Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification and crystallization of *Escherichia coli* pseudouridine synthase RluD

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Received 16 April 2003
Accepted 18 August 2003

RluD is the pseudouridine (Ψ) synthase responsible for forming Ψ1911, Ψ1915 and Ψ1917 in *Escherichia coli* 23S RNA. Out of the 11 Ψ synthases in *E. coli*, only cells lacking RluD show a severe growth defect. In addition, RluD belongs to the RluA family of Ψ synthases, one of the two remaining families without a representative crystal structure. In this paper, the crystallization of selenomethionine-substituted RluD by the hanging-drop method is reported. The crystals diffract to 1.9 Å and belong to space group $P4_32_12$, with unit-cell parameters $a = b = 75.14$, $c = 181.81$ Å. Synchrotron radiation was used on a single crystal to collect a complete multiwavelength anomalous dispersion (MAD) data set to 2.0 Å resolution.

1. Introduction

Approximately 100 different post-transcriptionally modified nucleosides have been identified in cellular RNA to date (Rozenski *et al.*, 1999). The most widespread and numerous of these modified nucleosides is pseudouridine (Ψ; the 5-ribosyl isomer of uridine). Ψ is found in rRNA, tRNA, tmRNA, snRNA and snoRNA in varying quantities and positions (reviewed in Charette & Gray, 2000; Ofengand *et al.*, 2001; Ofengand, 2002). Only mRNA and viral RNA appear to lack Ψ, although Ψ is made on a leader sequence that is spliced onto all trypanosome mRNAs (Liang *et al.*, 2002). The function of Ψ in RNA is unclear, although there is evidence to support a structural role involving hydrogen bonding through its NH1 group (Newby & Greenbaum, 2002). Biosynthesis of Ψ by the enzymes responsible for its synthesis is more clearly understood.

The enzymes termed Ψ synthases catalyze the isomerization of uridine to Ψ without the need for any cofactors. In general, Ψ synthases contain site-specificity determinants to make Ψ at one or more sites in one or more classes of RNA. In eukaryotic rRNA, where Ψ residues can number >100, and in some snRNAs, site-specificity is achieved by small guide RNAs that guide the activity of a single Ψ synthase (Kiss, 2001; Ofengand, 2002). The exact reaction mechanism of these enzymes is still under scrutiny, but clearly involves a conserved catalytic aspartate in a conserved sequence motif as shown by mutational (Del Campo *et al.*, 2001) and structural evidence (Hoang & Ferré-D'Amaré, 2001).

Using amino-acid sequence similarity, all known Ψ synthases can be grouped into the RsuA, RluA, TruA, TruB and TruD families; each named for the first *Escherichia coli* Ψ synthase in each family to be cloned and characterized. RsuA, RluA and TruB family members share three conserved sequence motifs (motif II contains the catalytic aspartate) and sufficient sequence similarity to be grouped into one superfamily (Koonin, 1996; Ofengand & Rudd, 2000). TruA family members have a detectable motif II but lack significant homology to the aforementioned superfamily; thus, they form a superfamily of their own (Ofengand & Rudd, 2000). The TruD family also represents its own superfamily and does not share any homology with the other four families (Kaya & Ofengand, 2003). Recent crystal structures of *E. coli* TruA (Foster *et al.*, 2000), TruB (Hoang & Ferré-D'Amaré, 2001) and RsuA (Sivaraman *et al.*, 2002) have shown that despite a lack of global sequence similarity between their respective families, their catalytic domains share a core α/β fold and their catalytic aspartates are in the same location (reviewed in Mueller, 2002). Thus, it appears that these families arose by divergent evolution from a common ancestor. Since the RluA family is most similar at the sequence level to the RsuA family, it is expected that RluA family members will have the same Ψ synthase fold, but this may not be the case for TruD members.

There are a total of 11 known Ψ synthases in *E. coli* that account for all of the Ψ positions known in *E. coli* tRNA and rRNA with no overlapping specificity. Of these, only cells lacking RluD show any obvious phenotype. RluD is a member of the RluA family that forms three Ψ (1911, 1915 and 1917) in helix 69 of 23S RNA, two of which are the most universally conserved Ψ in rRNA across all kingdoms of life (Ofengand, 2002). *E. coli* lacking an active Ψ synthase RluD have altered ribosomal particles (Ofengand *et al.*,

2001) and a growth rate 20% that of the wild type (Gutgsell *et al.*, 2001). Insights into function often come from atomic resolution structures, but no structure is available for RluD or any other RluA-family member. Thus, we have initiated a structural study of RluD. Here, we present the crystallization and preliminary X-ray analysis of *E. coli* RluD and a selenomethionine (SeMet) derivative.

2. Materials and methods

2.1. Cloning, expression and purification

The entire 981 bp *rluD* gene was cloned into the *Nhe*I and *Xho*I sites of the pET28a vector (Novagen Inc.) and transformed into strain BL21(DE3) (Novagen Inc.) as described previously (Raychaudhuri *et al.*, 1998). The RluD protein produced from this construct comprises 349 residues (326-amino-acid RluD with an N-terminal histidine tag of 23 amino acids), with a calculated molecular weight of 39.5 kDa. RluD was overexpressed by adding IPTG (1 m*M* final concentration) to 2 l of cells grown in Luria Broth (Invitrogen Inc.) to an OD_{600} of 0.6 at 298 K; the induction proceeded for 15 h. Cells were pelleted by centrifugation, resuspended in buffer *A* (20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 10 m*M* imidazole) and flash-frozen at 193 K. For RluD purification, the frozen cell suspension was thawed at 273 K, cells were lysed using a French press in the presence of protease inhibitors [0.7 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.7 µg ml^{-1} pepstatin A and two tablets of complete mini protease-inhibitor cocktail (Boehringer Mannheim Inc.)] and the lysate was clarified by centrifugation. DNA and DNA-binding proteins were removed from the soluble fraction by precipitation with polyethylenenimine [added to 0.24%(*v*/*v*)]. The soluble fraction was loaded onto a nickel metal-affinity column (Poros MC20 Media, Applied Biosystems Inc.) equilibrated in buffer *A* and RluD eluted at 80 m*M* imidazole with a linear gradient to buffer *B* (buffer *A* at 0.5 *M* imidazole). RluD fractions were pooled and EDTA pH 8.0 (2 m*M* final concentration) and dithiothreitol (DTT, 0.5 m*M* final concentration) were added. The sample was dialyzed overnight against buffer *C* (20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 2 m*M* EDTA, 0.5 m*M* DTT) and loaded onto a Mono-Q (Pharmacia Inc.) column equilibrated in buffer *C*; RluD eluted at 300 m*M* NaCl with a linear gradient to buffer *D* (buffer *C* at 0.5 *M* NaCl). RluD-containing fractions were pooled and dialyzed overnight against buffer *E* (20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 2 m*M* EDTA, 0.5 m*M* DTT, 10% glycerol), concentrated to a volume of 2 ml by Centricon centrifugation (Millipore Inc.) and loaded onto a Superdex 200 (Pharmacia Inc.) column equilibrated in buffer *E*. The RluD-containing fractions from gel filtration were pooled and concentrated by Microcon centrifugation (Millipore Inc.) to either 19 or 25 mg ml^{-1}, as measured by A_{280}, prior to setting up crystallization trials.

The purification described above yields approximately 18 mg of RluD, which appears to be over 95% pure as judged by SDS–PAGE and subsequent Coomassie staining (data not shown). Comparison with MW standards during gel filtration shows RluD to be a monomer (data not shown). RluD protein can be kept at 193 K after flash-freezing in liquid N_2. All purification, dialysis and concentration steps were carried out at 277 K.

SeMet-substituted RluD was overexpressed under conditions of methionine-pathway inhibition (Van Duyne *et al.*, 1993). Cells from a 2 ml overnight culture grown in rich medium were pelleted by centrifugation, resuspended in 10 ml of M9 minimal medium containing glucose [0.4%(*w*/*v*)] and grown for 1 h at 310 K. This culture was added to 2 l of pre-warmed M9 minimal medium supplemented with the following: 0.4% glucose, 50 mg l^{-1} SeMet, 40 mg l^{-1} amino-acid set I (Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, His, Pro, Ser), 100 mg l^{-1} amino-acid set II (Ile, Leu, Lys, Phe, Thr, Val), 40 mg l^{-1} amino-acid set III (Trp, Tyr), and 2 mg l^{-1} thiamine, thymine, niacinamide and pyridoxine monohydrochloride. The culture was grown to an OD_{600} of 0.6 at 310 K, IPTG (1 m*M*) was added and the induction proceeded for 8 h. SeMet-substituted RluD was purified as described above, except that DTT was used at 1 m*M* in buffers *C*, *D* and *E*.

2.2. Crystallization

The sitting-drop vapor-diffusion technique was used to screen 96 crystallization conditions in the Crystal Screen HT kit (Hampton Research Inc.) and subsequent trials used the hanging-drop technique. Each drop contained 1 µl of protein and 1 µl of reservoir solution. Regular crystals of RluD and SeMet RluD were observed in approximately 24 h using 25–40% ethylene glycol at room temperature (Fig. 1). Crystals grew to full size in 4–6 d, with maximum dimensions of 0.8 × 0.3 × 0.1 mm. Since ethylene glycol is a cryoprotectant, prior to data collection crystals were transferred to ethylene glycol solutions of the same percentage as used in crystallization and then transferred in steps to at least 35% ethylene glycol (if needed) and flash-frozen in liquid N_2.

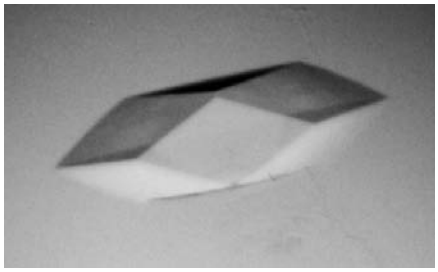

Figure 1
Typical crystal of *E. coli* Ψ synthase RluD.

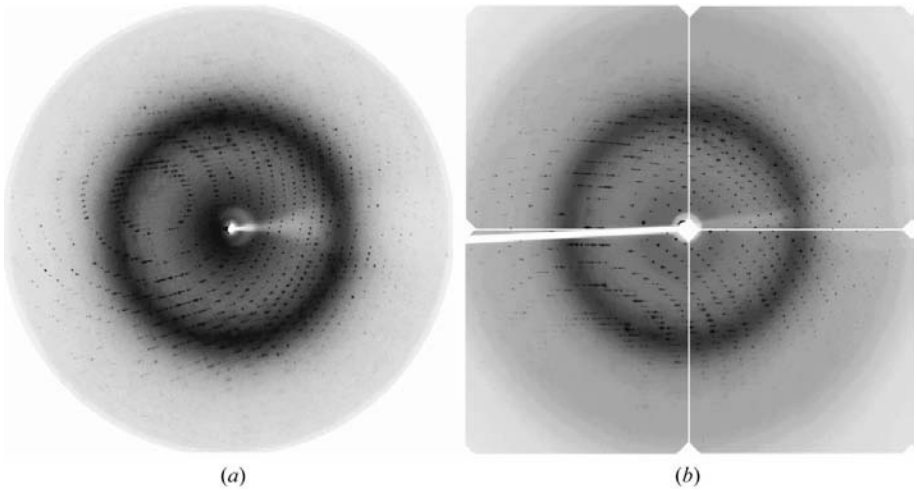

Figure 2
X-ray diffraction of RluD crystals. (*a*) A 1° oscillation image showing diffraction of Cu *Kα* radiation by a native RluD crystal taken on our in-house 30 cm MAR Research image plate: the edge of the plate is 2.2 Å. (*b*) A 1° oscillation image showing diffraction of synchrotron radiation by a SeMet RluD crystal taken on the CCD detector at the NSLS beamline X12-C: the left and right edges are 1.9 Å.

Table 1
Crystallographic parameters and data-collection statistics for SeMet-substituted RluD.

Values for the highest resolution shell (2.07–2.00 Å) are given in parentheses.

	Inflection	Peak	Remote
Space group	$P4_32_12$		
Unit-cell parameters (Å)	$a = b = 75.14$, $c = 181.81$		
X-ray source	X12-C, NSLS, BNL		
Wavelength (Å)	0.978795	0.978462	0.950037
Resolution (Å)	50.0–2.0	50.0–2.0	50.0–2.0
Mosaicity (°)	0.259	0.298	0.334
Total observations	441842	370551	349515
Unique reflections	34611	32497	31024
Completeness (%)	95.1 (69.6)	88.9 (51.2)	84.7 (45.1)
Overall $I/\sigma(I)$	38.3 (9.2)	31.5 (5.8)	31.1 (4.3)
$R_{\rm merge}$† (%)	5.3 (13.3)	7.3 (18.2)	5.8 (18.0)

† $R_{\rm merge} = \sum(|I_j - \langle I\rangle|)/\sum\langle I\rangle$, where I_j is the observed intensity of reflection j and $\langle I\rangle$ is the average intensity of multiple observations.

2.3. Data collection and processing

Preliminary diffraction data to screen crystals were collected using Cu $K\alpha$ radiation from our in-house Rigaku H3R rotating-anode X-ray generator with Osmic confocal optics equipped with a 30 cm MAR Research image plate and an ADSC cryosystem. Diffraction data were also collected using synchrotron radiation from the X12-C beamline at the National Synchrotron Light Source, Brookhaven National Laboratory (NSLS, BNL), which was equipped with a B4 CCD detector and an Oxford Cryostream low-temperature system. All diffraction intensities were integrated and scaled using the *HKL* software package (Otwinowski & Minor, 1997).

3. Results and discussion

RluD is a protein amenable to crystallization; crystals of RluD were easily and quickly formed in less than 1 d using only the common cryoprotectant ethylene glycol. To determine if our RluD crystals diffracted well and to sufficient resolution, a crystal (0.65 × 0.35 mm along its longest axis, 35% ethylene glycol condition) was mounted in a cryoloop, rapidly frozen to 93 K in a stream of cold N_2 and exposed to Cu $K\alpha$ radiation. The crystal diffracted well, with some spots extending to 2.2 Å (Fig. 2*a*). One data set was collected and processed to 2.4 Å. Diffraction data reduction and scaling showed that the crystal is tetragonal, with unit-cell parameters $a = b = 76.0$, $c = 181.7$ Å. The overall $I/\sigma(I)$ of this data set was approximately 12.

To improve the resolution limits of diffraction and to determine phases, synchrotron radiation was used on an SeMet derivative of RluD. A complete three-wavelength MAD data set was collected from a single crystal (0.45 × 0.25 mm, 38% ethylene glycol condition). The diffraction extended to 1.9 Å and a typical diffraction pattern is shown in Fig. 2(*b*). Data reduction and scaling to 2.0 Å showed the crystal to be tetragonal, with unit-cell parameters $a = b = 75.14$, $c = 181.81$ Å, and to belong to space group $P4_12_12$ or $P4_32_12$ based on symmetry and systematic absences of reflections. More details of the data-collection statistics are given in Table 1. Assuming a monomer in the asymmetric unit, the Matthews coefficient ($V_{\rm M}$; Matthews, 1968) was 3.2 Å^3 Da^{-1}, which corresponds to a solvent content of 60%.

Selenium sites in SeMet-substituted RluD were located by analyzing our scaled MAD data with the automated structure-determination program *SOLVE* (Terwilliger & Berendzen, 1999). Using data to 2.1 Å and space group $P4_12_12$, *SOLVE* located eight of the 14 expected selenium sites and generated an initial electron-density map with an overall figure of merit of 0.55. Using data to 2.1 Å and space group $P4_32_12$, *SOLVE* located nine selenium sites and generated an initial electron-density map with an overall figure of merit of 0.56. Based on examination of both maps, $P4_32_12$ was chosen as the final space group because the map using $P4_12_12$ contained left-handed α-helices. Subsequent density modification with *RESOLVE* (Terwilliger, 2000) improved the figure of merit to 0.64. In addition to improving the quality of our initial map, *RESOLVE* modeled 150 amino acids into the electron density using its automated building function. Further model building and refinement are in progress.

We would like to thank Anand Saxena at the X12-C beamline, NSLS, BNL for assistance with data collection. This work was supported in part by NIH grant GM58879 (to JO), NIH fellowship GM66374 (to MD), Florida Biomedical Research Foundation grant BM030 (to AM) and an American Heart Association Florida/Puerto Rico Affiliate award SDG-0130456B (to AM).

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