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Crystals of a mutant form of ribosomal protein L22 rendering bacterial ribosomes resistant to erythromycin

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A mutant form of *Thermus thermophilus* ribosomal protein L22 responsible for erythromycin resistance has been overexpressed in *Escherichia coli*, purified to homogeneity and crystallized using the hanging-drop vapour-diffusion technique. While several different crystallization conditions were found, only one set of conditions yielded crystals suitable for X-ray diffraction analysis. These crystals grow as thick plates, with unit-cell parameters a = 31.8, b = 86.59, c = 38.96 Å, β = 104.47°. The crystals belong to the space group $P2_1$ and diffract to 1.8 Å resolution. On the basis of density calculations, two monomers are predicted per asymmetric unit (V_M = 2.06 Å^3 Da^{-1}), with a solvent content of 40%.

1. Introduction

The translational functions of the bacterial ribosome are the targets for a large number of antimicrobial agents. Erythromycin, a 14-membered macrolide antibiotic, has an equivalent inhibitory effect on the formation of the 50S ribosomal subunit in growing bacterial cells and on the blocking of the nascent peptide elongation after two to five residues (Champney & Burdine, 1995). The majority of antibiotic sites are formed by rRNA segments located distantly in the 23S rRNA sequence but brought together in the tertiary structure of the ribosome. There is considerable evidence of the participation of ribosomal proteins in establishing the three-dimensional fold of rRNA into a catalytically active structure (Green & Noller, 1997). The ribosomal protein L22 is one of the five proteins necessary for the formation of an early intermediate of the 23S rRNA in the assembly of the large ribosomal subunit (Nierhaus, 1990). Furthermore, a deletion of three amino-acid residues Met82-Lys83-Arg84 in *E. coli* L22 renders the ribosome resistant to erythromycin (Chittum & Champney, 1994). The effect of the mutation is that erythromycin can still bind to the ribosomes but cannot inhibit translation (Cooperman *et al.*, 1990).

The crystal structures of the native bacterial L22 from *T. thermophilus* (Unge *et al.*, 1998) and of the archaeal L22 within the large ribosomal subunit of *Haloarcula marismortui* (Ban *et al.*, 2000) have been solved at atomic resolution, clarifying important details of the interactions between rRNA and ribosomal proteins. It became obvious that the predictions about the functional and structural importance of an unusually long β-hairpin in the L22 structure were correct. However, the mechanism of erythromycin action is still unclear.

Residues deleted in the mutant protein are well conserved in the known sequences and located on one strand of the β-hairpin. Even if the protein could incorporate this deletion without any major changes of the main-chain hydrogen bonds, this mutation would nevertheless change the twist and strongly distort the structure of the hairpin. Since the β-hairpin is postulated to be involved in RNA binding, the mutation could lead to a local distortion or even larger rearrangement of parts of the ribosomal RNA (Unge *et al.*, 1998).

Crystallization and preliminary X-ray analysis of the L22 mutant reported here may lead to a better understanding of the interplay between erythromycin, L22 and 23S RNA in the ribosome.

2. Materials and methods

2.1. Gene cloning and expression

The three-amino-acid deletion was introduced into the *rpl22* gene by the polymerase chain reaction (PCR) with the help of oligonucleotide primers corresponding to the region of the mutation. The plasmid pTthL22 (Davydova *et al.*, 1995), which contains the *rpl22* gene from *T. thermophilus*, was used as a PCR template. The resulting mutant gene was cloned into the pET 11c expression vector using *Bam*HI and *Nde*I restriction sites and transformed into *E. coli* strain TG1. After PCR screening, the plasmid with the correct-sized insert was checked by direct sequencing and transformed into *E. coli* strain BL21 (DE3). A starter culture on two LB agar plates with

Table 1
Features and crystal parameters of native and mutant *T. thermophilus* L22 proteins.

	Native	Mutant
Stability	1 week, 277 K	1 month, 277 K
Crystal shape	Needles	Thick plates
Diffraction quality (Å)	2.1	1.8
Space group	$P2_12_12_1$	$P2_1$
Unit-cell parameters (Å,°)	a = 32.6, b = 66.0, c = 67.7	a = 31.88, b = 86.59, c = 38.96, β = 104.47
No. of molecules in asymmetric unit	1	2
Solvent content (%)	56	40
Crystallization conditions	2.0 *M* $NH_4H_2PO_4$, 100 m*M* $MgCl_2$, 0.1 *M* Tris–HCl pH 4.5	1.9 *M* NaCl, 3% ethanol

100 µg ml^{-1} ampicillin was prepared from frozen cell stock and grown overnight at 310 K. This overnight culture was added to 800 ml of 2 × LB media containing 100 µg ml^{-1} ampicillin and incubated in a shaker (180 rev min^{-1}) at 310 K until an OD_{600} reading of 0.6–0.8. At this point, protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 m*M*. The cells were harvested 3–4 h post-induction by centrifugation (4000 rev min^{-1}, Beckman JA-10 rotor, 277 K, 20 min). The recombinant protein accumulated to a level of 15% of the total cellular protein.

2.2. Protein purification

The mutant protein was purified by column chromatography similar to that in Davydova *et al.* (1995). 3 g of wet cells were disintegrated by grinding with alumina and resuspended in 30 ml 50 m*M* sodium acetate pH 5.5, 150 m*M* $MgCl_2$, 800 m*M* NaCl. The suspension was centrifuged for 20 min at 15 000*g* to remove cellular debris and heated at 328 K for 10 min. After pelleting of precipitated *E. coli* proteins, the supernatant was diluted with 50 m*M* sodium acetate pH 5.5, 50 m*M* NaCl to a final NaCl concentration of 200 m*M* and loaded onto a CM-Sepharose column. After washing, the protein was eluted with a linear gradient of 200–1000 m*M* NaCl in 50 m*M* sodium acetate pH 5.5. The fractions homogeneous by Coomassie-blue stained SDS–PAGE analysis were pooled and concentrated to 15 mg ml^{-1}.

2.3. Crystallization

Screening for crystallization conditions was performed using commercially purchased sparse-matrix screens (Jancarik & Kim, 1991; Cudney *et al.*, 1994) from Hampton Research (Crystal Screens I and II) using the hanging-drop vapour-diffusion technique. All drops were set up by mixing 2 µl of protein in 50 m*M* sodium acetate pH 5.5, 200 m*M* NaCl with 2 µl of precipitant solution on siliconized cover slides and equilibrated against 1.0 ml of the same precipitant solution. Crystallization trays were incubated at 293 K.

Table 2
Details of data collection and processing.

Values in parentheses are for the highest resolution shell (1.91–1.8 Å).

Wavelength	Cu $K\alpha$, 1.54179 Å
Resolution range (Å)	23.2–1.8
No. of measured reflections	64827
No. of unique reflections	16162 (1334)
Completeness (%)	85.6 (47.3)
Multiplicity	4 (3)
R_{merge} (%)	7.4 (33.1)
$I/\sigma(I)$	74.7 (64.0)

2.4. Data collection

Selected crystals were mounted in thin-walled quartz capillaries, which were sealed with wax after filling both ends with reservoir solution. X-ray diffraction data were collected at room temperature using a MAR 345 image plate. A total of 203 frames of 0.5° $\Delta\varphi$ oscillation were collected. Indexing, integration and scaling of data sets were carried out using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

In the initial experiments four different crystallization conditions of the mutated protein were found (Figs. 1*a*–1*d*). Optimization of the first precipitant solution (1 *M* ammonium sulfate, 12% glycerol, 0.1 *M* Tris pH 8.5) had no positive effect on the size of the crystals (Fig. 1*a*). Crystals suitable for diffraction analysis formed within 2 d in 1.5 *M* NaCl, 10% ethanol (Fig. 1*d*). After optimization of conditions, single plate-like 1.0 × 0.5 × 0.13 mm crystals grew in 1.9 *M* NaCl, 3% ethanol in drops containing 5 µl of protein and 5 µl of reservoir solution. Under these conditions, crystals formed within 24–36 h and diffracted to at least 1.75 Å resolution. The crystals belong to space group $P2_1$, with two monomer molecules in the asymmetric unit. The quality of L22 mutant crystals appears to be higher than that for the native species (Table 1). Only one crystal form could be obtained in 2.0 *M* ammonium dihydrogen phosphate, 100 m*M* $MgCl_2$, 100 m*M* Tris pH 4.5 (Fig. 2*b*) for the native protein, while the mutant L22 crystallized easily under several conditions. The

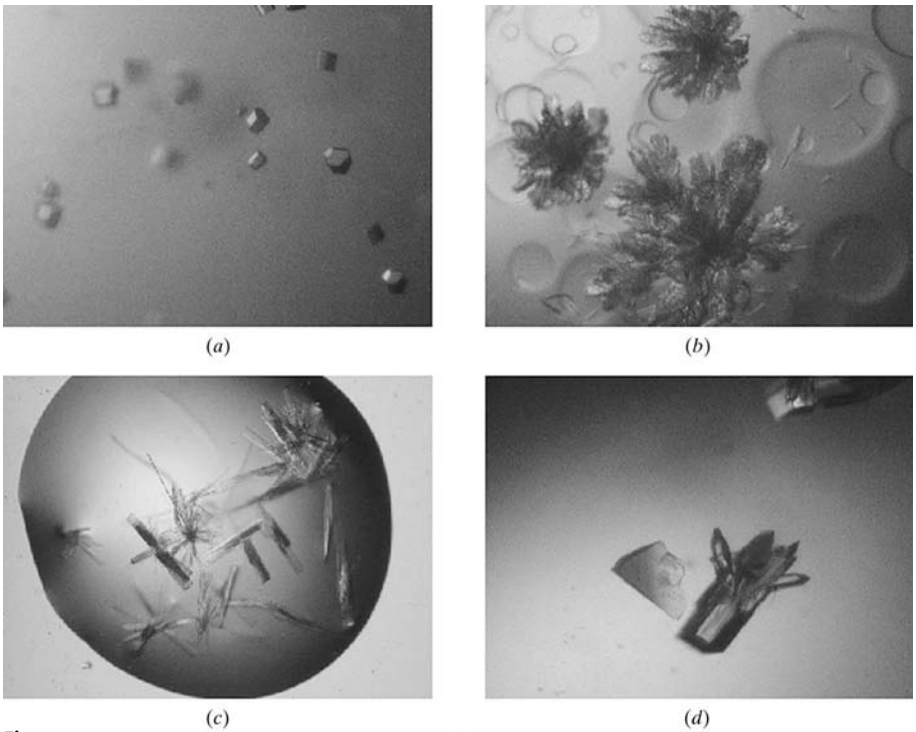
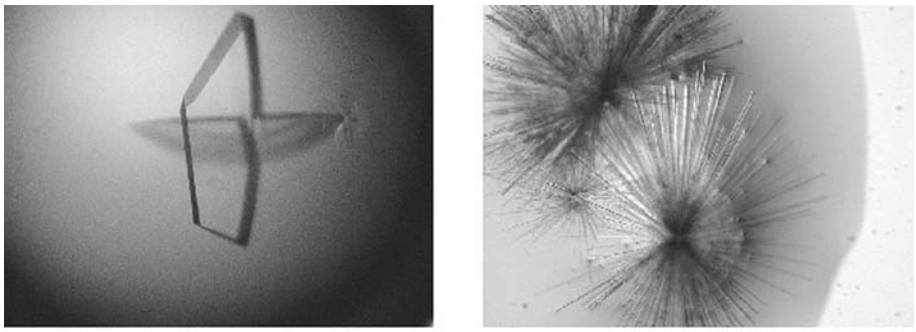


Figure 1
Crystals of mutant L22 protein formed under different conditions: (*a*) 1 *M* ammonium sulfate, 12% glycerol, 0.1 *M* Tris pH 8.5, (*b*) 30% PEG MME 2000, 0.2 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6, (*c*) 1.6 *M* ammonium sulfate, 10% dioxane, 0.1 *M* MES pH 6.5, (*d*) 1.5 *M* NaCl, 10% ethanol.



(*a*) (*b*)

Figure 2
Crystals used for X-ray diffraction: (*a*) mutant L22 grown in 1.9 *M* NaCl, 3% ethanol, (*b*) native L22 protein grown in 2.0 *M* ammonium dihydrogen phosphate, 0.1 *M* $MgCl_2$, 0.1 *M* Tris pH 4.5.

solvent content in the X-ray quality crystals of the mutant L22 is 40% and the Matthews coefficient V_M is 2.06 Å^3 Da^{-1} (Matthews, 1968), compared with the corresponding values of 56% and 2.86 Å^3 Da^{-1} for the native protein crystals. Details of data collection and processing are presented in Table 2. The structure was determined through a combination of molecular replacement and electron-density difference map calculations. As a search model, a truncated version of the water-free native L22 structure (PDB code 1bxe) was generated by removing the non-conserved β-loop region. A rotation search resulted in an almost unique solution with the correlation coefficient between squared normalized structure factors of 0.165, which also suggested that two molecules with differences in coordinates and *B* factors are present in the asymmetric unit. The translation search was then used to determine which of the rotation solutions correspond to the two monomers. There was one solution with a correlation coefficient (in this case E2E2) which was significantly higher than all other solutions (0.299, with the next closest peak 0.209). The packing value was 0.24. The translation solution obtained was used in the second translation search. The coordinates for the top translation-search solution were used for the first molecule, the location of which was fixed and the starting model was used for the second molecule, the location of which was tested for each rotation peak. The correlation coefficient of 0.577 and the packing value of 0.63 increased significantly compared with the first translation search with only one molecule. The high packing value indicates minimal overlap for this solution compared with the others. The positions of the two molecules without β-loops in the asymmetric unit were optimized to R = 0.39 using rigid-body refinement. Further structural refinement is in progress and will be published elsewhere.

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