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Crystallization and characterization of a fragment of pseudouridine synthase RluC from *Escherichia coli*

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RluC from *E. coli* is the enzyme responsible for catalyzing the isomerization of uridines 955, 2504 and 2580 in 23S rRNA to pseudouridine. Histidine-tagged RluC was cloned, overexpressed and purified by nickel-affinity chromatography. A proteolytically derived fragment of the enzyme consisting of residues 89–319 has been shown to retain catalytic activity. Crystals of this fragment, grown by precipitation with sodium acetate at pH 8.0, belong to space group *P*321, with unit-cell dimensions $a = b = 97.1$, $c = 86.3$ Å and have two molecules in the crystallographic asymmetric unit. The flash-frozen crystals diffract X-rays to at least 2.3 Å resolution and appear suitable for crystal structure determination.

1. Introduction

Pseudouridine (5-β-D-ribofuranosyluracil) is a common modified nucleoside found in tRNA (Sprinzl *et al.*, 1996), rRNA (Maden, 1990) and sn(o)RNA (Gu & Reddy, 1997; Massenet *et al.*, 1998) but not so far in mRNA or viral RNAs. Enzyme-catalyzed isomerization of uridine to pseudouridine takes place at the polynucleotide level and requires neither cofactors nor an external energy source such as ATP. Small subunit rRNA from *Escherichia coli* contains one pseudouridine (Bakin, Kowalak *et al.*, 1994), while the large subunit rRNA contains nine (Bakin & Ofengand, 1993; Bakin, Lane *et al.*, 1994). In *E. coli*, several distinct synthases have been identified and characterized with respect to their specificities for isomerization of particular uridine residues in tRNAs and rRNAs (Kammen *et al.*, 1988; Nurse *et al.*, 1995; Wrzesinski, Bakin *et al.*, 1995; Wrzensinski, Nurse *et al.*, 1995). For a recent review, see Ofengand & Fournier (1998).

Amino-acid sequences of the four known *E. coli* pseudouridine synthases (Kammen *et al.*, 1988; Nurse *et al.*, 1995; Wrzesinski, Bakin *et al.*, 1995; Wrzensinski, Nurse *et al.*, 1995) were used to establish the existence of a relationship among them and to search sequence databases for homologous open reading frames (ORFs) in *E. coli* and other organisms (Koonin, 1996; Gustafsson *et al.*, 1996). A number of ORFs were found which could be subdivided into four families on the basis of three conserved sequence segments. These segments are believed to be components of the active sites of these enzymes (Koonin, 1996). One of the conserved segments shows sequence similarity with a conserved motif in *E. coli* deoxycytidine triphosphate deaminase and several species of uridine triphosphatases, and contains a conserved aspartic acid that has been suggested to play a role in uridine binding and to be at the active site (Koonin, 1996). Indeed, when this Asp was mutated to Ala, Asn, Gln, Lys or Ser in TruA, an *E. coli* tRNA pseudouridine synthase, all activity was lost (Huang *et al.*, 1998). The crystal structure of a dUTPase from *E. coli* has been reported to be a trimer in which each 152-residue subunit resembles a jelly-roll fold (Cedergren-Zeppezauer *et al.*, 1992).

One of the homologous ORFs in *E. coli* is *yce*C (SWISS-PROT, PIR P23851). Gene deletion has recently shown it to be the sole synthase in *E. coli* responsible for synthesis of pseudouridine at positions 955, 2504 and 2580 in 23S RNA (Conrad *et al.*, 1999). The 319 amino-acid enzyme, renamed RluC, belongs to group I of the four families of pseudouridine synthases. Other *E. coli* synthases belonging to this group include RluA (Wrzesinski, Nurse *et al.*, 1995) and RluD, previously called SfhB or YfiI (Ray Chaudhuri *et al.*, 1999), which are specific for the formation of other pseudouridine residues in 23S RNA.

The unique nature of the reaction catalyzed by pseudouridine synthases, namely cleavage of an N1—C1′ glycosyl bond and reformation of a C5—C1′ bond, makes the structure determination of the active site of this class of enzyme of particular interest. To our knowledge, *E. coli* TruA, a pseudouridine synthase from group 4, is the only other enzyme of this type for which crystals have previously been reported (Foster *et al.*, 1997). In this paper, we describe the cloning and expression of RluC. Crystals of a proteolytically cleaved, catalytically active C-terminal fragment of the enzyme have been characterized and shown to be suitable for X-ray crystal structure determination to at least 2.3 Å resolution.

2. Methods and results

2.1. Cloning of the *rluC* gene

The *yce*C ORF was amplified and prepared for insertion into pET28a by PCR. The N-terminal primer extended from −16 to +22, where the A of the initiating AUG is +1, with base changes at −1, −2 and −3 to create an *Nde*I site which included the initiating AUG. The C-terminal primer, in the reverse orientation, extended from +955 to +993, where the last sense nucleotide is +957, and contained mismatches at +981 and +982 to create a *Bam*HI site. The PCR product was isolated from a 1.0% agarose gel using DE81 paper. The DNA was eluted from the paper and precipitated overnight with ethanol. The purified PCR product as well as the pET vector were digested with *Bam*HI and *Nde*I (New England Biolabs, Beverly, MA). The digested products, purified as above, were combined in a ligation mixture containing 50 m*M* Tris–HCl pH 7.5, 10 m*M* $MgCl_2$, 25 µg ml^{-1} bovine serum albumin, 10 m*M* DTT, 1 m*M* ATP, 20000 units ml^{-1} T4 ligase (New England Biolabs), 10 µg ml^{-1} vector and 53 µg ml^{-1} insert and were incubated overnight at 289 K. Transformation into Novablue cells (Novagen) was performed according to the manufacturers directions. Plasmids were isolated from positive clones and subsequently re-transformed into BL21(DE3) cells. DNA sequencing across the ligation junction verified the construction.

2.2. Overexpression and purification

Transformed BL21/DE3 cells were grown in 2 l of LB medium (Zyskind & Bernstein, 1992) containing 30 µg ml^{-1} kanamycin. Cells were grown at 303 K to an A_{600} of 0.6 before induction with 1 m*M* IPTG and further growth for 2.5 h. Harvested cells were washed with 50 m*M* sodium phosphate pH 7.0 and repelleted. Cells suspended in 50 m*M* Tris–HCl, 2 m*M* EDTA, pH 7.0, were incubated with 100 µg ml^{-1} hen egg-white lysozyme at 303 K for 15 min before sonication. Cell debris was pelleted by centrifugation at 17000*g* for 1.5 h and the supernatant applied directly to a 20 ml column of His-bind resin. Conditions for the preparation and operation of this column were as described in the pET System Manual (Novagen Inc., 1997).

2.3. Crystallizations

Since RluC was found to precipitate from solution at low ionic strength, protein from the His-bind column was dialyzed against 0.2 *M* ammonium sulfate before concentrating to an A_{280} of 14.0 for crystallization experiments. The sparse-matrix method (Jancarik & Kim, 1991) was used to search for crystallization conditions using crystal screen reagents (Hampton Research, Riverside, CA). Hanging droplets were set up at 277 K in Linbro tissue-culture trays (Flow Labs. Inc., McLean, Virginia) by mixing 2 µl of the protein solution with an equal volume of reservoir solution.

The first microcrystals appeared after about two months in a droplet with reservoir composition 1.4 *M* sodium acetate, 50 m*M* piperazine-*N*,*N*′-bis-2-ethanesulfonic acid (PIPES) buffer pH 6.5. Use of different buffers in the pH range 4.6–8.0 resulted in larger crystals at pH 8.0 with *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine) buffer and a sodium acetate concentration of 1.6 *M*. In all cases at least six weeks elapsed before the appearance of crystals, and use of acetate concentrations greater than 1.7 *M* in the reservoirs resulted in rapid precipitation of the protein. In view of these observations, we decided to investigate the effects of enzyme storage under conditions approximating those used for crystallization (0.7 *M* sodium acetate, 50 m*M* Bicine, pH 8.0). A sample of enzyme stored for two weeks at 277 K under these conditions was analyzed by hydrophobic interaction chromatography on a phenyl Sepharose column using an FPLC apparatus (Pharmacia). Elution using a linearly decreasing concentration gradient of ammonium sulfate in 50 m*M* Bicine pH 8.0 separated three major protein fractions. SDS–PAGE of samples from the separated protein fractions revealed a major fragment with apparent molecular weight 25 kDa. It was hypothesized that the crystals we had grown could consist of this fragment rather than the intact enzyme.

2.4. Fragment characterization

A single large crystal was washed in protein-free substitute mother liquor, dissolved in distilled water and subjected to N-terminal sequencing in an Applied Biosystems Procise Sequencer. The first ten cycles of this gave the sequence AALADVILYE, which could be readily identified as residues 89–98 of RluC. The N-terminal 88 residues, together with the histidine tag appear to have been cleaved from the enzyme by hydrolysis of the V88–A89 peptide bond. Although we have not attempted to analyze the C-terminus of the fragment, the molecular weight calculated from the sequence assuming an unmodified C-terminus would be 25969 Da, in agreement with the size of the fragment purified by phenyl Sepharose chromatography as estimated by SDS–PAGE.

The catalytic activity of a single crystal which had been washed with protein-free mother liquor and dissolved by the addition of distilled water was determined using the assay described by Wrzesinski, Nurse *et al.* (1995). The results shown in Fig. 1 indicate that the fragment retains catalytic activity. Because the amount of protein from the dissolved crystal was too low to measure, we are unable to compare the specific activity of the fragment to that of the intact enzyme. However, an upper-level estimate of the protein concentration yielded a minimum value for the specific activity equal to at least 80% of that of the intact enzyme.

2.5. Preliminary X-ray analysis

A crystal was washed briefly in substitute mother liquor consisting of 0.1 *M* ammonium sulfate, 1.0 *M* sodium acetate, 0.1 *M* Bicine pH 8.0 and 20% 2-methyl-2,4-pentanediol (MPD) as cryoprotectant, before flash freezing in a stream of nitrogen at 93 K from a cryogenic crystal-cooling system (Area Detector Systems Corp., Poway, CA). X-ray data were recorded using a 30 cm MAR Research image-plate detector on a Rigaku RU300 generator equipped with a

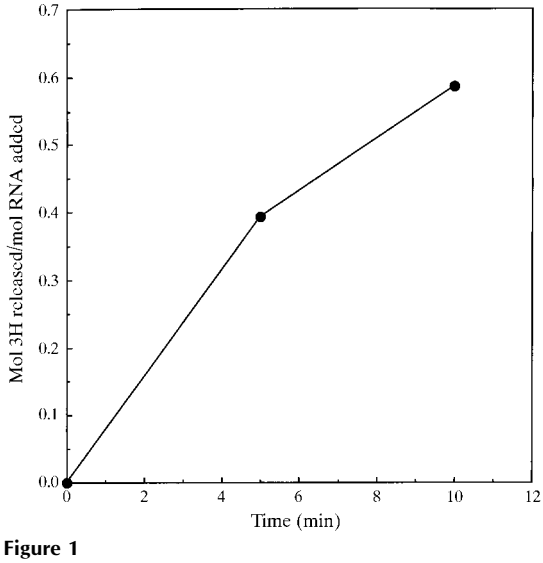

Figure 1
Pseudouridine synthase activity of the C-terminal fragment of RluC. Synthase activity was measured using 5-(^{3}H)-uracil-labelled 23S RNA as substrate. Pseudouridine synthesis is accompanied by release of ^{3}H from the C5 position of the uracil ring. Results are expressed as moles of ^{3}H released (equal to moles of pseudouridine formed) per mole of RNA substrate.

Table 1
Data-collection statistics.

Resolution range (Å)	20–2.3	2.38–2.30
Number of intensities	134913	6824
Number unique	20016	1809
Completeness (%)	95.0	87.9
Average $I/\sigma(I)$	13.2	6.4
$R_{\rm merge}$	0.046	0.191

helium-purged large double focusing mirror system (Area Detector Systems Corp.). The generator was operated at 4.5 kW using a copper target and 0.3 × 3 mm focusing cathode. The crystal-to-detector distance was 100 mm, and 231 images were recorded at 1° rotation intervals. Image processing and data reduction were performed using the *DENZO* and *SCALEPACK* programs (Otwinowski, 1993).

The autoindexing option of *DENZO* suggested a trigonal unit cell which was further characterized as space group *P*321 during data scaling. The unit-cell dimensions $a = b = 97.1$, $c = 86.3$ Å for the frozen crystal give a unit-cell volume of 704660 Å^3 which can accommodate 12 molecules of molecular weight 25969 Da with a reasonable value for V_m (Matthews, 1968) of 2.26 Å^3 Da^{-1}, indicating that there are two molecules of the RluC fragment in the crystallographic asymmetric unit. The frozen crystals appear to be suitable for diffraction data collection to at least 2.3 Å resolution, as indicated by the data-reduction statistics for the native data set given in Table 1. A search for suitable isomorphous heavy-atom derivatives for use in phase determination is under way. Since the fragment contains two cysteines which are not believed to form a disulfide, mercury compounds are expected to be particularly effective for this purpose.

3. Discussion

The cleavage of recombinant RluC to yield a C-terminal fragment may be catalyzed by an *E. coli* protease that remains associated with the His-tagged enzyme during purification on the His-bind nickel column. Crystallizations using intact RluC further purified by phenyl Sepharose chromatography did not yield crystals, suggesting that the protease may have been separated from the enzyme by this procedure. In contrast, crystallizations using the C-terminal fragment purified by phenyl Sepharose chromatography yielded crystals rapidly in 2–3 d.

All three of the conserved sequence segments found in the pseudouridine synthases are present in the C-terminal fragment of RluC for which crystals have been obtained. The conserved aspartic acid, which has been suggested to be essential for uridine binding and for catalytic activity, also occurs in the C-terminal fragment of RluC at position 144 of the intact enzyme. It is anticipated that knowledge of the structure of this fragment will facilitate the identification of other residues that participate in catalysis of this unusual reaction.

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