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Expression, purification, crystallization and preliminary X-ray diffraction studies of bacterial and archaeal L4 ribosomal proteins

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Ribosomal protein L4 is implicated in the peptidyltransferase activity of the ribosome and in certain bacteria it regulates the transcription and translation of the 11-gene S10 operon. The genes for the L4 ribosomal proteins from the hyperthermophilic bacterium *Thermotoga maritima* and the halophilic archaeon *Haloarcula marismortui* have been PCR amplified from genomic DNA and cloned under the control of a T7 promoter to generate overexpressing *Escherichia coli* strains. For both proteins, efficient purification procedures were developed to yield material suitable for crystallization trials. Crystals of *T. maritima* L4 were obtained in the orthorhombic space group $P2_12_12_1$, with one molecule per asymmetric unit, diffracting to 1.7 Å resolution with synchrotron radiation. Crystals of *H. marismortui* L4 belonged to the trigonal space group $P3_121$ or $P3_221$ and diffracted to 3.2 Å resolution with a rotating-anode source, presumably containing three molecules per asymmetric unit. The results demonstrate that for certain halophilic proteins the same purification and crystallization procedures can be employed as for conventional proteins.

1. Introduction

Ribosomal (r-) L4 proteins belong to the largest polypeptides in the prokaryotic translational machineries. The conserved bacterial variants are the best characterized L4 proteins among the three kingdoms of life. They are primary binding r-proteins with an essential role in the early folding process of 23S rRNA (Nierhaus, 1991). *E. coli* L4 was shown to be important for structuring a substantial portion of the 23S rRNA, presumably by connecting RNA segments which are distant in the primary sequence (Maly *et al.*, 1980; Gulle *et al.*, 1988; Nierhaus, 1991). Some biochemical data indicate that L4 is located near the peptidyltransferase centre of the ribosome and may be actively involved in the catalysis of peptide-bond formation (Hampl *et al.*, 1981; Sumpter *et al.*, 1991). *E. coli* L4 was also shown to be a feedback regulatory protein, adjusting the expression of its own S10 operon at the level of both transcription and translation (Yates & Nomura, 1980; Zengel *et al.*, 1980). The mechanism of transcriptional control seems to be mediated by the stabilization of a pre-termination complex consisting of RNA polymerase paused at a NusA-dependent terminator (Zengel & Lindahl, 1990, 1996). It was demonstrated that L4 proteins from other bacteria share this transcriptional regulatory feature and are able to control the S10 operon in *E. coli* (Zengel *et al.*, 1995).

There is less information available on archaeal or eukaryotic L4. Sequence alignments reveal that the proteins from these kingdoms show clear homologies to each other, whereas there are only subtle similarities to the bacterial equivalents. However, the equivalent positioning of the corresponding genes in the archaeal and bacterial operons suggests similar functions for the proteins (Auer *et al.*, 1989; Arndt *et al.*, 1990; Bult *et al.*, 1996). In order to determine whether the L4 proteins share a common fold across kingdoms, we set out to solve the three-dimensional structures of a bacterial and an archaeal variant from *T. maritima* (TmaL4) and *H. marismortui* (HmaL4), respectively. Both proteins have a molecular weight of about 26 kDa and exhibit 28% sequence identity.

2. Experimental

2.1. Cloning and expression

The genes for TmaL4 and HmaL4 were extracted from the respective genomic DNA preparations by PCR (Saiki *et al.*, 1985), using primers with overhanging restriction sites. The PCR products were cloned into the T7-promoter based pET22b(+) and pET11c expression vectors (Novagen, Abingdon, England), respectively. PCR, restriction digests, ligation reactions and bacterial transformations were performed according to

standard protocols. The correct insert sequences of the plasmids were verified by automated dideoxynucleotide sequencing (PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit, Perkin Elmer, Überlingen, Germany).

Expression of both proteins was performed in 12 l cultures of *E. coli* strain BL21(DE3)/pLysS. Freshly transformed cells were grown to an OD_{595} of 0.6 and expression was induced by the addition of 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG). In order to reduce the formation of inclusion bodies, cells were cultivated at room temperature for 6 h after induction. The bacteria were harvested by centrifugation and resuspended in 20 ml harvest buffer (20 m*M* HEPES pH 7.0, 50 m*M* LiCl, 10 m*M* $MgCl_2$ for TmaL4; 20 m*M* Tris–HCl pH 7.6, 50 m*M* NaCl, 2 m*M* EDTA for HmaL4). Cells were frozen in liquid nitrogen and stored at 203 K.

2.2. Protein purification

The cell suspensions were thawed at room temperature and, after addition of phenylmethylsulfonyl fluoride to 100 µg ml^{-1}, were subjected to two rounds of sonification (Branson macrotip sonifier, Danbury, CT, USA; 100% output, 50% interval, 5 min). Cell debris were removed by ultracentrifugation (UC; 3 h, 50 000 rev min^{-1}; 55.2 Ti rotor, Beckman Instruments, Palo Alto, CA, USA).

The UC supernatant of the TmaL4 expression was brought to 3 *M* LiCl with a buffered 8 *M* stock solution. The solution was kept on ice for 1 h and separated from the precipitate by centrifugation. The soluble fraction was heated to 343 K for 20 min to denature the host proteins, the precipitate was spun down and the supernatant was dialyzed against buffer *A* (10 m*M* HEPES pH 7.0, 50 m*M* LiCl, 3 m*M* EDTA). The dialysate was applied to a 300 ml DEAE-Sepharose FF column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer *A*. The eluate of a 2 l gradient to buffer *A* plus 0.9 *M* LiCl was checked for TmaL4 by SDS–PAGE and the pooled fractions were loaded onto a 150 ml hydroxylapatite column (Biorad, Hercules, CA) equilibrated with buffer *B* (50 m*M* Tris–HCl pH 7.6, 2 *M* KCl). TmaL4 was eluted in a 1 l gradient to buffer *B* supplemented with 250 m*M* potassium phosphate, pH 7.6. The relevant fractions were pooled and concentrated with Centriprep-3 concentrators (Amicon, Beverley, MA, USA). The concentrated pool was buffer-exchanged to 10 m*M* HEPES pH 7.0, using NAP-25 columns (Amersham Pharmacia Biotech). Protein at a concentration of 10 mg ml^{-1} was frozen in liquid nitrogen and stored at 203 K. About 3–4 mg of pure protein was obtained per 1 l culture.

For HmaL4, the clarified lysate was applied to a 300 ml DEAE-Sepharose FF column equilibrated with buffer *C* (20 m*M* Tris–HCl pH 7.6, 50 m*M* NaCl). Bound material was eluted with a 700 ml linear gradient to buffer *C* plus 400 m*M* NaCl. The eluate was inspected by SDS–PAGE and fractions containing the protein were pooled. The pool was adjusted to 1 *M* $(NH_4)_2SO_4$ and applied to a 75 ml phenyl Sepharose HP column (Amersham Pharmacia Biotech) in buffer *D* [20 m*M* Tris–HCl pH 7.6, 1 *M* $(NH_4)_2SO_4$, 50 m*M* NaCl]. The efflux of a 700 ml gradient to buffer *C* was inspected as before. The combined fractions were dialyzed against buffer *E* (20 m*M* MES pH 6.0, 10 m*M* NaCl) and again loaded onto a DEAE column. A 700 ml gradient to buffer *E* plus 400 m*M* NaCl eluted HmaL4, which was pooled and dialyzed against 20 m*M* NaOAc pH 5.0, 10 m*M* KCl. The precipitate formed during the dialysis was harvested by centrifugation, dissolved in 2 ml 20 m*M* MES pH 6.0, 400 m*M* NaCl (buffer *F*) and applied to a 20 ml hydroxylapatite column. A 200 ml gradient to 100 m*M* potassium phosphate buffer was applied and HmaL4-containing fractions were pooled and dialyzed against 10 m*M* HEPES pH 7.0, 1 *M* KCl. The solution was concentrated to 4 mg ml^{-1} using Centricon-10 concentrators, frozen in liquid nitrogen and stored at 203 K. The purity of the final material was greater than 95% as judged by SDS–PAGE and N-terminal peptide sequencing. The typical yield was around 5 mg of protein per 1 l culture.

2.3. Crystallization and X-ray data collection

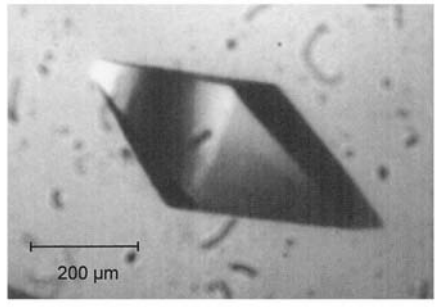
Crystals of TmaL4 were grown *via* sitting-drop vapor diffusion within 3–4 d by mixing 3 µl protein solution (10 mg ml^{-1} in 10 m*M* HEPES pH 7.0) with 1.5 µl reservoir [0.1 *M* citrate pH 3.8–4.5, 35–38%(*v*/*v*) polyethylene glycol (PEG) 400 and 0.2 *M* ammonium acetate]. They reached maximum dimensions of 400 × 200 × 200 µm within one week. The PEG 400 in the reservoir served as a cryo-protectant and allowed data collection at 100 K.

For crystallization of HmaL4, frozen aliquots were thawed on ice, buffer-exchanged to 10 m*M* HEPES pH 7.0 using NAP-25 columns and the protein concentration was adjusted to 6 mg ml^{-1} (Centriprep-10 concentrators). Crystals were obtained by sitting-drop vapor diffusion (3 µl protein solution, 1.5 µl reservoir) at room temperature within 2–3 d, employing reservoirs of 0.1 *M* HEPES pH 7.2, 0.2 *M* $CaCl_2$, 25% PEG 4000 or 0.1 *M* MES pH 6.4, 0.2 *M* calcium acetate, 18% PEG 8000. The crystals grew to maximum dimensions of 300 × 300 × 300 µm.

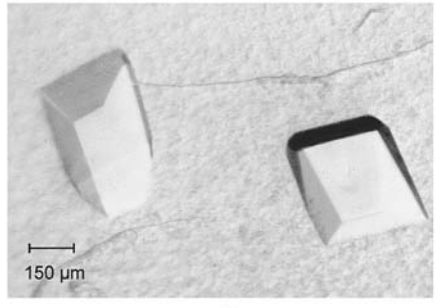
X-ray data for both crystal forms were initially collected at room temperature on a MAR Research (Hamburg, Germany) imaging-plate system mounted on a Rigaku (Tokyo, Japan) RU-200 rotating-anode X-ray generator, which produced Cu $K\alpha$ radiation (λ = 1.5418 Å) at 50 kV and 100 mA. In the case of TmaL4, a high-resolution data set was subsequently recorded at the Deutsche Elektronen Synchrotron (DESY), Hamburg, Germany, on a MAR Research CCD detector. Data sets were processed with the *HKL* program package (Otwinowski & Minor, 1997).

3. Results and discussion

Under non-denaturing conditions, some bacterial L4 species are difficult to purify and high salt concentrations are necessary to extract these primary binding r-proteins from the rRNA (Dijk & Littlechild, 1979). For the purification of TmaL4, we successfully exploited its inherent thermostability in



(*a*)



(*b*)

Figure 1
(*a*) Orthorhombic crystal of TmaL4. (*b*) Trigonal crystals of HmaL4.

Table 1
Data-collection statistics.

RT, room temperature. Values in parentheses are statistics for the highest resolution shell (3.29–3.2 Å for HmaL4, 1.73–1.70 Å for the cryo data set of TmaL4 and 2.56–2.5 Å for the RT data set of TmaL4).

Data set	Resolution range (Å)	Completeness (%)	R_{merge}† (%)	No. of unique reflections	Multiplicity	Unit-cell dimensions (Å)		
						a	*b*	*c*
HmaL4 (RT)	25–3.2	83.9 (87.0)	9.8 (42.9)	10413	2.5	79.4	79.4	196.1
TmaL4 (100 K)	15–1.7	92.8 (88.0)	5.4 (34.4)	23468	3.4	43.0	48.6	112.0
TmaL4 (RT)	20–2.5	92.8 (93.8)	5.2 (21.7)	7999	3.7	43.0	48.6	112.0

† $R_{merge} = \sum|I_i - \langle I\rangle|/\sum I$, in which I_i is an individual intensity measurement and $\langle I\rangle$ is the averaged intensity for this reflection.

combination with high concentrations of LiCl. Phenyl Sepharose columns had to be avoided because TmaL4 could not be recovered intact from the matrix. A nucleic acid fraction strongly co-purified with TmaL4 and could only be removed by hydroxylapatite chromatography in the presence of 2 *M* KCl. HmaL4 was treated at salt concentrations normally employed in the purification of non-halophilic proteins. Under these conditions, it either withstood a loss in solubility and folding or it refolded under the crystallization conditions. However, the crystallizability of the HmaL4 preparations varied from batch to batch.

Crystals of TmaL4 were obtained under high [35–38%(*v*/*v*)] PEG 400 concentrations, allowing their direct mounting in a liquid-nitrogen stream without additional cryoprotectants (Fig. 1). While the crystals diffracted to about 2.5 Å resolution on a rotating-anode X-ray source, high-quality data could be collected to 1.7 Å resolution using synchrotron radiation (Table 1). The crystals belonged to the primitive orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 43.0, b = 48.6, c = 112.0 Å, suggesting one monomer per asymmetric unit (Matthews coefficient 2.2 Å^3 Da^{-1}; Matthews, 1968). Crystals of HmaL4 diffracted to about 3.2 Å resolution using a rotating-anode generator when conventionally mounted in a special glass capillary with mother liquor at one end. They belonged to one of the enantiomorphic primitive trigonal space groups $P3_121$ or $P3_221$ (Fig. 1). The unit-cell parameters were determined to be $a = b$ = 79.4, c = 196.1 Å. Three monomers per asymmetric unit yielded a reasonable Matthews coefficient (2.1 Å^3 Da^{-1}), but self-rotation searches failed to detect a non-crystallographic threefold symmetry. The slow deterioration of the crystals in the beam prevented the collection of data sets with a completeness greater than 84% from single crystals. So far, no suitable cryoprotectant has been found for the HmaL4 crystals. Data-collection statistics for both crystals are summarized in Table 1. Because TmaL4 crystals are easier to handle and are more reproducible than their HmaL4 counterparts, we set out to solve the TmaL4 crystal structure first. It might function afterwards as a model in a Patterson rotation/translation search for HmaL4 (Hoppe, 1957; Huber, 1965).

The present communication is only the second crystallization report of an archaeal ribosomal protein (Tishchenko *et al.*, 1998). Novelly, HmaL4 stems from a halophilic species, promising insights into the protein–RNA recognition features under high salt conditions. The prospective crystal structure should also have immediate relevance for the ongoing model building of the entire *H. marismortui* 50S ribosomal subunit (von Bohlen *et al.*, 1991; Ban *et al.*, 1999). TmaL4, conversely, is a representative of the bacterial L4 group. Its structure will hopefully shed some light on the protein's incorporation into the ribosome *via* rRNA binding and on the mechanisms of the transcriptional and translational control of the S10 operon. A bacterial large ribosomal subunit has previously been crystallized (Mussig *et al.*, 1989; Volkmann *et al.*, 1990) and for the eventual construction of bacterial 50S atomic models the corresponding r-protein structures will be invaluable.

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