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Purification, crystallization and preliminary X-ray diffraction study of human ribosomal protein L10 core domain

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Eukaryotic ribosomal protein L10 is an essential component of the large ribosomal subunit, which organizes the architecture of the aminoacyl-tRNA binding site. The human L10 protein is also called the QM protein and consists of 214 amino-acid residues. For crystallization, the L10 core domain (L10CD, Phe34–Glu182) was recombinantly expressed in *Escherichia coli* and purified to homogeneity. A hexagonal crystal of L10CD was obtained by the sitting-drop vapour-diffusion method. The L10CD crystal diffracted to 2.5 Å resolution and belongs to space group $P3_121$ or $P3_221$.

1. Introduction

The ribosome, which is an RNA–protein complex composed of two subunits, is the molecular machinery for protein synthesis in all living cells. Ribosomal proteins and ribosomal RNAs (rRNAs) vary in length and number among the three phylogenetic domains eukaryota, archaea and bacteria (Woese *et al.*, 1990). Ribosomal protein L10 is conserved between eukaryota and archaea (called L10e in archaea) and recent X-ray crystallographic studies of ribosomes have shown that its bacterial counterpart is the ribosomal protein L16 (Ban *et al.*, 2000; Yusupov *et al.*, 2001; Harms *et al.*, 2001). In the ribosome crystal structures, the structure of archaeal L10 is similar to that of bacterial L16 and both are located at the aminoacyl-tRNA binding site in the vicinity of the peptidyl transferase centre (Ban *et al.*, 2000; Yusupov *et al.*, 2001; Harms *et al.*, 2001). The eukaryotic large ribosomal subunit is almost completely matured in the nucleus, while L10 is one of the four ribosomal proteins that are assembled in the cytoplasm, in contrast to the other members, which are incorporated in the nucleus (Kruiswijk *et al.*, 1978). L10 incorporation is a prerequisite for ribosomal subunit association and translation initiation (Dick, Eisinger *et al.*, 1997; Eisinger, Dick & Trumpower, 1997; Eisinger, Dick, Denke *et al.*, 1997). In addition, it is also important for the nuclear-export process of the large ribosomal subunit (Johnson *et al.*, 2002; see below). L10 has a genetic interaction with the nuclear-export adapter NMD3p, which provides the nuclear-export signal to the large ribosomal subunit (Karl *et al.*, 1999; Gadal *et al.*, 2001). The latest studies show that L10 incorporation triggers the dissociation of NMD3p from the large ribosomal subunit for the next cycle of the export process (Hedges *et al.*, 2005; West *et al.*, 2005). Several mutations of L10 have been reported to cause a translation defect and nuclear accumulation of the large ribosomal subunit (Tron *et al.*, 1995; Eisinger, Dick & Trumpower, 1997; Eisinger, Dick, Denke *et al.*, 1997; Dick, Karamanou *et al.*, 1997; Koller *et al.*, 1996; Karl *et al.*, 1999; Gadal *et al.*, 2001). Thus, L10 is an essential factor not only for protein synthesis but also for large-subunit maturation and assembly in the eukaryotic cell.

The L10 protein is well conserved among eukaryota and has a C-terminal extension that is lacking in archaeal and bacterial homologues (Farmer *et al.*, 1994). Structural elucidation of the L10 protein will provide clues as to how these characteristics are related to the unique aspects of eukaryotic L10 function and how the L10 mutations reported in yeast studies cause functional defects in the translation system. It will also allow us to examine the structural divergence of this ribosomal protein by examining its similarities to

and differences from the previously determined structures of archaeal L10e (Ban *et al.*, 2000; Klein *et al.*, 2004) and bacterial L16 (Nishimura *et al.*, 2004). In this study, we focused on human L10, which is also called QM protein (Dowdy *et al.*, 1991), and examined *Escherichia coli* expression vectors in order to obtain a sufficient quantity of the L10 protein of suitable quality for crystallization. We succeeded in overproducing and purifying the L10 core domain (L10CD, Phe34–Glu182). The crystallization procedure and the preliminary X-ray crystallographic analysis are reported here.

2. Cloning and construction of plasmids

Human L10 (molecular weight 25 kDa) is comprised of 214 amino-acid residues. An *E. coli* vector for the expression of a glutathione *S*-transferase (GST) fusion form of full-length human L10 was supplied by the RIKEN Structural Genomics Initiative (Yokoyama *et al.*, 2000). This vector has the open reading frame of human L10 (gene ID 15718685) at the *Bam*HI–*Eco*RI site of pGEX4T-1 (Amersham Pharmacia) and is named pGEX4T1-L10. Alternatively, we constructed an expression vector for the thioredoxin-fusion form of full-length human L10, named pET32b-L10. For the construction of pET32b-L10, the coding region of full-length human L10 was amplified from pGEX4T1-L10 by PCR using primer 1, 5′-GAG GTA CCC CTC CAA AAT CGG ATC TGG TTC, and primer 2, 5′-GAG GCA GAT CGT CAG TCA G, and then inserted into the *Kpn*I–*Eco*RI site of pET32b (Novagen). Preparation of the full-length L10 protein using these expression vectors failed owing to aggregation and fragmentation during the purification procedure. Therefore, we analyzed a sequence alignment between archaeal L10e and eukaryotic L10 using the program *ClustalW* (Thompson *et al.*, 1994) and constructed a truncated L10 variant containing residues 34–182 corresponding to the core fold part of archaeal L10e (PDB code 1s72, chain *H*, residues Lys35–Ser167; Klein *et al.*, 2004; see Fig. 1). An expression vector for the L10 core domain, named pET32b-L10CD, was constructed by replacing the *Bam*HI–*Eco*RI region of pET32b-L10 with the L10CD coding fragment amplified from pGEX4T1-L10 using primer 3, 5′-AGG ATC CTT TGA CCT GGG GCG GAA AAA GGC AAA AGT G, and primer 4, 5′-AGG ATC CGA GTT TCC GCT TTG TGG CCA TGG TG. The pET32b-L10CD expression vector is designed to express a polypeptide consisting of an N-terminal thioredoxin, a His_6 tag and a thrombin cleavage site followed by the L10CD sequence. The L10CD construct (17.2 kDa, 149 residues) retains part of the eukaryote-specific C-terminal extension in addition to the core fold part (Fig. 1).

3. Overexpression and purification

E. coli strain BL21(DE3) was transformed by the pET32b-L10CD vector and cultured at 310 K in 6.4 l M9 medium supplemented with 100 µg µl^{-1} ampicillin. When the cell concentration reached an OD_{600} (optical density at 600 nm) of 0.2, the culture temperature was lowered to 289 K. Protein expression was induced at an OD_{600} of 0.45 by the addition of 0.2 m*M* IPTG (isopropyl *β*-D-1-thiogalactopyranoside). After further cultivation for 20 h, cells were harvested by centrifugation. Purification steps were performed at 277 K unless specified otherwise. Harvested cells were suspended in 70 ml buffer *A* (22 m*M* sodium phosphate pH 7.0, 500 m*M* NaCl, 5% glycerol, 5 m*M* 2-mercaptoethanol). Cells were lysed ultrasonically and the cell debris was removed by centrifugation at 50 000 rev min^{-1} for 2 h using a 70 Ti rotor (Beckman Coulter). The supernatant was applied onto a 10 ml Ni–NTA agarose column equilibrated with buffer *A*. Weakly bound proteins were washed out with 200 ml of 20 m*M* imidazole in buffer *A* and the target protein was eluted with 70 ml of 200 m*M* imidazole in buffer *A*. Thioredoxin and the His_6 tag were separated from the L10CD protein by thrombin treatment, leaving Gly-Ser residues as a remnant at the N-terminus. 1.0 unit of thrombin (Amersham Pharmacia) was added per 2 ml sample solution; the concentration was adjusted to 1.0 at A_{280} (absorbance at 280 nm) and incubated at 283 K for 48 h. After reducing the thrombin activity by lowering the temperature to 277 K, the tag moiety was trapped using an Ni–NTA agarose column. The L10CD solution was concentrated with an Amicon Ultra-15 (Millipore) and dialyzed in buffer *B* (100 m*M* sodium acetate pH 6.5, 5 m*M* 2-mercaptoethanol). After clarification by centrifugation, 5 m*M* $ZnCl_2$ was added to the L10CD solution, considering the report that L10 binds zinc ion (Inada *et al.*, 1997). For the purpose of the removal of thrombin and further purification, gel filtration was performed using a 100 ml Superdex 75 column equilibrated with buffer *B*. SDS–PAGE analysis showed that the L10CD protein was >95% pure. Approximately 2 mg L10CD protein was obtained per litre of culture.

4. Crystallization and preliminary X-ray analysis

Crystallization was performed by the sitting-drop vapour-diffusion method using a 96-well Intelliplate (Art Robbins Instruments). The L10CD concentration was adjusted to 1.5 mg ml^{-1} in buffer *B* supplemented with 170 µ*M* $ZnCl_2$. 1 µl of the L10CD solution was mixed with 1 µl precipitant solution in the drop well and 100 µl precipitant solution was placed in the reservoir. The crystallization plate was then incubated at 293 K. Crystal Screens I and II (Hampton Research) and Wizard Screens I and II (Emerald Biostructures) were used in the first screen. Hexagonal crystals appeared using solution No. 35 of Wizard Screen II (0.8 *M* sodium dihydrogen phosphate, 1.2 *M* dipotassium hydrogen phosphate and 100 m*M* sodium acetate pH 4.5) in 1–2 d. An additive-screening experiment on this crystallization condition using the Additive Screen kit (Hampton Research) showed that TCEP [tris(2-carboxyethyl)phosphine] improved the crystal size. Thus, 8 m*M* TCEP was added to the L10CD solution for subsequent crystallization experiments. The optimized precipitant solution was 1.0 *M* sodium dihydrogen phosphate, 1.0 *M* dipotassium hydrogen phosphate and 100 m*M* sodium acetate pH 4.5. The microseeding technique (Stura, 1999) was used for further improvement of crystal quality as follows. Crystallization drops containing L10CD crystals were collected in a Seed Bead tube (Hampton Research) and then supplemented with an equal volume of a seed-stabilizing solution which was prepared by mixing 1.5 mg ml^{-1} L10CD solution (supplemented with 8 m*M* TCEP) and precipitant solution (0.8 *M* sodium dihydrogen phosphate, 0.8 *M* dipotassium hydrogen phosphate and 100 m*M* sodium acetate pH 4.5) in a 1:4

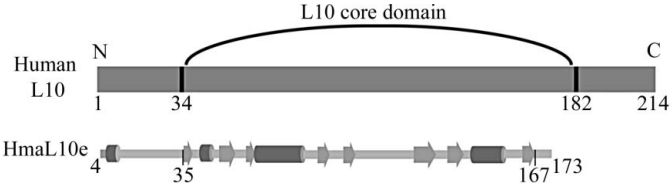

Figure 1
Human L10 core domain designed for crystallization in this study. For reference, the secondary-structure diagram of the *Haloarcula marismortui* L10e structure (HmaL10e; PDB code 1s72, chain *H*; Klein *et al.*, 2004) is aligned beneath. The *β*-strands and *α*-helices/3_{10}-helices are represented by arrows and cylinders, respectively. HmaL10e has a core fold comprised of residues Lys35–Ser167.

Table 1
Statistics of the diffraction data of the hexagonal L10CD crystal.

Values in parentheses are for the outermost resolution shell.

Resolution range (Å)	50–2.50 (2.59–2.50)
No. of observations	64123 (6012)
No. of unique reflections	10870 (1019)
Completeness (%)	98.1 (97.0)
Redundancy	5.9 (5.9)
Average $I/\sigma(I)$	36.6 (3.6)
R_{sym}† (%)	5.0 (43.8)
Space group	$P3_121$‡
Unit-cell parameters (Å, °)	$a = b = 52.72$, $c = 185.49$, $\alpha = \beta = 90.0$, $\gamma = 120.0$

† $R_{sym} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I_{hkl}\rangle$ is the mean intensity of multiple $I_i(hkl)$ observations of symmetry-related reflections. ‡ The enantiomorph $P3_221$ is also possible.

ratio. After mixing on a vortex mixer for 5 min and centrifuging at 20 000*g* for 2 min, the supernatant solution was collected as the seed solution. The seed solution was serially diluted with the seed-stabilizing solution to between 1/4 and 1/10 000 and 0.2 µl of the diluted seed solutions were then added to crystallization drops which had been incubated in advance at 293 K for 1 h. The crystals were grown for one week, reaching dimensions of approximately 100 × 100 × 30 µm (Fig. 2). An antifreeze solution for the L10CD crystal was prepared by mixing 3.4 *M* sodium malonate pH 7.0 and the reservoir solution in a 3:7 ratio. The crystal was briefly harvested into the antifreeze solution using a cryoloop and was then stored in liquid nitrogen. Diffraction data were collected using synchrotron radiation at the SPring-8 BL41XU beamline equipped with an ADSC Quantum 315 detector. The crystal-to-detector distance was set to 350 mm and the diffraction experiment was carried out using radiation of wavelength 1.0000 Å under a nitrogen stream at 100 K. The oscillation angle was 2.0° per image, with an exposure time of 0.5 s; the total oscillation range was 122.0°. Data processing was performed using the *HKL*-2000 program package (Otwinowski & Minor, 1997). Statistics of the diffraction data are summarized in Table 1. The L10CD hexagonal crystal diffracted to 2.5 Å resolution. The most plausible space group is $P3_121$ or its enantiomorph $P3_221$ and the unit-cell parameters are $a = b = 52.72$, $c = 185.49$ Å. Given the presence of two molecules in the asymmetric unit, the Matthews coefficient V_M (Matthews, 1968) and the solvent content were calculated to be 2.16 Å^3 Da^{-1} and 43.1%, respectively. We could not detect the presence of zinc ion in the L10CD crystal by an XAFS experiment (data not shown). Although the L10 solution after gel filtration contains zinc ions according to chemical detection using 4-(2-pyridylazo)-resorcinol (PAR, Dojindo; data not shown), the affinity of L10 for zinc ion reported by Inada *et al.* (1997) may weaken in the L10CD construct or under the crystallization conditions. The L10CD structure could be solved by the molecular-replacement method using the archaeal L10 structure (PDB code 1s72, chain *H*; Klein *et al.*, 2004) as a model. Model refinement of the L10CD structure is now in progress.

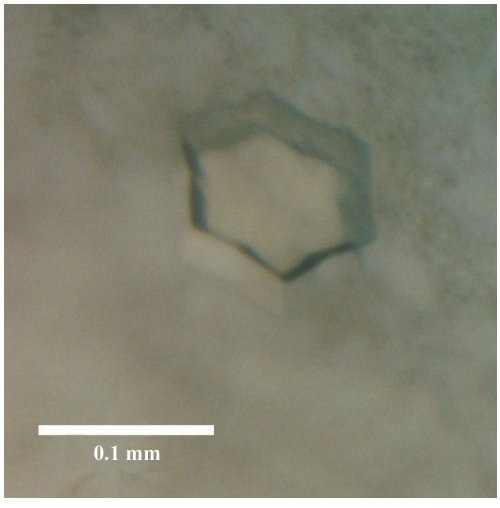

Figure 2
Hexagonal crystal of L10CD.

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