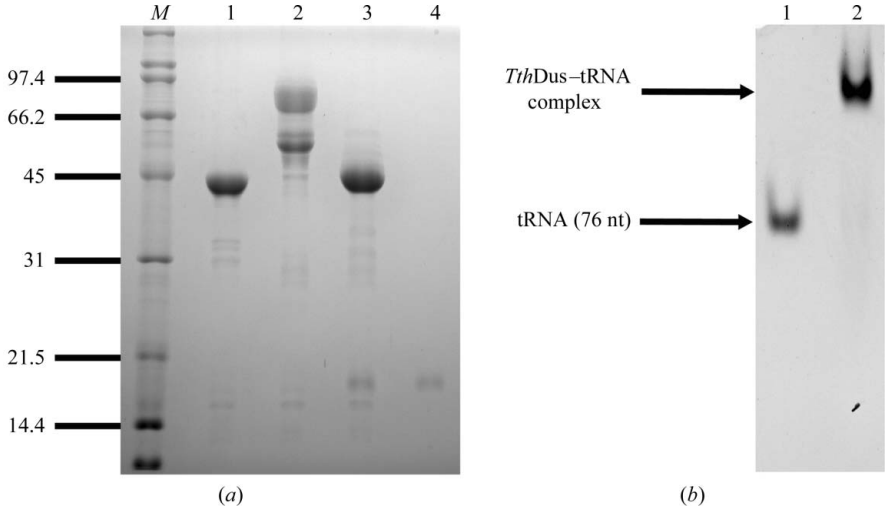


Figure 2
The results of polyacrylamide gel electrophoresis. (*a*) SDS–PAGE of the purified *Tth*Dus protein (lane 1), *Tth*Dus–tRNA complex (lane 2), RNase A-treated *Tth*Dus–tRNA complex (lane 3) and RNase A (lane 4) stained with Coomassie Brilliant Blue. Lane *M* contains molecular-weight markers (labelled in kDa). (*b*) Native PAGE of tRNA with 76 nucleotides (lane 1) and the purified *Tth*Dus–tRNA complex (lane 2) stained with ethidium bromide.

*Tth*Dus with a His_6 tag attached at either the C-terminus or the N-terminus was used in crystallization of the *Tth*Dus–tRNA complex, but crystals of the complex were only obtained using N-terminally His_6-tagged *Tth*Dus. The crystal of the SeMet-*Tth*Dus–tRNA complex diffracted to a resolution of 3.51 Å. We attempted to determine the crystal structure by the molecular-replacement method with *MOLREP* (Vagin & Teplyakov, 2010) using the structure of *Tth*Dus as a search model. Although two definite solutions corresponding to the molecules in an asymmetric unit were obtained with translation-function contrast values of 14.19 and 12.37, respectively, we could not construct the model of tRNA because of the unclear electron density for the bound tRNA. Therefore, we attempted to determine the structure using the SAD method. *SHELXD* (Sheldrick, 2008, 2010) determined ten of the 11 Se atoms, which were completely consistent with the Met sites in *Tth*Dus determined by the molecular-replacement method. The sites were refined and initial phases with an overall FOM of 0.649 were calculated with *SOLVE* and *RESOLVE* (Terwilliger & Berendzen, 1999). The calculated initial electron density showed that the asymmetric unit contains two *Tth*Dus–tRNA complexes, which is consistent with the result of molecular replacement. Substantial electron density corresponding to tRNA was observed around the protein in the initial map. Phase improvement and model building of the complex are currently under way.

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