

Sam Griffiths, Robert T. Byrne,‡
Alfred A. Antson and Fiona
Whelan*

York Structural Biology Laboratory,
Department of Chemistry, The University of
York, Heslington, England

‡ Current address: Division of Structural Biology
Laboratory, Institute of Cancer Research,
Chester Beatty Laboratories, London, England.

Correspondence e-mail:
fiona.whelan@york.ac.uk

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Crystallization and preliminary X-ray crystallographic analysis of the catalytic domain of human dihydrouridine synthase

Dihydrouridine synthases catalyse the reduction of uridine to dihydrouridine in the D-loop and variable loop of tRNA. The human dihydrouridine synthase *HsDus2L* has been implicated in the development of pulmonary carcinogenesis. Here, the purification, crystallization and preliminary X-ray characterization of the *HsDus2L* catalytic domain are reported. The crystals belonged to space group $P2_1$ and contained a single molecule of *HsDus2L* in the asymmetric unit. A complete data set was collected to 1.9 Å resolution using synchrotron radiation.

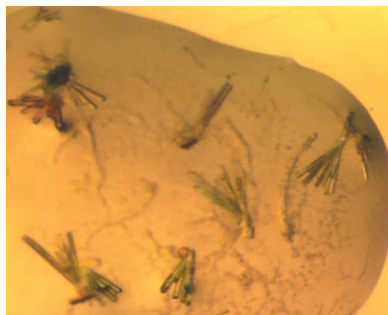
1. Introduction

Post-transcriptional modifications influence the three-dimensional structure of tRNA, rendering regions either more flexible or more rigid in order to modulate and reinforce the structure of the core (Motorin & Helm, 2010). Dihydrouridine synthase (Dus) enzymes principally target the D-loop of tRNA, named for incorporation of dihydrouridine (D), which is understood to increase regional flexibility (Fig. 1*a*). Residues within the D-loop are involved in conserved tertiary base pairs which stabilize the fold of tRNA (Shi & Moore, 2000). Psychrophilic bacteria living at temperatures of 268–285 K possess 40–70% more D than the mesophilic *Escherichia coli*. This modification is almost absent from hyperthermophilic bacterial tRNAs (Dalluge *et al.*, 1997).

Little is known of the function of the human Dus2-like (*HsDus2L*) enzyme. Increased incorporation of D in tRNA^{Phe} has been identified in malignant tissues (Kuchino & Borek, 1978). *HsDus2L* expression is upregulated in non-small-cell lung carcinoma (NSCLC), potentiates hyperproliferation and correlates with a poorer prognosis (Kato *et al.*, 2005). *HsDus2L* is known to interact with the apoptotic regulator dsRNA-activated protein kinase PKR (Mittelstadt *et al.*, 2008).

In *Saccharomyces cerevisiae* Dus2p (*ScDus2p*), D-loop modification has been characterized at position 20 and other sites (Fig. 1*a*; Xing *et al.*, 2004). *HsDus2L* has moderate sequence identity to *ScDus2p* and low identity to two structurally characterized enzymes (Table 1). The first crystal structure of a Dus enzyme, that of TM0096 from *Thermotoga maritima* (*TmDUS*; PDB entry 1vhn; Park *et al.*, 2004), identified a flavin mononucleotide (FMN) cofactor binding in the TIM barrel. FMN is used as a cofactor to perform an NADPH-dependent redox cycle in which the oxidative half-reaction reduces target uracil (Rider *et al.*, 2009). The crystal structure of *Thermus thermophilus* Dus–tRNA (*TthDus*; PDB entry 3b0p) identified the mechanism of specific modification (U20) (PDB entries 3b0u and 3b0v; Yu *et al.*, 2011). Binding involves recognition at the nexus of D-loops and T-loops of folded tRNA. Given that D is more important for tRNA flexibility in psychrophiles and mesophiles, a complete understanding of the panoply of Dus enzymes awaits structural characterization of these groups.

The low sequence identity of the *HsDus2L* Dus domain to *TmDus* and *TthDus* suggests that while the overall fold may be moderately conserved, the C-terminal α -helical domain (253–340) could vary substantially (Table 1). Hence, the structure of *HsDus2L* should



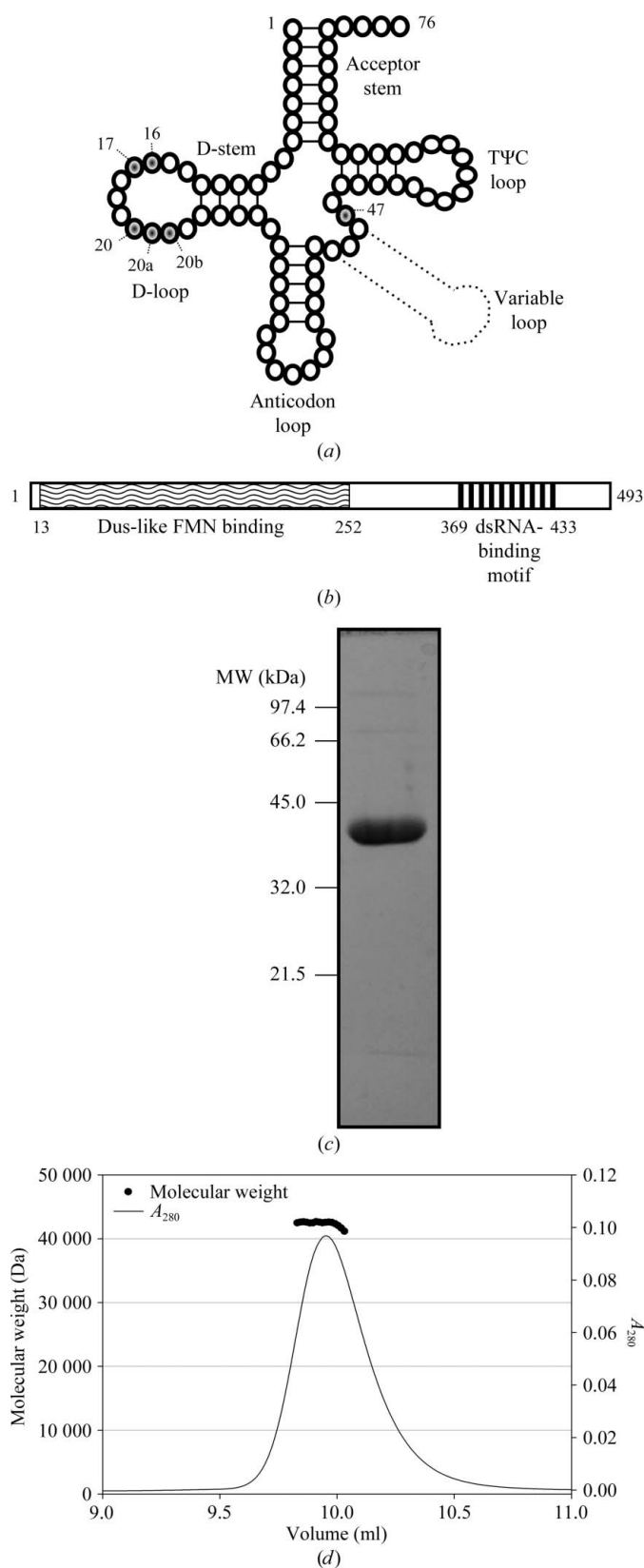


Figure 1
 Secondary structure of tRNA, domain topology of *HsDus2L*, and SDS-PAGE and SEC-MALLS of *H₆-HsDus2L* 1–340. (a) Secondary structure of tRNA with D target sites (U16, 17, 20, 20a, 20b and 47) shaded. (b) Domain topology of *HsDus2L*. Lines representing expression constructs are annotated with the results of expression tests. (c) SDS-PAGE and (d) SEC-MALLS analysis of SEC-purified *H₆-HsDus2L* 1–340.

Table 1

Sequence identity of *ScDus2p*, *TthDus* and *TmDus* to *HsDus2L* (PSIBLAST; Altschul *et al.*, 1997).

Protein	Region	<i>HsDus2L</i>	Identity (%)
<i>ScDus2p</i>	2–301	8–289	39
<i>TthDus</i>	5–237	13–254	22
<i>TmDus</i>	4–248	13–270	23

generate novel mechanistic details for eukaryotic Dus enzymes. The structure of the enzymatic domain will be crucial to address the structure-based design of specific inhibitors of D incorporation as potential NSCLC therapeutics. Here, we describe the purification and crystallization of the Dus domain of *Dus2L* from *Homo sapiens*.

2. Methods and materials

2.1. Cloning

Bioinformatic analysis with *JPred* was utilized to predict secondary structure (Cole *et al.*, 2008). Three constructs were designed based on predicted domain boundaries (Fig. 1*b*). The cDNA of *HsDus2L* was accessed through the DNASU plasmid repository (clone HsCD00303989; Cormier *et al.*, 2011). The coding sequences (accession code AAH06527) were PCR-amplified, digested and ligated into the *NheI/XhoI* sites of the pET28a vector, generating an N-terminal fusion with a His₆ tag/thrombin cleavage site.

2.2. Overexpression and purification

Clones were transformed into *Escherichia coli* BL21 (DE3) pLysS competent cells. An LB culture containing 30 $\mu\text{g ml}^{-1}$ kanamycin and 40 $\mu\text{g ml}^{-1}$ chloramphenicol was inoculated and incubated at 310 K to an OD₆₀₀ of 0.6. Expression was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside followed by incubation at 290 K for 20 h. The cells were harvested and resuspended in 20 mM Tris pH 8, 500 mM NaCl, 20 mM imidazole, 2 mM DTT, sonicated for 3 min and the lysate was cleared by centrifugation at 40 000g for 1 h at 277 K. The lysate was loaded onto a charged HisTrap HP column (GE Healthcare) and protein was eluted using an imidazole gradient (20–500 mM, 20 column volumes). The protein was concentrated using an Amicon centrifugal filter (Millipore). The protein concentrate was diluted in Tris/DTT to an NaCl concentration of 50 mM, loaded onto a pre-equilibrated MonoQ 5/50 GL column (GE Healthcare) and eluted with an NaCl gradient (50 mM–1 M, 20 column volumes). *H₆-HsDus2L* 1–340 (molar mass 40 326 Da) was purified by size-exclusion chromatography (SEC) as described below. The protein was digested using thrombin and incubated at 277 K for 16 h. Untagged *HsDus2L* 1–340 (molar mass 38 605 Da) was separated from uncleaved material by passage through a charged HisTrap HP column. Cleaved and uncleaved *HsDus2L* 1–340 were concentrated and purified by SEC using a Superdex 75 HR 10/30 column (GE Healthcare) equilibrated in 20 mM Tris pH 8, 100 mM NaCl, 5 mM DTT (Fig. 1*c*). The protein concentration was determined from the absorbance at 280 nm using molar extinction coefficients of 21 890 $\text{M}^{-1} \text{cm}^{-1}$ (untagged) and 22 515 $\text{M}^{-1} \text{cm}^{-1}$ (hexahistidine tagged). The tagged and untagged proteins were concentrated to 10 mg ml⁻¹. *H₆-HsDus2L* 1–340 (2.5 mg ml⁻¹) was analysed by SEC multi-angle laser light scattering (SEC-MALLS) using a BioSep-SEC-s3000 column (Phenomenex), a Dawn HELEOS-II 18-angle light-scattering detector and an Optilab rEX refractive-index monitor (Wyatt). Purified tagged protein was characterized using matrix-

Table 2

 Data-collection statistics for *HsDus2L*.

Values in parentheses are for the highest resolution shell.

X-ray source	Beamline I04, Diamond
Wavelength (Å)	0.9795
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 46.3, b = 49.6, c = 69.6,$ $\beta = 95.7$
Resolution limits (Å)	49.6–1.9 (2.0–1.9)
Unique reflections	24244 (3515)
Completeness (%)	97.6 (97.8)
Multiplicity	3.0 (2.8)
$\langle I/\sigma(I) \rangle$	10.4 (1.7)
$R_{\text{merge}}^{\dagger}$ (%)	6.3 (58.5)
$R_{\text{p.i.m.}}^{\ddagger}$ (%)	4.0 (41.4)
$CC_{1/2}^{\S}$	0.997 (0.716)
No. of molecules in asymmetric unit	1
V_M (Å ³ Da ⁻¹)	2.1
Solvent content (%)	40.2

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I(hkl)$ is the integrated intensity of a given reflection. $\ddagger R_{\text{p.i.m.}} = \sum_{hkl} (1/[N(hkl) - 1])^{1/2} \times \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. $\S CC_{1/2}$ is the half-data-set correlation coefficient.

assisted laser desorption/ionization mass spectrometry (MALDI-MS).

2.3. Crystallization and data collection

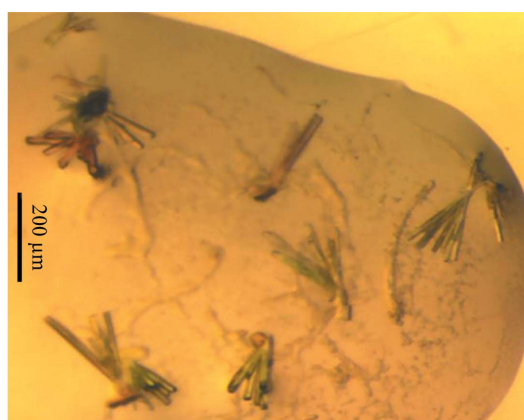
Initial screening for crystallization conditions for tagged and untagged proteins was performed by the sitting-drop vapour-diffusion method using the PACT (Newman *et al.*, 2005; Qiagen), Ammonium Sulfate (Qiagen), MPD (Qiagen), Morpheus (Gorrec, 2009; Molecular Dimensions) and Index (Hampton Research) screens. 0.30 μ l sample solution and 0.15 μ l reservoir solution were aliquoted using a Mosquito robot into an MRC/Wilden 96-well plate with 54 μ l reservoir solution and incubated at 292 K. Crystals were cryoprotected in protein buffer and reservoir supplemented with PEG 1500 to 32% (w/v) and flash-cooled in liquid N₂. X-ray data for untagged *HsDus2L* 1–340 crystals were collected on beamline I04 at Diamond Light Source, Didcot, England using an ADSC Q315r CCD detector at 100 K over 180° (crystal-to-detector distance of 274 mm, 0.5° oscillation, 0.5 s exposure for high-resolution data and crystal-to-detector distance of 424 mm, 1° oscillation and 0.5 s exposure for low-resolution data). Indexing and integration was performed with *XDS* (Kabsch, 2010). The Laue group was confirmed by *POINTLESS* and scaling and merging of the data were performed by *SCALA* (Evans, 2006). Data-collection statistics are summarized in Table 2.

3. Results

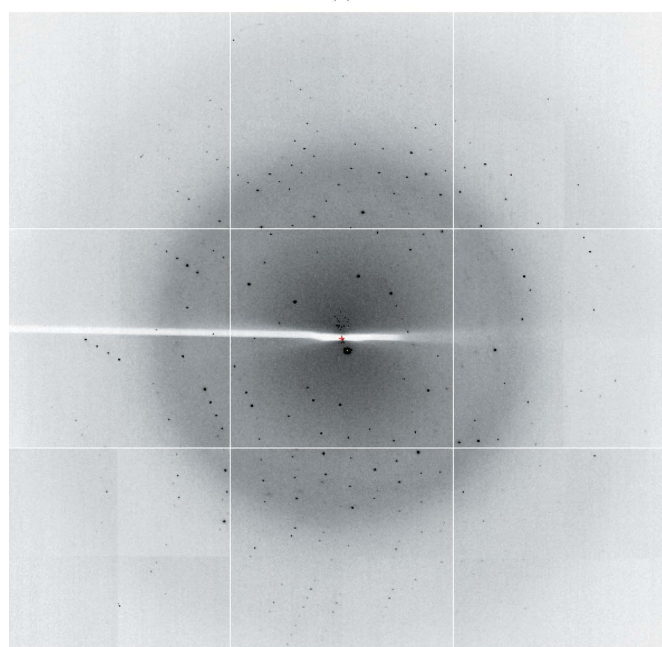
Expression trials were performed for three constructs in small-scale cultures (Fig. 1*b*). Purification of the full-length enzyme resulted in the isolation of a degraded product. *HsDus2L* 1–340 was expressed in *E. coli* and purified by nickel-affinity, anion-exchange and size-exclusion chromatography to >95% purity as estimated by SDS-PAGE analysis (Fig. 1*c*). Purified *HsDus2L* 1–340 had an absorption peak at 450 nm and was yellow-coloured, which was suggestive of the presence of oxidized FMN, a cofactor that is critical for enzymatic reduction of uracil and that has also been identified in *TthDus* and *TmDus* (Park *et al.*, 2004; Yu *et al.*, 2011). MALDI-MS of H₆-*HsDus2L* 1–340 identified a single peak of 40 370 Da consistent with the predicted mass. The molar mass in solution estimated using SEC-MALLS was 40 700 ± 2850 Da, indicating a monomeric species (Fig. 1*d*).

Crystals of H₆-*HsDus2L* 1–340 grew in 2 d in PACT screen conditions B11, C11 and D11 [0.2 M CaCl₂, 20% (w/v) PEG 6000 and 0.1 M MES pH 6/HEPES pH 7/Tris pH 8, respectively]. These crystals grew in clumps, proved impossible to separate and were difficult to reproduce in optimization screens. Crystallization screening with untagged enzyme resulted in the growth of rod-like clusters that could be mechanically separated. These crystals grew in 2 d from PACT screen condition D1 in a reservoir consisting of 0.1 M MES/malic/Tris buffer pH 4 (Newman, 2004) and 25% (w/v) PEG 1500. Crystals of untagged *HsDus2L* 1–340 (Fig. 2*a*) diffracted to 1.9 Å resolution (Fig. 2*b*) and belonged to space group $P2_1$, with unit-cell parameters $a = 46.2, b = 49.6, c = 69.6$ Å, $\beta = 95.7^\circ$. The Matthews coefficient estimated that the asymmetric unit comprised a single molecule of *HsDus2L* (Matthews, 1968).

We attempted to phase the data by molecular replacement using the structures of *TthDus* and *TmDus*. MR experiments were performed using *MOLREP*, *Phaser* and *BALBES* (Vagin & Teplyakov, 2010; McCoy *et al.*, 2007; Long *et al.*, 2008); however, no solutions were identified. We are performing selenomethionine



(a)



(b)

Figure 2

Crystals and diffraction of untagged *HsDus2L* 1–340. (a) Rod-like crystals from PACT screen (condition D1). (b) Diffraction recorded using synchrotron radiation with the crystal rotated by 0.5°. The resolution at the edge is 1.9 Å.

labelling to attempt to determine the structure of the *HsDus2L* Dus domain by multiwavelength anomalous dispersion phasing.

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