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Thermus thermophilus L4 ribosomal protein: purification and sensitivity alteration against erythromycin of *E. coli* cells harboring a single amino acid mutant of TthL4 within its extended loop

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Summary. Protein L4 from the thermophilic bacterium *Thermus thermophilus* (TthL4) was heterologously overproduced in *Escherichia coli* cells and purified under native conditions by using ion exchange chromatography. Although it's known strong binding to RNA (23S rRNA as well as mRNA) the yield of the purified protein was 6 mg per 10 g of cells and it is similar to that referred for *Thermotoga maritima* L4 ribosomal protein.

In addition, *E. coli* cells harboring the wild type *Thermus thermophilus* L4 (wtTthL4) ribosomal protein as well as its mutant having changed the highly conserved glutamic acid 56 by alanine (TthL4-Ala 56) were incorporated into *E. coli* ribosomes after transformation of the host cells with the recombined vector. The cells having incorporated the mutant TthL4-Ala56 are more sensitive against erythromycin related to that containing the wtTthL4 protein.

The resistance to the drug indicates that the mutated amino acid Glu56 is probably critical for the local ribosomal conformation and that its mutation induces conformational disturbances that are "transferred" to the entrance of the major exit tunnel, the place where the drug does bind.

Keywords: Ribosomal proteins – Mutations – Erythromycin sensitivity

Introduction

The prokaryotic ribosome consists of two subunits, 50S (large) and 30S (small), containing over than 50 different proteins in complex with RNA molecules. Recently, complete atomic structures for the large (Ban et al., 2000, Harms et al., 2001) and small (Wimberly et al., 2000) ribosomal subunit from bacteria were determined by X-ray crystallography, and it is evidenced that the ribosomal primary activities, the decoding process and peptide bond formation, occur in the interior ribosomal compartment (Ban et al., 2000; Nissen et al., 2000) which is composed largely of rRNA.

Ribosomal (r) L4 proteins 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).

Protein L4 from *Thermus thermophilus* shows clear homology to the other L4 proteins from bacteria. As it mentioned before it is a highly important protein for the translational function. Together with the L22 protrudes into the exit tunnel region, where their extended tips along with rRNA residues form that it appears to be a gate opening (Jenni and Ban, 2003). It has been suggested that the exit tunnel, whose existence has been confirmed by crystallographic data (Nissen et al., 2000), is the normal exit path for nascent polypeptides before emerging from the ribosome (Tenson and Ehrenberg, 2002). However, there is also evidence suggesting that some proteins may leave the PTase center through other routes (Komar et al., 1997; Gabashvili et al., 2001). Interestingly, even if a growing peptide has decided to enter the tunnel, several exit possibilities remain, since the exit tunnel branches at the backside of the large subunit (Gabashvili et al., 2001).

The role of the exit tunnel gate is still under investigation; it might function as a sensor recognizing special features of the nascent peptide chain and transmitting messages to the PTase center through L22 and L4 proteins (Nissen et al., 2000), it might monitor alternative exits for the nascent peptides (Komar et al., 1997; Gabashvili et al., 2001; Nakatogawa and Ito, 2002), or it might regulate the protein elongation cycle by stopping or modulating

the traffic (Tenson and Ehrenberg, 2002; Gabashvili et al., 2001).

Antibiotics that contain an extending arm, reaching the A-site, inhibit directly the PTase reaction (Hansen et al., 2002; Dinos and Kalpaxis, 2000), whereas antibiotics that lack such extensions, like erythromycin, inhibit protein synthesis by blocking the exit-tunnel and inducing premature dissociation from the ribosome of short peptidyl-tRNAs with six, seven or eight amino-acid residues (Tenson et al., 2003; Lovmar et al., 2004)

In this work we present the purification of *Thermus thermophilus* L4 protein (TthL4) – heterologous overproduced in *E. coli* – in native form without using chemicals that would destroy its conformation. The protein is purified in quite high amount although it's strong basic character as well as its property to interact primarily with RNA that made its purification steps difficult. Namely, 6 mg protein have been purified from 10 g *E. coli* cells and have been identified by 2D-electrophoresis PAGE.

In addition, in order to investigate its extended loop function that protrudes into the exit tunnel region the highly conserved Glutamic acid 56 was changed to alanine (TthL4-Ala56) and after incorporation within the *E. coli* ribosomes the host *E. coli* cells were tested for the erythromycin sensitivity. Our results clearly show a hypersensitivity to erythromycin which probably means that the local conformation of the protein itself enhances the sensitivity against the drug or that some 23S rRNA nucleotides near to the drug binding region are ''delocalized'' leading to a stronger binding.

Materials and methods

Materials

Restriction endonucleases and dNTPs were purchased from New England Biolabs. Expand^{TM} Long Template PCR System and the Rapid Ligation Kit were from Boehringer Mannheim. IPTG was from Appligene. Oligonucleotides used as primers in the PCRs, were synthesized by TIB MOLBIOL Berlin.

PCR amplification, isolation and cloning of TthL4 gene

Based on the known sequence of TthL4 gene (Pfeiffer et al., 1995), two oligonucleotides with suitable cloning sites at their 5′-end were utilized for the amplification of TthL4 gene by PCR, using genomic DNA from *T. thermophilus* as template. The wild-type sequence of the amplified TthL4 was identified by nucleotide sequence and cloned into pET11a plasmid.

In vitro mutagenesis of TthL4

The mutant of TthL4 gene was produced, using a three-step PCR mutagenesis protocol (Picard et al., 1994). The codon for glutamic acid at position 56 was replaced by the codon for alanine. The template used, was the recombinant plasmid pET11a/wtTthL4. The PCR product was purified, cloned into pET11a, and screened for the desired mutation by nucleotide sequencing.

Overexpression of wild-type or mutant TthL4 in E. coli *cells*

E. coli BL21 (DE3) cells were transformed with recombinant pET11a plasmid carrying either the wtTthL4 or the mutant. The transformed cells were grown at 37 °C until absorption of 0.6–0.7 at 600 nm was achieved. The expression of the heterologous protein was induced by the addition of 1 mM IPTG to the growth medium. The cells were harvested 3 h after IPTG induction, and then disrupted and their protein content was analyzed by SDS-12% polyacrylamide gel electrophoresis. The proteins were visualized by staining with Coomassie Blue R-250.

Purification of the recombinant wtTthL4

E. coli cells (10 g) expressing wtTthL4, were harvested by centrifugation and resuspended in 40 ml of lysis buffer containing 20 mM Tris–HCl pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$, 0.8 M NaCl, 1 mM PMSF and 7 mM 2-mercaptoethanol. The resuspended cells were disrupted by sonication (Dupont Instruments; Sorvall, Omni-mixer 17106) and the cell lysate was centrifuged first at $10{,}000 \times g$ for 20 min, and then at $100{,}000 \times g$ for 2 h to remove cell debris and ribosomes. The final supernatant (S100) was dialyzed against lysis buffer containing 0.4 M NaCl, and then against the same buffer containing 50 mM NaCl. The dialysate after treatment with RNase, was loaded onto a 20-ml pre-equilibrated DEAE-Sepharose CL 6B column with buffer A (20 mM Tris–HCl pH 7.5) containing 50 mM NaCl, and eluted with a 0.05–2 M linear gradient of NaCl in buffer A. Fractions were characterized by SDS-12% polyacrylamide gel electrophoresis. The peak fractions containing the heterologous protein were pooled and dialyzed against buffer B (20 mM Tris–HCl pH 6.5) containing 100 mM NaCl. The resulting dialysate was loaded onto a 20-ml pre-equilibrated CM-Sepharose CL-6B column with the same solution. TthL4 protein was eluted with a 100–650 mM linear gradient of NaCl in buffer B. The peak fractions containing TthL4 protein were further purified by chromatography on a second CM-Sepharose CL-6B column, eluted as above. The final product was subjected to SDS-12% polyacrylamide electrophoresis, followed by electroblotting onto PVDF membranes (Immobilon-P, Millipore) and sequencing at a ProciseHT sequenator (Choli et al., 1989).

Incorporation of the TthL4 into E. coli *ribosomes*

The incorporation of wild type or mutant TthL4 into *E. coli* 70S ribosomes was examined by a 2D-urea PAGE electrophoresis, according to Geyl et al. (1981). Briefly, total proteins from 70S ribosomes (TP-70) extracted with two volumes of acetic acid (Hardy et al., 1969) were dialyzed against water, dried and then subjected to electrophoresis followed by electroblotting onto PVDF membranes (Choli et al., 1989). After Coomassie Blue staining, the spots corresponding to TthL4 species were identified by *N*-terminal protein sequencing, as described above. The degree of incorporation was estimated by Image Analysis of the stained gels (Molecular Dynamics Densitometer and Image Quant 5.0 Software for scanning and quantitating wet gels and X-ray plates, University of Virginia, ITC-Academic computing Health Sciences).

Sensitivity to erythromycin of E. coli *cells expressing wild-type or mutant TthL4 protein*

Cells (70 µl of a 0.900 OD_{600} pre-culture) were added in 7 ml of LB medium containing 50 µg/ml ampicillin and grown at 37 °C in the presence or in the absence of erythromycin until the optical density of the control culture (*E. coli* cells expressing wtTthL4 protein and grown in the absence of erythromycin) reached the value 1.000 at 600 nm. The concentration of erythromycin ranged from 0 to 180 µg/ml. A 10 µg/ml concentration is equivalent to 13.62 µM erythromycin.

Results and discussion

Purification and characterization of the recombinant wtTthL4 protein

After successful overproduction of TthL4 protein in *E. coli* cells (Fig. 1, lane 2), the cellular lysate was discharged from cell debris and ribosomes by two sequential centrifugation, the later one run at 100,000 × *g*.

The purification of ribosomal proteins would be expected to be in generally an easy task taking into account the high number of the published ribosomal proteins. However, as it is clearly shown the purification of *Thermus thermophilus* L4 ribosomal protein was unexpected not so easy. Therefore, the next steps of purification were designed, taking seriously into account the highly basic character of TthL4 that possesses a pI of 10.03.

Due to our experience the purification of ribosomal proteins (Triantafillidou et al., 1999; Tsiboli et al., 1994, 1998; Katsani et al., 2000), that bind primarily to rRNA, is quite difficult in high yields although their good overproduction. Therefore, the supernatant achieved by ultracentrifugation, S100, (Fig. 1, lane 3) was analyzed by a DEAE-Sepharose column, in order to remove the negatively charged molecules, such as acidic proteins and traces of nucleic acids. Unfortunately, only a small amount of the loaded protein was identifiable in the flow through of the column. Evidently, the bulk of TthL4 protein was trapped on the column, probably via nonspecific binding to coexisting nucleic acids. Pre-incubation of the sample with DNase, free of RNase, did not substantially improve the recovery of TthL4. In contrast, pre-treatment of S100 with RNase was more effective. Here it has to be underlined that after this treatment the final protein yield was indeed increased but a part of it was denatured and should be removed by centrifugation after the dialysis of the protein against 50 mM NaCl–20 mM Tris–HCl pH 7.5. The dialysate after treatment with RNase, was loaded onto a 20-ml pre-equilibrated DEAE-Sepharose CL 6B column with buffer A (20 mM Tris–HCl pH 7.5) containing 50 mM NaCl, and eluted with a 0.05–2 M linear gradient of NaCl in buffer A. Fractions were characterized by SDS-12% polyacrylamide gel electrophoresis. Most of the bound TthL4 was eluted at a concentration NaCl equal to 0.25 M (Fig. 1, lane 4). The fractions containing the TthL4 protein were pooled, dialyzed against 100 mM NaCl-20 mM

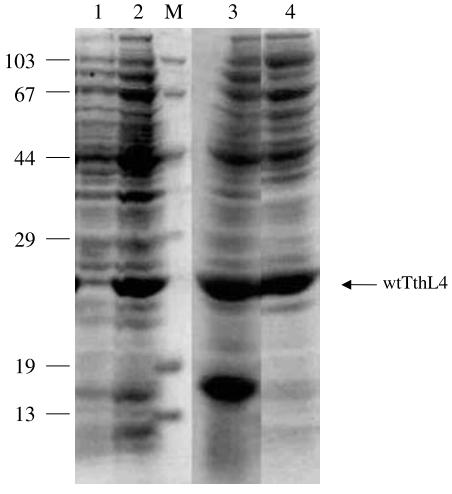

Fig. 1. SDS-PAGE analysis of protein samples (10 μg) after purification steps. *1* Cellular extract of non-induced BL21 *E. coli* cells transformed with the recombinant plasmid pET11a/wtTthL4; *2* cellular extract of the same cells after induction with 1 mM IPTG; *M* molecular weight markers; *3* supernatant of lysed cells after ultracentrifugation at 100,000 × *g* (S100) applied on the DEAE-sepharose ion exchange column; *4* DEAE fractions applied on CM-sepharose column. The TthL4 protein is indicated

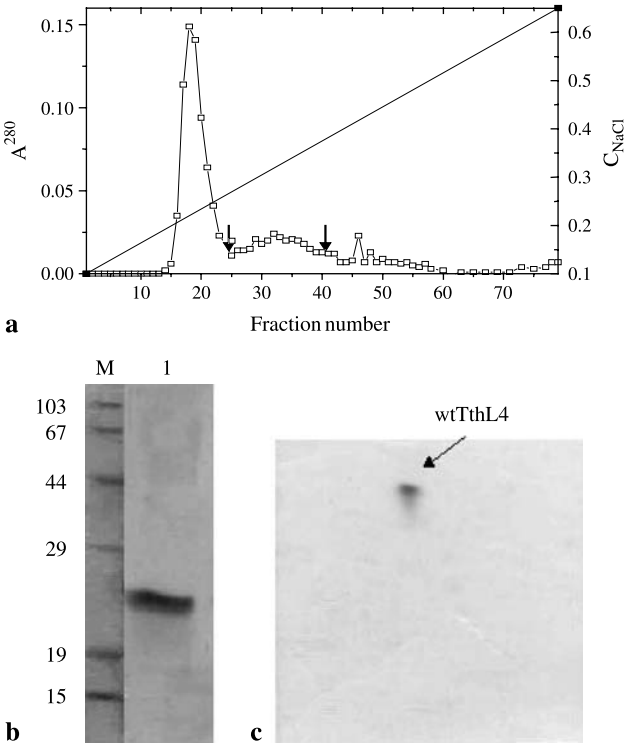

Fig. 2. Elution profile of CM-sepharose column and identification of the purified TthL4 protein by electrophoresis. **a** Elution profile of the CM-sepharose column. After equilibration with 20 mM Tris–HCL pH 6.5 and 0.1 M NaCl and washing with the same buffer, applying a linear gradient of 0.1–0.65 M NaCl eluted the bound protein. The white squares (□) indicate absorption at 280 nm. The arrows indicate the elution position of the pure TthL4 as well as the fractions (25–40) that were collected and analysed as described below. **b** SDS-PAGE electrophoresis of purified TthL4 from the collected fractions of L4 peak as indicated in **a**. **c** 2D-electrophoresis of purified TthL4 protein

Tris pH 6.5, loaded onto a 20-ml pre-equilibrated CM-Sepharose column and the protein was eluted with a 100–650 mM linear gradient of NaCl in 20 mM Tris pH 6.5 (Fig. 2a). The peak fractions, indicated by an arrow, were found by one- (Fig. 2b) or two-dimensional urea PAGE electrophoresis (Fig. 2c) to contain TthL4 in high purity. The purified protein was further characterized by electro blotting onto PVDF membrane and *N*-terminal sequencing at a ProciseHT sequenator (Choli et al., 1998). The purity of the final material was greater than 95% as judged by SDS-PAGE and its yield 6 mg per 10 g of cells, similar to that referred for *Thermotoga maritima* L4 ribosomal protein (Worbs and Wahl, 2000).

Ribosome preparation and evaluation of wild-type or mutant TthL4 incorporation

E. coli BL21 (DE3) cells transformed with the appropriate recombinant plasmids, were prepared according to Kalpaxis et al. (1986). The isolated ribosomes were further purified by two sequential centrifugations at 100,000 × *g* through a 0.5 M sucrose cushion made up in association buffer (100 mM Tris–HCl pH 7.2, 100 mM NH_4Cl, 6 mM $MgCl_2$, 100 μM spermine, 6 mM 2-mercaptoethanol) in order to remove excess of the recombinant proteins non specifically bound to the ribosome surface.

As described in Materials and methods, wtTthL4 as well as the mutant TthL4-Ala56 were cloned into pET11a plasmid and over expressed, after induction by isopropyl b-D-thiogalactopyranoside (IPTG), in *E. coli* BL21 (DE3) cells. Both of them were over expressed at high levels (Fig. 2b and c, respectively). The growth in the presence of IPTG was monitored for all transformed cells. *E. coli* BL21 (DE3) cells, had generation time, ranged between 30 and 35 min and cells overproducing wtTthL4 as well as TthL4-Ala56 had a minor but statistically significant effect on the doubling time (45 min).

In order to examine the incorporation of TthL4 protein into the ribosomes total protein amount (TP-70) was extracted with two volumes of acetic acid, and their identification was performed by electro blotting onto PVDF membranes and N-terminal sequencing, as described in Materials and methods.

As shown in Fig. 3b, the endogenous *E. coli* L4 and the thermophilic TthL4 exhibit different charges, namely *E. coli* L4 has a pI equal to 9.72 while TthL4 a pI of 10.03 and thereby appear at distinct positions onto 2D-Urea gels. In Figs. 3b and c an extra spot is apparent at the right of the EcL4 spot, corresponding to the incorporated wtTtL4 and TthL4-Ala56, respectively. In contrast, the

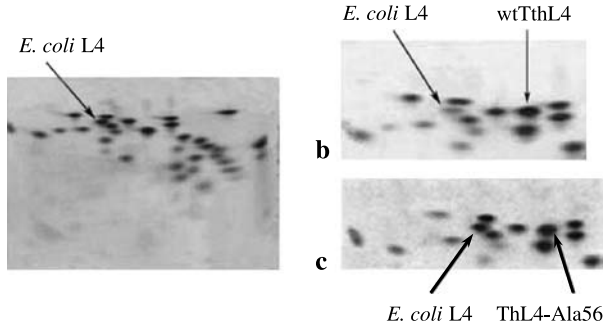

Fig. 3. 2D-gel electrophoresis of total proteins (TP-70) extracted from the 70S ribosomes. **a** TP-70 ribosomal proteins isolated from ribosomes of *E. coli* BL21 (DE3) cells. **b** TP-70 ribosomal proteins isolated from *E. coli* cells over producing wtTthL4. **c** TP-70 ribosomal proteins isolated from *E. coli* cells over producing TthL4-Ala56 mutant. The arrows indicate the spots corresponding to endogenous ribosomal protein L4 (ECL4) as well as the spots corresponding to the incorporated wild type or mutant TthL4

electrophoretic pattern illustrated in Fig. 3a differs remarkably due to the absence of any kind of TthL4 spot.

The present investigation was carried out by using TthL4 instead of *E. coli* L4 (EcL4). In an attempt to incorporate His-tagged EcL4 into *E. coli* ribosomes, it was realized that the overproduced EcL4 protein could not be identified by 2D-PAGE (data not shown) and, therefore the use of another L4 was required. The degree of incorporation for both the wild type TthL4 as well as the TthL4-Ala56 mutant was estimated by Image Analysis of the stained gels as described in Materials and methods.

Sensitivity to erythromycin of E. coli *cells expressing wild-type or mutant TthL4-Ala56 protein*

The target site for macrolide interaction is the bacterial ribosome. Erythromycin destabilizes the peptidyl-t-RNA: ribosome interaction causing release of the peptidyl-tRNA into the cytoplasm. In addition, since macrolides are not bound covalently to the ribosomes, they likely dissociate in vivo at some frequency, thus freeing up the ''engaged'' ribosome to complete polypeptide chain synthesis normally. It is likely that most nascent peptides exit the ribosome through a tunnel in the 50S subunit (Nissen et al., 2000; Tenson and Ehrenberg, 2002). The binding sites for the microlides are located in the beginning of this tunnel, before it is constricted by the ribosomal proteins L4 and L22 (Hansen et al., 2002; Schlunzen et al., 2001). Three 23S rRNA nucleotides, namely A2058, A2059 and A2062 (*E. coli* numbering) are essential for binding of the macrolides. One explanation for the effects of these drugs could therefore be that they prevent nascent peptides from

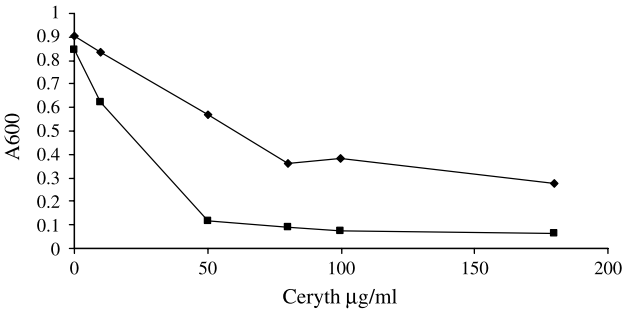

Fig. 4. Inhibition of growth of *E. coli* cells by erythromycin expressing wild-type or mutant TthL4 protein. Cells were grown at 37 °C in LB medium containing 50 μg/ml ampicillin and erythromycin at the indicated concentrations until the optical density of the control culture (cells expressing wtTthL4 and grown in the absence of erythromycin) reached the value 1.000 at 600 nm. This optical density was taken as 100%. Overproduced protein: (◆) wtTthL4, (■) TthL4-Ala56

entering the tunnel. When this happens one would expect that further peptide elongation is inhibited, and that the only way a peptidyl-tRNA can leave the ribosome is in a drop-off event. Although the target of the drug has been found by crystallographic data to be the above mentioned bases of the 23S rRNA several works have been published about the appearance of resistance to erythromycin due to single mutations or deletions within the ribosomal proteins L4 and L22 (Gabashvili et al., 2001; Pardo and Rosset, 1977; Malbruny et al., 2002).

In Fig. 4 we show the different behaviour of wtTthL4 and its mutant TthL4-Ala56 incorporated within *E. coli* ribosomes against different concentrations of erythromycin. The aim to mutate the amino acid Glu-56 to Ala was based on the fact that Glu-56 is a highly conserved amino acid that has been found to be essential for the loop stabilization of TthL4 (Leontiadou et al., 2003). By taking into account the importance of this region that is extended into the entrance of the major tunnel we suggested another indirect function which would be related to the erythromycin binding. Therefore MIC of cells transformed with the recombined vectors pET11a with wtTthL4 and/or the mutant TthL4-Ala56 was done in the absence and in the presence of the drug at different concentrations, as described in Materials and methods. Thus, cells harbouring the TthL4-Ala56 showed a greater sensitivity against erythromycin even at 50 μg/ml in contrast to the cells having incorporated the wtTthL4 protein. One possible explanation for this effect would therefore be the distortion of the nucleotides of 23S rRNA that bind to the drug in a way to allow perhaps its binding for longer time or their greater exhibition to the drug due to the transferred conformational changes of the protein loop to the local region of the erythromycin binding. In any case, this in vivo observation leads to several suggestions concerning the ability of cells to be resistant or sensitive against antibiotics. Here we have to underline that until now several works even with clinical isolates of other bacteria point out the resistance of cells against macrolides due to mutations on ribosomal proteins and not their sensitivity against the same antibiotics.

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