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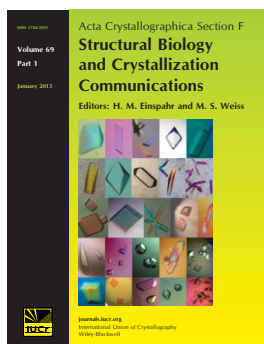
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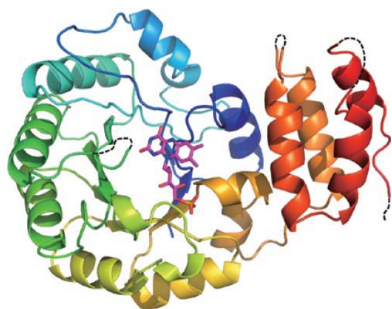
PDB Reference: EcoDusC, 3w9z

Structure of dihydrouridine synthase C (DusC) from *Escherichia coli*

Dihydrouridine (D) is one of the most widely conserved tRNA modifications. Dihydrouridine synthase (Dus) is responsible for introducing D modifications into RNA by the reduction of uridine. Recently, a unique substrate-recognition mechanism using a small adapter molecule has been proposed for *Thermus thermophilus* Dus (*Tth*DusC). To acquire insight regarding its substrate-recognition mechanism, the crystal structure of DusC from *Escherichia coli* (*Eco*DusC) was determined at 2.1 Å resolution. *Eco*DusC was shown to be composed of two domains: an N-terminal catalytic domain and a C-terminal tRNA-binding domain. An L-shaped electron density surrounded by highly conserved residues was found in the active site, as observed for *Tth*Dus. Structure comparison with *Tth*Dus indicated that the N-terminal region has a similar structure, whereas the C-terminal domain has marked differences in its relative orientation to the N-terminal domain as well as in its own structure. These observations suggested that Dus proteins adopt a common substrate-recognition mechanism using an adapter molecule, whereas the manner of tRNA binding is diverse.

1. Introduction

Post-transcriptional modification is an indispensable step in the maturation of noncoding RNA. To date, more than 100 modifications have been reported of transfer RNA (tRNA). These modifications play various important cellular roles, such as in stabilization of the tRNA structure, in translational fidelity control and in metabolic responses to the environment. Dihydrouridine (D) is one of the most widely conserved tRNA modifications found in bacteria, some archaea and eukaryotic organisms (Sprinzl *et al.*, 1998), and is mostly found in the D-loop of tRNAs, which is named after it. Individual tRNAs have varying numbers of D modifications. It has been suggested that D modification destabilizes the C3'-*endo*-form ribose conformation associated with base stacking and consequently increases the flexibility of the tertiary structure of tRNA (Dalluge *et al.*, 1996). D is formed by the reduction of the double bond between positions 5 and 6 of the uridine base by dihydrouridine synthase (Dus). Genes encoding four Dus isozymes have been found in the genome of *Saccharomyces cerevisiae* and their modification sites have been identified (Xing *et al.*, 2004). Site specificity and nonredundant catalytic functions have been confirmed for three Dus enzymes from *Escherichia coli* (Bishop *et al.*, 2002). In a previous study, a unique substrate-recognition mechanism in which basic residues located around the active site recognize uridine bases indirectly through a small adapter molecule was proposed based on structural analysis of *Thermus thermophilus* Dus (*Tth*Dus) in complex with tRNA and mutational analyses (Yu *et al.*, 2011). However, the adapter molecule has yet to be identified. Therefore, information regarding the adapter molecule is important to gain further understanding of the reaction mechanism. Furthermore, relevance of D modification to cancer has been reported, suggesting that elucidation of the mechanism underlying the introduction of D modifications will have important medical implications (Kato *et al.*, 2005).



In the present study, to gain insight into the substrate base- and/or tRNA-recognition mechanism, the crystal structure of DusC from *E. coli* (*EcoDusC*) was determined at a resolution of 2.1 Å. The revealed structure suggests that an adapter molecule is commonly used by Dus enzymes. The structural differences between *EcoDusC* and *TthDus* are also discussed.

2. Materials and methods

2.1. Preparation of *EcoDusC* and selenomethionine-substituted *EcoDusC*

A DNA fragment encoding *EcoDusC* was cloned into the *NdeI* and *XhoI* sites of the pET-26b(+) vector (Merck, Darmstadt, Germany). In the resultant plasmid, the His tag was attached at the C-terminus. The recombinant plasmid was introduced into *E. coli* strain B834 (DE3). The transformed cells were incubated on LB-agar plates containing 25 mg l⁻¹ kanamycin overnight at 310 K. A single colony was inoculated into 100 ml LB preculture containing 25 mg l⁻¹ kanamycin and was incubated overnight at 310 K with shaking at 130 rev min⁻¹. A 100 ml aliquot of the preculture was transferred into 1 l LB culture containing 25 mg l⁻¹ kanamycin and incubated at 310 K until an *A*₆₀₀ of 0.6 was reached. After adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, the culture was incubated at 297 K for an additional 18 h. The cells were collected by centrifugation at 4500g for 15 min and were resuspended in sonication buffer consisting of 50 mM HEPES pH 7.6, 1 mM magnesium chloride, 500 mM potassium chloride, 7 mM β-mercaptoethanol, 10% glycerol. The cells were sonicated for 20 min on ice and the cell debris was removed by centrifugation at 40 000g for 30 min at 283 K. The supernatant was filtrated and loaded onto a HisTrap HP column (GE Healthcare, Waukesha, Wisconsin, USA) pre-equilibrated with sonication buffer. After washing with sonication buffer, the adsorbed protein was eluted with a 0–0.5 M gradient of imidazole in purification buffer (20 mM HEPES pH 7.6, 1 mM magnesium chloride, 200 mM potassium chloride, 7 mM β-mercaptoethanol, 10% glycerol). Fractions containing *EcoDusC* were dialyzed against purification buffer containing 50 mM imidazole and loaded onto a HiTrap Heparin HP column (GE Healthcare). The adsorbed protein was eluted with a 100–800 mM 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 from the absorption at a wavelength of 280 nm with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using a molar extinction coefficient of 56 104 M⁻¹. Purified native *EcoDusC* was concentrated to 9.63 mg ml⁻¹ (the yield was 8 mg per litre of LB culture). The selenomethionine derivative (*SeMet-EcoDusC*) was obtained by the same method as described above except that M9 medium supplemented with 1 mM selenomethionine was used instead of LB medium.

2.2. Crystallization

Initial screening for crystallization conditions of native *EcoDusC* was performed using The Classics, Classics II, MPD, PEGs, PEGs II and ProComplex Suites and JCSG Core Suites I–IV (Qiagen, Hilden, Germany) by the sitting-drop vapour-diffusion method, in which 0.2 µl sample and 0.2 µl reservoir solution were mixed and equilibrated against 50 µl reservoir solution in 96-well MRC plates 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. Well diffracting crystals of native

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Native <i>EcoDusC</i>	<i>SeMet-EcoDusC</i>
Data collection		
Wavelength (Å)	1.00000	0.97914
Crystal-to-detector distance (mm)	189.80	269.46
Exposure time (s)	2	1
No. of frames	180	180
Unit-cell parameters (Å)	$a = b = 93.3, c = 115.5$	$a = b = 94.5, c = 116.6$
Space group	$P4_32_12$	$P4_32_12$
Resolution (Å)	50.00–2.10 (2.14–2.10)	50.00–2.80 (2.85–2.80)
Unique reflections	30448	13445
Multiplicity	15.3 (14.4)	13.2 (9.7)
Completeness (%)	99.9 (100)	99.2 (92.6)
$\langle I/\sigma(I) \rangle$	71.72 (5.30)	58.79 (2.71)
R_{merge}	0.063 (0.703)	0.096 (0.649)
Refinement		
Resolution (Å)	50.00–2.10	
No. of reflections	29181	
$R_{\text{work}}/R_{\text{free}}$ (%)	22.1/23.5	
Model composition		
No. of protein atoms/waters	2335/112	
Stereochemistry		
R.m.s.d., bond lengths (Å)	0.0078	
R.m.s.d., bond angles (°)	1.172	
Ramachandran plot, residues in		
Favoured regions	267 [95.04%]	
Allowed regions	15 [5.32%]	
Outlier regions	0 [0%]	

EcoDusC were obtained using a reservoir solution consisting of 0.1 M Tris pH 7.9, 0.2 M sodium acetate, 12% PEG 4000, while crystals of *SeMet-EcoDusC* were obtained using a reservoir solution consisting of 0.1 M imidazole pH 8.0, 15% (v/v) 2-propanol, 20% (v/v) glycerol.

2.3. X-ray data collection

Data collection was 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-EcoDusC* crystals to a resolution of 2.8 Å on beamline BL41XU at SPring-8, Harima, Japan. The wavelength of 0.9791 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 *SeMet-EcoDusC* crystal belonged to space group $P4_32_12$, with unit-cell parameters $a = b = 94.5, c = 116.6$ Å. A data set was collected from a native crystal to a resolution of 2.1 Å on beamline BL5A at the Photon Factory, Tsukuba, Japan. The data were indexed, integrated and scaled with *HKL-2000*. The crystal of native *EcoDusC* belonged to space group $P4_32_12$, with unit-cell parameters $a = b = 93.3, c = 115.5$ Å. Data-collection statistics are summarized in Table 1.

2.4. Structure determination and refinement

The structure of *EcoDusC* was determined by the SAD method. The selenium sites were determined with *SHELX* (Sheldrick, 2010). The programs *phenix.autosol* (Terwilliger *et al.*, 2009) and *phenix.autobuild* (Terwilliger *et al.*, 2008) improved the initial phase and the constructed structure model, respectively. One molecule of *EcoDusC* was located in the asymmetric unit. The structure was refined using the native data at 2.1 Å resolution. To monitor the refinement, a random 5% subset of all reflections was set aside for calculation of the R_{free} factor. After several cycles of refinement with the program *phenix.refine* (Adams *et al.*, 2010) and manual fitting with *Coot* (Emsley & Cowtan, 2004), the crystallographic R_{work} and R_{free} factors converged to 22.1 and 23.5%, respectively.

3. Results and discussion

3.1. Overall structure

Although *EcoDusC* has a sequence identity of 16% to *TthDus*, no obvious solution was obtained in molecular replacement using *TthDus* as a search probe. Although the crystal structure of *DusC* from *E. coli* has been deposited in the Protein Data Bank with PDB codes 4bf9 and 4bfa (R. T. Byrne, F. Whelan, A. Konevega, N. Aziz, M. Rodnina & A. A. Antson, unpublished work), these depositions have yet to be released. Therefore, the structure was determined by the Se-SAD method. Five of the six selenium sites were determined by *SHELX*. The initial structure model consisting of 206 residues was

automatically constructed by *phenix.autosol* and *phenix.autobuild*. After refinement, atomic models of 282 of the 321 residues, one FMN molecule and 112 water molecules were constructed. Owing to poor electron density, the regions Pro99–Thr111, Lys261–Tyr268, Lys297–Asn299 and Leu315–His322 were not modelled.

EcoDusC consists of two domains: an N-terminal catalytic domain with a TIM-barrel fold and a C-terminal tRNA-binding domain composed of four helices (Fig. 1*a*). The N-terminal catalytic domain consists of eight helices and eight strands. The eight strands form a parallel β -barrel at the centre of the catalytic domain, surrounded by eight helices. As observed for *TthDus*, a clear electron-density map of FMN was observed at the centre of the N-terminal catalytic domain.

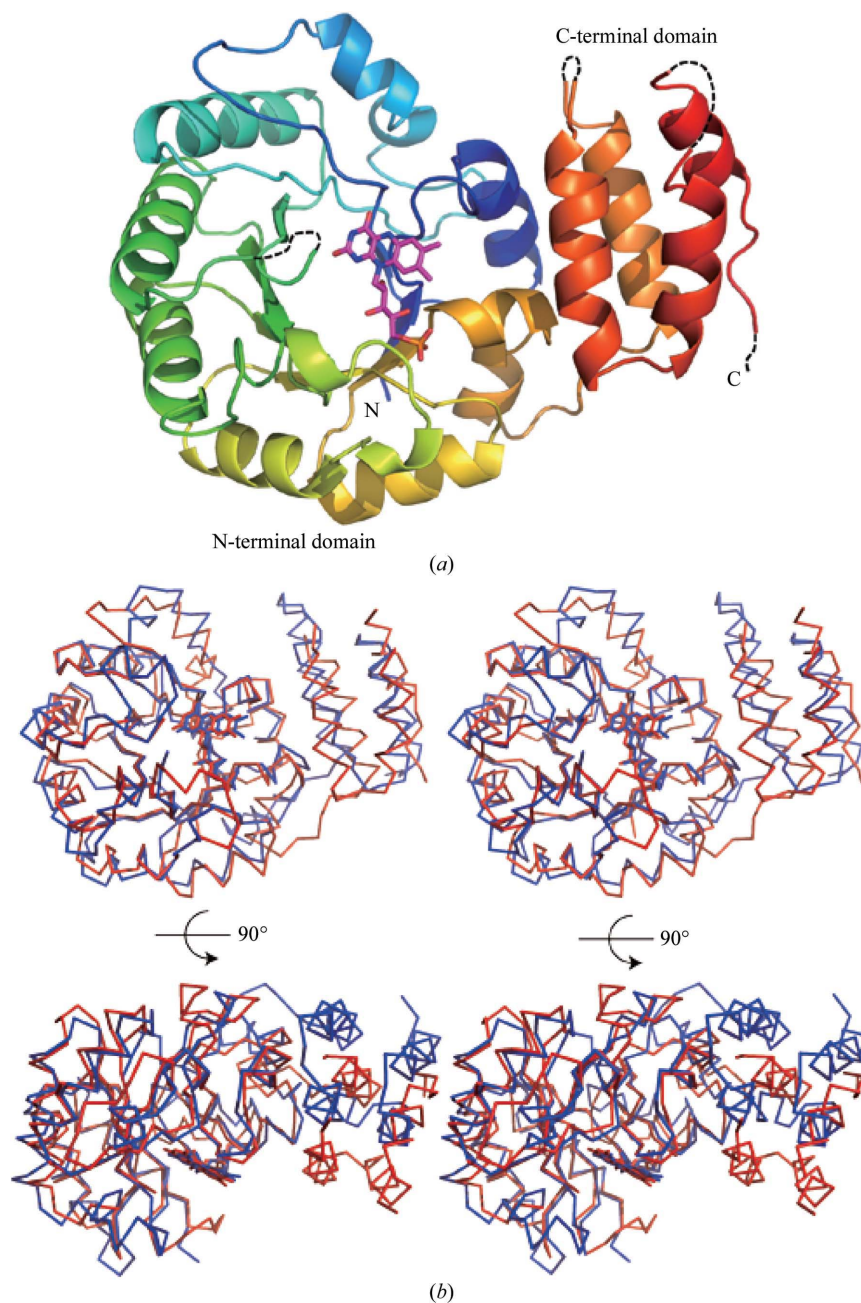


Figure 1

Overall structure of *EcoDusC*. (*a*) Ribbon diagram of *EcoDusC*. The ribbon model is coloured according to the sequence from blue at the N-terminus to red at the C-terminus. FMN is shown as sticks. The dotted lines indicate disordered regions. (*b*) Stereo diagram of the structure of *EcoDusC* (red) superposed onto that of *TthDus* (blue). The N-terminal domain was superposed.

The C-terminal tRNA-binding domain is formed by four parallel helices. This domain is commonly found in Dus-family proteins and is responsible for binding to the substrate tRNA.

The N-terminal catalytic domain of *EcoDusC* superposed well on the same region of *TthDus* (r.m.s.d. of 1.39 Å for 240 C α atoms), whereas the C-terminal domain did not (Fig. 1*b*). The domains of both Dus proteins were composed of four α -helices (r.m.s.d. of 2.50 Å for 70 C α atoms). Furthermore, the relative location of the C-terminal domain and the N-terminal domain differed between *EcoDusC* and *TthDus*, suggesting that the catalytic mechanism using the N-terminal domain is similar but the mechanism of tRNA recognition using the C-terminal domain differs between these two Dus proteins.

3.2. Active site of *EcoDusC*

We proposed previously that *TthDus* recognizes substrate uridine bases using an adapter molecule captured by highly conserved basic residues in the active site (Yu *et al.*, 2011). In the *EcoDusC* structure, an obvious L-shaped electron density was observed at a position corresponding to the cofactor-binding site of the *TthDus*-tRNA complex (Fig. 2). Furthermore, the residues that participate in binding to the adapter molecule in *TthDus* are all structurally conserved in *EcoDusC* (*i.e.* Asn95, Lys139, Arg141, His168 and Arg170). In addition, the catalytic cysteine residue (Cys98) was located at the same position as in *TthDus*. These residues are completely conserved among Dus-family proteins. These structural similarities to *TthDus* suggest that the catalytic mechanism proposed for *TthDus* is common to other Dus enzymes, although the molecule that is captured at the active site has yet to be identified. This was further supported by the observation that substitution of the residues surrounding the electron density resulted in a lack of tRNA-binding activity (Yu *et al.*, 2011). The as yet unidentified adapter molecule is expected to interact with the side of the uridine base opposite to the double bond reduced by Dus. It may act to position the substrate base at the correct position and/or to activate the reaction by pushing or pulling delocalized electrons of the uridine base.

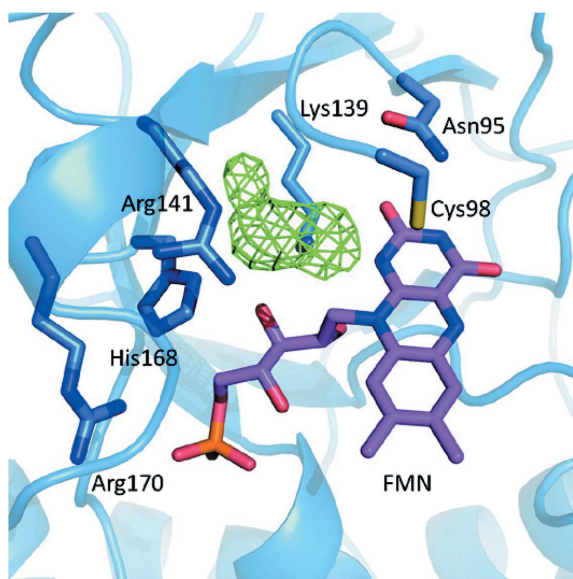


Figure 2
Active-site structure. Electron density ($F_o - F_c$ map) of the adapter molecule is shown as a green mesh (contoured at 2.5σ). Conserved basic residues surrounding the adapter are also shown.

3.3. tRNA recognition

In the *TthDus*-tRNA complex structure, tRNA was captured in the negatively charged cavity between the N-terminal and C-terminal domains. Structure comparison of *EcoDusC* with *TthDus* showed that the relative orientations of the N-terminal and C-terminal domains differ between these two Dus proteins and that the structure of the C-terminal domain itself is also different. These observations suggest that *EcoDusC* recognizes tRNA in a different manner from *TthDus*. *TthDus* recognizes the tRNA shoulder region, which causes flipping of the target uridine base (U20; Yu *et al.*, 2011). Although the target base of *EcoDusC* has not been identified, these structural dissimilarities suggest that *EcoDusC* would recognize tRNA from a different direction from *TthDus*. The genome of *E. coli* carries three *dus* genes: *dusA*, *dusB* and *dusC*. These three Dus proteins would recognize tRNA in different orientations to introduce D modification at different sites. The degree of sequence similarity of the C-terminal domain is lower than that of the N-terminal domain, suggesting that each Dus protein adopts its own manner of tRNA recognition.

In the structure of *TthDus*, a flexible loop located above the active site (Pro94–Cys106) forms interactions with tRNA (Yu *et al.*, 2011). In the structure of *EcoDusC*, the adjacent loop above the active site (Pro99–Thr111) was disordered instead of this loop, suggesting that a different loop is used for tRNA recognition in *TthDus* and *EcoDusC*. This would be owing to the difference in the orientation of tRNA to place the substrate base into the active site correctly.

4. Conclusion

In the present study, we determined the crystal structure of *EcoDusC* at a resolution of 2.1 Å by the Se-SAD method. In the revealed structure an L-shaped electron density was located at the position corresponding to the adapter-binding site of *TthDus*, and the residues recognizing the adapter were all structurally conserved in *EcoDusC*. In contrast, the C-terminal domain showed obvious differences in its relative orientation to the N-terminal domain as well as in its own structure. These structural similarities and dissimilarities suggest that Dus proteins may adopt a common substrate-recognition mechanism using an adapter molecule in the active site, whereas the manner of tRNA binding of *EcoDusC* would differ from that of *TthDus*. Further studies are currently under way in our laboratory to identify the adapter molecule in order to gain further insight into the mechanism underlying D modification. Carboxy-S-adenosyl-L-methionine has recently been identified as a novel cofactor molecule for tRNA-modification enzymes based on high-resolution crystal structures (Byrne *et al.*, 2013; Kim *et al.*, 2013). Determining the high-resolution structure of Dus enzymes may be helpful in identifying the adapter molecule.

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