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Purification, crystallization and preliminary X-ray studies of *Mycobacterium tuberculosis* RRF

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The ribosome recycling factor from *Mycobacterium tuberculosis* has been crystallized. The monoclinic crystals, with 52.5% solvent content, contain one protein molecule in the asymmetric unit.

1. Introduction

With the re-emergence of tuberculosis providing the impetus, research on *Mycobacterium tuberculosis* at the molecular level has registered considerable progress in recent years (Ratledge & Dale, 1999). In particular, the availability of the complete genome sequence of the pathogen has facilitated such research (Cole *et al.*, 1998). The sequence indicates the presence of about 4000 tuberculosis proteins, but the three-dimensional structures of only a few dozen of them are currently known. As part of a continuing effort in this laboratory to characterize the structures of these proteins (Datta *et al.*, 2000, 2003; Saikrishnan *et al.*, 2003), the crystallization and preliminary studies of the ribosome recycling factor (RRF) from this organism have been carried out.

RRF is an essential factor which in concert with elongation factor G (EFG) and in the presence of GTP disassembles the post-termination complex consisting of 70S ribosome bound to deacylated tRNA and mRNA (Janosi *et al.*, 1996). This factor is essential for eubacterial growth (Janosi *et al.*, 1996). RRF is also present in the organelles of eukaryotic cells. However, it has been shown to be nonessential, at least for the survival of yeast (Kaji *et al.*, 1998). Therefore, this makes RRF a promising target for antibacterial drugs (Kaji *et al.*, 1998). The three-dimensional structure of RRF determined from a number of Gram-negative bacteria (Selmer *et al.*, 1999; Kim *et al.*, 2000; Toyoda *et al.*, 2000; Yoshida *et al.*, 2001; Nakano *et al.*, 2002, 2003) indicates strong conservation of its structure. The protein has a tRNA-like 'L'-shaped structure. The arms of the 'L' are formed by two domains: domain I is a triple-helix bundle and domain II is a three-layer β–α–β sandwich structure. However, the structure of RRF from either Gram-positive bacteria or eukaryotes has yet to be deduced.

Of the Gram-positive bacteria, RRF from *M. tuberculosis* (MtRRF) has been biochemically studied (Rao & Varshney, 2001, 2002). This protein, which is 184 residues long, shares a sequence identity of 40% with its homologue in *Escherichia coli* (EcRRF). Despite the strong sequence similarity and overlap of many biochemical properties, cross-species complementation experiments have demonstrated the failure of MtRRF to function in *E. coli* (Rao & Varshney, 2001). Instead, simultaneous expression of MtEFG and MtRRF complements a temperature-sensitive RRF strain of *E. coli* (Rao & Varshney, 2001). These results not only indicate the necessity of a 'cross-talk' between RRF and EFG for the disassembly of the post-termination complex, but also that this interaction in *M. tuberculosis* is species-specific. Structural information on this protein is a necessary platform to gain further insights into the origin of this specificity.

2. Materials and methods

2.1. Overexpression and purification of MtRRF

MtRRF was overexpressed and purified to apparent homogeneity using a protocol described by Rao & Varshney (2002) with modifications. *E. coli* BL21 (DE3) harbouring pET11dMtRRF was used for overexpression of the protein. The transformants were inoculated into Luria–Bertani medium (Sambrook *et al.*, 1989) and induced with 0.5 m*M* IPTG at the mid-exponential phase of growth (OD_{600} value between 0.3 and 0.4). The culture was induced at 310 K. The harvested cells were sonicated in a buffer consisting of 20 m*M* phosphate pH 7.6, 10% glycerol, 2 m*M* β-mercaptoethanol and 1 m*M* EDTA and centrifuged at 100 000*g* to prepare the S100 extract. The S100 extract was subjected to streptomycin sulfate (0.9%) precipitation and the supernatant was loaded onto a hydroxyapatite column (Bio-Rad). The proteins were eluted with a linear gradient of 20 m*M*–1 *M* phosphate pH 7.6 and 10% glycerol. The fractions enriched in RRF were pooled and subjected to ammonium sulfate precipitation (70% saturation). The precipitate was recovered by centrifugation, dissolved in 1 ml of

20 m*M* Tris–HCl pH 7.5 and 10% glycerol buffer and loaded onto a Superdex 75 column (Amersham Pharamacia Biotech). The protein was eluted with a buffer containing 20 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl and 10% glycerol buffer. The fractions enriched in RRF were pooled and diluted with 20 m*M* Tris–HCl pH 7.5 and 10% glycerol and loaded onto a Mono Q column (Amersham Pharmacia Biotech). The proteins were eluted with a linear gradient of 250–750 m*M* NaCl in 20 m*M* Tris–HCl pH 7.5 and 10% glycerol buffer. The fractions enriched in RRF were pooled and diluted with 20 m*M* phosphate pH 7.6 and 10% glycerol and loaded onto a hydroxyapatite column. The proteins were eluted with a linear gradient of 100–300 m*M* phosphate pH 7.6 and 10% glycerol. A final preparation containing 10–15 mg ml^{-1} of this homogeneous protein in 20 m*M* Tris–HCl buffer pH 7.5 was used for crystallization (Fig. 1*a*).

2.2. Crystallization and data collection

Crystals were grown using the hanging-drop vapour-diffusion method. The drop size varied from 5–9 µl, with a well volume of 500 µl. The drops consisted of 4–8 µl of protein solution and 1 µl of well solution. The crystallization trays were stored at 298 K. The crystal from which the working data set was collected was grown in 20 m*M* Tris–HCl buffer pH 7.5 containing 6% PEG 8000 and 0.6 m*M* cadmium acetate (Fig. 1*b*). The diffraction data were collected at room temperature (298 K) with a MAR Research imaging plate mounted on a Rigaku RU-200 X-ray generator. The crystal-to-image plate distance was 100 mm. The data were processed and scaled using the *HKL* package (Otwinowski & Minor, 1997). Intensities were converted to structure factors using *TRUNCATE* (Collaborative Computational Project, Number 4, 1994). Structure solution was obtained by the molecular-replacement method using *AMoRe* (Navaza, 1994). The structure was refined using *CNS*1.1 (Brünger *et al.*, 1998) involving iterations of rigid-body and positional refinement and simulated annealing.

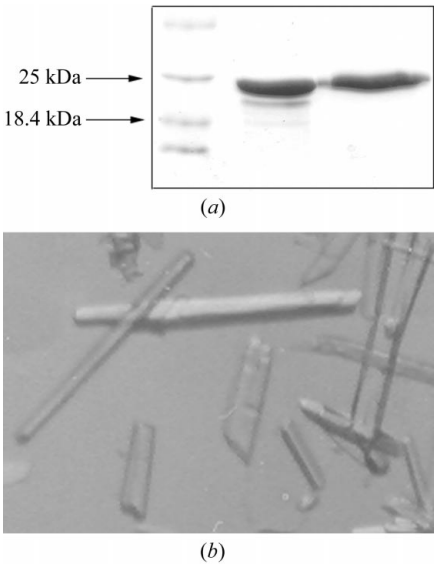

Figure 1
(*a*) 15% SDS–PAGE of purified MtRRF stained with Coomassie brilliant blue. Lane 1, marker; lane 2, protein sample used for crystallization; lane 3, protein recovered from the MtRRF crystals. (*b*) Crystals of MtRRF.

3. Results and discussion

3.1. Crystallization

Initial attempts at crystallizing the protein using commercially available screening kits failed to produce any positive results. Efforts were then directed towards screening strategies involving commonly used precipitating agents (McPherson, 1990). In particular, divalent cations such as zinc, calcium, magnesium, manganese and cadmium that have been known to be effective precipitating agents (McPherson, 1982, 1990; Trakhanov & Quiocho, 1995) were tested for their ability to induce crystallization of the protein. Eventually, crystals of MtRRF were obtained from 20 m*M* Tris–HCl pH 7.5 with 6% PEG 8000 and cadmium acetate as the precipitating agents. The other divalent cations did not yield any crystals. Although chloride and sulfate salts of cadmium were also tried, crystallization occurred only with the acetate salt. The quality of the crystals depended significantly on the concentration of cadmium acetate. The best quality crystals grew when the concentration of cadmium acetate was 0.6 m*M*. Showers of tiny crystals appeared with increasing concentrations of the salt. The size and quality of the crystal improved further with the addition of 10% glycerol to the crystallization condition. The crystals grew to approximate dimensions of 0.6 × 0.08 × 0.08 mm.

The role of cadmium ion in influencing crystallization has been systematically investigated by Trakhanov and coworkers (Trakhanov & Quiocho, 1995; Trakhanov *et al.*, 1998). We also have had considerable success with cadmium salts as the precipitating agent, as in the crystallization of single-stranded DNA-binding protein from *Mycobacterium tuberculosis* (Saikrishnan *et al.*, 2002) and DPS from *M. smegmatis* (Roy *et al.*, 2003). Cadmium ions appear to facilitate crystallization by cross-linking neighbouring molecules through coordination with side chains of various residues, water and main-chain carbonyl groups (Trakhanov *et al.*, 1998).

Table 1
Crystal data and data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters	
a (Å)	56.3
b (Å)	33.8
c (Å)	62.4
β (°)	112.5
Packing density V_M (Å^3 Da^{-1})	2.6
Solvent content (%)	52.5
No. molecules in the AU	1
Resolution range (Å)	20.00–2.80 (2.90–2.80)
Observed reflections	15988
Unique reflections	5273 (505)
Completeness (%)	94.2 (94.9)
R_{merge} (%)	11.3 (58.4)

3.2. Structure solution and refinement

The crystals of MtRRF belong to space group $P2_1$, with unit-cell parameters $a = 56.3$, $b = 33.8$, $c = 62.4$ Å, $\beta = 112.5°$ (Table 1). The crystals diffracted to better than 3 Å resolution. The Matthews coefficient for this crystal corresponds to a solvent content of 52.5%. A partial structure solution was obtained by molecular replacement using *AMoRe* (Navaza, 1994). Molecular replacement was tried separately with all the structures of RRF available in the Protein Data Bank as search models. The models were divided into three different sets: (i) full molecules, (ii) domain I and (ii) domain II. A single solution with a correlation coefficient of 43.6% and an *R* factor of 44.0% was obtained by using domain I (residues 1–35 and 105–184) of *Vibrio parahaemoliticus* RRF (PDB code 1is1; Nakano *et al.*, 2003; non-identical residues converted to alanine) as the search model in the resolution range 8.0–4.0 Å. The other search models failed to provide a solution. The structure thus obtained was then subjected to alternate cycles of refinement and model building using the programs *CNS*1.1 (Brünger *et al.*, 1998) and *FRODO* (Jones, 1978).

With the progress in refinement, clear electron densities for the linker chains that connect the two domains and the terminal residues of domain II (residues 36–40 and 98–104) were obtained. However, the electron densities for domain II remained extremely poor, hindering the modelling of this region. Earlier structural studies of RRF had shown that the two domains move with respect to each other with the linker region acting as a hinge (Nakano *et al.*, 2003). This

motion is approximately a rotation of domain II about the axis of the helical bundle constituting domain I. Based on this information and using the position of the terminal residues of domain II, this domain from *V. parahaemoliticus* (non-identical residues converted to alanine) was placed in the unit cell. Subsequent refinement resulted in a considerable decrease in R and $R_{\rm free}$ and in a clear electron density for the newly added region, giving enough confidence to pursue this composite model. The refinement is in progress, the current R and $R_{\rm free}$ values being 27.4 and 36.9%, respectively, in the resolution range 20–2.90 Å.

X-ray intensity data were collected at the X-ray Facility for Structural Biology, supported by the Department of Science and Technology (DST) and Biotechnology (DBT), Government of India. Computations were performed at the Supercomputer Education and Research Centre at the Institute and the Bioinformatics Centre and the Interactive Graphics Facility, both funded by the DBT. The work forms part of a programme on Structural Genomics of Microbial Pathogens supported by the DBT.

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