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Crystallization and preliminary X-ray crystallographic studies of a mutant of ribosome recycling factor from *Escherichia coli*, Arg132Gly

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Ribosome recycling factor (RRF) plays a central role during the recycling of ribosomes in the final step of protein biosynthesis in prokaryotes and is therefore a favourable target for the development of new antibiotics. The crystal structure of *Escherichia coli* RRF has been reported to have an open L-shaped conformation, while other RRFs from thermophilic bacteria have a strict L-shaped conformation [Yun *et al.* (2000), *Acta Cryst.* D**56**, 84–85]. Wild-type *E. coli* RRF has so far not been crystallized free from bound detergent. Here, a mutant of RRF, Arg132Gly, has been crystallized without any detergent. A complete data set from a crystal of this mutant obtained by the hanging-drop vapour-diffusion method has been collected at 2.2 Å resolution using synchrotron radiation at 100 K. The crystal belongs to the monoclinic space group $P2_1$, with unit-cell parameters a = 46.02, b = 49.27, c = 49.37 Å, β = 110.1°. The currently refined structure indicates that RRF has a tRNA-like L-shaped conformation.

1. Introduction

Various soluble protein factors are involved in each step of polypeptide biosynthesis. At the termination step, release factors RF1 or RF2 recognize the stop codon on mRNA at the A-site of the ribosome and accelerate the hydrolysis of peptidyl-tRNA located in the P-site of the ribosome to release the nascent polypeptide chain. Release of these factors from the A-site is then promoted by RF3 in a GTP-dependent reaction (Pavlov *et al.*, 1997). As a result of these reactions, the so-called post-termination complex, which is composed of 70S ribosome, deacylated tRNA and mRNA, remains. For the next translation cycle, ribosome recycling factor (RRF) catalyzes the breakdown of the post-termination complex into its components. This reaction requires the existence of an elongation factor EF-G and is GTP dependent (Kaji *et al.*, 1998). Because it has been shown that RRF is essential for bacterial growth (Janosi *et al.*, 1994) and is not essential for eukaryotic cells (Kaji *et al.*, 1998), RRF is regarded as a promising target for a new class of antibiotics. Furthermore, RRF is also involved in preventing errors during peptide elongation (Janosi *et al.*, 1996).

Recently, the three-dimensional structures of RRFs from several bacteria, *Thermotoga maritima* (Selmer *et al.*, 1999), *Escherichia coli* (Kim *et al.*, 2000), *Thermus thermophilus* (Toyoda *et al.*, 2000) and *Aquifex aeolicus* (Yoshida *et al.*, 2001), have been determined by X-ray crystallography and NMR spectroscopy. All of these RRFs consist of two domains; domain I assumes a triple-helix bundle structure and domain II displays a three-layer $\beta/\alpha/\beta$ sandwich structure. The two domains of the thermophilic RRFs from *Thermotoga maritima*, *Thermus thermophilus* and *A. aeolicus* are arranged in an L-shaped conformation, so that the overall structures are very similar to that of tRNA in terms of shape and are of nearly the same dimensions. This characteristic tRNA-like conformation of RRF suggests that RRF mimics the function of tRNA. It has been proposed that RRF first binds to the A-site of the ribosome and is then translocated by EF-G to the P-site in a similar manner to tRNA, leading to the mechanistic disassembly of the post-termination complex (Selmer *et al.*, 1999).

On the other hand, the three-dimensional structure of *E. coli* RRF, which has been determined by X-ray crystallography, shows a remarkably different profile from thermophilic RRFs (Kim *et al.*, 2000). The two domains, the secondary-structure elements of which are almost the same as those of other RRFs, are placed at an obtuse angle so that the overall structure is an open L-shape and does not show similarity to that of tRNA. Thus, it appears that there may be a structural difference in RRFs depending on the source. Indeed, *E. coli* is a mesophilic bacterium and the other three are thermophilic bacteria. However, the crystal structure of *E. coli* RRF contains a detergent molecule, decyl-β-D-maltopyranoside, which

was required for crystallization (Yun *et al.*, 2000). The detergent was found to bind at the hinge region between the two domains (Kim *et al.*, 2000). Thus, the binding of the detergent molecule may be the cause of the difference in the relative orientation of the two domains. It is noted that the structure of *A. aeolicus* determined in the solution state still has a strict L-shaped conformation resembling those of other RRFs from thermophilic bacteria as determined in the crystalline state. Another possibility for the structural difference is that the *E. coli* structure may be a snapshot of its movement when trapped in the crystal and may only reflect its greater flexibility. The possibility that the domain movement might play an important role in its interaction with ribosome has been suggested and attracted our interest (Yoshida *et al.*, 2001).

Therefore, we attempted to obtain a crystal of wild-type *E. coli* RRF without any additive. Since we have so far failed to crystallize the wild-type *E. coli* RRF, we tried to crystallize several mutants that have been biochemically investigated in our laboratory. We have succeeded in crystallizing one such mutant without any detergent. In this mutant, R132G, the Arg132 residue is replaced by a Gly residue. The circular dichroism measurement showed that this mutant maintains the native-like conformation (Ishino *et al.*, 2000). Although it contains only one substitution, it does not show ribosome recycling activity either *in vivo* (Janosi *et al.*, 2000) or *in vitro* (Ishino *et al.*, 2000). Using the surface plasmon technique, we have shown that Arg132, which is conserved in all bacterial RRFs, plays a crucial role in the binding to ribosome (Ishino *et al.*, 2000). Arg132 is located at the helix region of domain I. The position is far from the hinge region as well as from the tip region corresponding to the anticodon loop or the CCA end of tRNA (Jack *et al.*, 1976; Fujiwara *et al.*, 2001). The reason why this mutant loses ribosome recycling activity is not well understood. Therefore, it is important to determine the tertiary structure of the R132G mutant and to compare it with that of the wild-type *E. coli* RRF. In this paper, we report the crystallization and preliminary X-ray analysis of the R132G mutant of *E. coli* RRF in its free state.

2. Materials and methods

2.1. Protein preparation

The gene for the *E. coli* RRF mutant R132G was generated using a QuickChange site-directed mutagenesis kit (Stratagene) using the sense oligonucleotide 5′-CGTGTTGCAGTACGTAACGTGGGCCGTGACGCGAACGAC-3′ and its antisense oligonucleotide. The plasmid pET22b(+) containing *E. coli* RRF was used as a template. After confirmation of the DNA sequence, the mutant plasmid was introduced into *E. coli* strain BL21(DE3) grown at 310 K in LB medium containing 100 μg ml^{-1} ampicillin. When the absorbance of the culture reached 0.6 at 660 nm, protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1.0 m*M*. 3 h after induction, cells were harvested and disrupted by sonication in buffer *A* (20 m*M* Tris pH 8.0, 10 m*M* $MgCl_2$ and 2 m*M* 2-mercaptoethanol). After centrifugation of the homogenate at 300 000*g* for 3 h, the supernatant was mixed with polyethyleneimine to remove nucleic acids. After further centrifugation at 20 000*g* for 1 h, the supernatant was applied to a 5 ml Hi-Trap Q column (Amersham Pharmacia Biotech) equilibrated with buffer *A*. The eluted fractions containing RRF were dialyzed against buffer *B* (20 m*M* phosphate buffer pH 6.0 and 2 m*M* 2-mercaptoethanol) and applied to a 5 ml Hi-Trap SP column (Amersham Pharmacia Biotech) equilibrated with buffer *B*. The RRF-containing fractions were concentrated using a Centricon YM-10 (Millipore) and further purified by a Superdex 75pg column (Amersham Pharmacia Biotech) equilibrated with buffer *A* containing 150 m*M* sodium acetate. The product homogeneity of the purified preparation was judged by SDS–PAGE.

2.2. Crystallization and data collection

The R132G mutant was concentrated to 10 mg ml^{-1} in 20 m*M* Tris buffer at pH 8.0 with 1 m*M* 2-mercaptoethanol and subjected to crystallization trials. The initial crystallization screening was performed with Hampton Crystal Screens I and II using the hanging-drop vapour-diffusion method at 277 K by mixing 2 μl protein solution with 2 μl reservoir solution (Jancarik & Kim, 1991; Cudney *et al.*, 1995). Hampton Crystal Screen condition I-43 (30% PEG 1500) produced several small cube-shaped or thin rod-shaped crystals (0.025 × 0.02 × 0.1–0.2 mm). Although the cube-shaped crystals grew to more suitable dimensions, 0.5 × 0.4 × 0.4 mm, the diffraction quality of these crystals was poor owing to their high mosaicity and the resolution was low. On the other hand, the thin rod-shaped crystals, which were smaller in size, diffracted to higher resolution. After optimization of crystallization conditions, the best crystals, which had dimensions of about 0.1 × 0.02 × 0.02 mm, were obtained from 50 m*M* MES buffer at pH 5.8 and 15–20% PEG 1500 in 2 d (Fig. 1). Attempts to crystallize the R132G mutant using the crystallization conditions for wild-type *E. coli* RRF (0.1 *M* MES buffer pH 6.5, 10% PEG 350 MME, 12–14% PEG 400 and decyl-β-D-maltopyranoside; Yun *et al.*, 2000) were unsuccessful. Wild-type *E. coli* RRF did not crystallize in the crystallization conditions of the R132G mutant.

The crystal was transferred into a cryoprotectant consisting of 50 m*M* MES buffer pH 6.2, 30%(*w*/*v*) PEG 1500, 30%(*v*/*v*) glycerol. Several minutes later, it was scooped up in a cryoloop and frozen in liquid nitrogen. It was then mounted on the goniometer in a nitrogen stream at 100 K. X-ray diffraction was detected on a Weissenberg camera with an imaging plate for macromolecular crystallography (Sakabe, 1991) with a crystal-to-detector distance of 573 mm at Station BL6A of the Photon Factory of the High Energy Accelerator Research Organization (Tsukuba, Japan). Data were collected to 2.2 Å resolution and were indexed, integrated and scaled with *DENZO* and *SCALEPACK* from the *HKL* package (Otwinowski & Minor, 1997).

3. Results and discussion

The crystal of the R132G mutant belongs to the monoclinic space group $P2_1$, with unit-cell parameters a = 46.02, b = 49.27, c = 49.37 Å, β = 110.1°. The crystal diffracted to a 2.2 Å resolution. Assuming one molecule per asymmetric unit, the calculated Matthews coefficient V_M value was 2.56 Å^3 Da^{-1} (Matthews, 1968). The solvent content of the crystal was therefore calculated to be 51.9%. Data-collection statistics

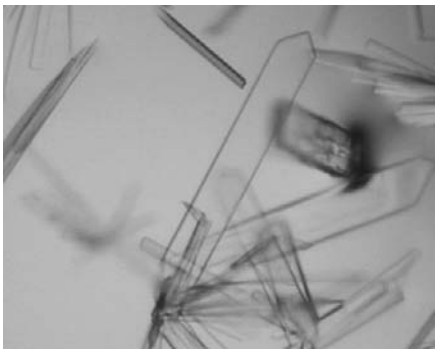

Figure 1
Typical thin rod-shaped crystals of the R132G mutant. The dimensions of the crystals were approximately 0.025 × 0.02 × 0.2 mm.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.24–2.20 Å).

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 46.02, b = 49.27, c = 49.37, β = 110.1
Resolution (Å)	40.0–2.20 (2.24–2.20)
No. of measurements	39303
No. of unique reflections	10611
Redundancy	3.7
$R_{\rm merge}$† (%)	4.8 (18.4)
Completeness (%)	98.4 (93.1)
$I/\sigma(I)$	12.6 (2.1)
$I > 3\sigma(I)$ (%)	68.9

† $R_{\rm merge} = \sum |I - \langle I \rangle| / \sum I$.

are given in Table 1. The space group and unit-cell parameters of the R132G mutant are different from those of the wild-type *E. coli* RRF. It has been reported that the wild-type *E. coli* RRF crystal belongs to the trigonal space group $P3_121$, with unit-cell parameters $a = b = 48.06$, $c = 142.27$ Å. The calculated Matthews coefficient ($V_{\rm M}$) of the R132G mutant, 2.56 Å^3 Da^{-1}, is larger than that of the wild-type *E. coli* RRF (2.30 Å^3 Da^{-1}).

Molecular-replacement calculations were performed on the R132G mutant using the program *AMoRe* (Navaza, 1994). Three initial search models, domain I (residue 2–29 and 109–185), domain II (residue 30–106) and the whole structure of *T. maritima* RRF (PDB code 1dd5; Selmer *et al.*, 1999), were subjected to calculations of the rotation and translation functions. When domain I was used, a single solution was obtained with a correlation coefficient of 0.297 and an R factor of 55.0% (8.0–4.0 Å) after the translation-function calculation. The first electron-density map after positional refinement of domain I revealed some secondary structures of the remaining domain II. With many cycles of model fitting and refinement using the programs *X-PLOR* (Brünger, 1992) and *TOM* (Cambillau *et al.*, 1984), it was possible to trace almost the entire domain II. The preliminary model has a strictly L-shaped conformation with two domains. The relative orientation of domains I and II is nearly the same as that in *Thermotoga maritima* (Selmer *et al.*, 1999), *Thermus thermophilus* (Toyoda *et al.*, 2000) and *A. aeolicus* (Yoshida *et al.*, 2001). The current structure is quite different from that of wild-type *E. coli* RRF with a detergent molecule (Kim *et al.*, 2000) and the structural difference is likely to be caused by the binding of the detergent. Further refinements of the model structure are currently in progress.

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