Acta Crystallographica Section D Biological Crystallography ISSN 0907-4449 Editors: E. N. Baker and Z. Dauter

# Expression, purification, crystallization and preliminary X-ray crystallographic studies of *Mycobacterium tuberculosis* thioredoxin reductase

## Mohd Akif, Radha Chauhan and Shekhar C. Mande

Copyright © International Union of Crystallography

Author(s) of this paper may load this reprint on their own web site provided that this cover page is retained. Republication of this article or its storage in electronic databases or the like is not permitted without prior permission in writing from the IUCr.

Received 27 January 2004

Accepted 25 February 2004

Acta Crystallographica Section D Biological Crystallography

ISSN 0907-4449

# Mohd Akif, Radha Chauhan and Shekhar C. Mande\*

Centre For DNA Fingerprinting and Diagnostics, Hyderabad, India

Correspondence e-mail: shekhar@cdfd.org.in

# Expression, purification, crystallization and preliminary X-ray crystallographic studies of *Mycobacterium tuberculosis* thioredoxin reductase

*Mycobacterium tuberculosis* (H37Rv), the causative agent of the dreaded disease tuberculosis, contains three thioredoxins and a single thioredoxin reductase. Thioredoxin reductase is a member of the pyridine-nucleotide disulfide oxidoreductase family of flavoenzymes. The thioredoxin reductase gene with a His tag at the C-terminus was expressed in *Escherichia coli* and purified. The dimeric (70 kDa) protein was incubated with 10 mM DTT for 30 min and then crystallized using the hanging-drop vapour-diffusion method in the presence of 15% PEG 3350 and phosphate–citrate buffer pH 5 at room temperature (298 K). A diffraction data set complete to 3 Å resolution has been collected under cryoconditions and the space group was determined to be  $P4_{1}2_{1}2$ , with unit-cell parameters a = 107.4, c = 118.2 Å. Matthews coefficient calculations revealed the presence of two monomers in the asymmetric unit.

### 1. Introduction

Mycobacterium tuberculosis (H37Rv) has infected more than one third of the world's population (Dolin et al., 1994). M. tuberculosis spends much of its life cycle in the strong oxidative environment of macrophages and therefore requires effective oxidative stress defences (Schorey et al., 1997). The thioredoxin system is a key player in the oxidative-defence system of the cell and helps in its survival in the presence of oxidative stress (Takemoto et al., 1998). Reactive oxygen species have many harmful effects on cells. For example, they can oxidize cysteine residues, leading to the formation of undesirable disulfide bridges. Such reactions often inactivate cellular proteins. Reduction of these products may be accomplished in microbial systems by the thioredoxin/thioredoxin reductase (TrxR; Farr & Kogoma, 1991) or glutathione/glutathione reductase systems. In addition to this, in many organisms peroxiredoxins catalyse the reduction of peroxides and thereby prevent oxidative stress and the induction of apoptosis. Peroxiredoxins require reduction by thioredoxin and thioredoxin reductase (Chae et al., 1999). TrxR is a flavoprotein that catalyses the reduction of Trx by NADPH (Moore et al., 1964; Williams, 1995). The substrate Trx in its dithiol state plays a key role in maintaining the redox environment of the cell (Holmgren, 1989). M. tuberculosis has three Trx genes and a single TrxR-encoding gene and surprisingly no glutathione/glutathione reductase-encoding genes in its genome sequence (Cole et al., 1998). In the absence of a glutathione/ glutathione reductase system, the thioredoxin/ thioredoxin reductase system is obviously

critical for this mycobacterium to maintain its intracellular redox environment. In order to determine the three-dimensional structure of mycobacterial TrxR, we have crystallized *M. tuberculosis* thioredoxin reductase (TrxR). This paper describes the preliminary crystallographic characterization of the mycobacterial TrxR crystals.

## 2. Material and methods

#### 2.1. Expression and purification

BACRv13 cosmid DNA of M. tuberculosis (H37Rv), kindly provided by Stewart Cole, was used as a template for PCR amplification of the 1005 bp trxR gene (Rv3913). The forward primer 5'-TTATTCCATATGACCGCCCCG-CCTCTCCA-3' and the reverse primer 5'-AACTAAGCTTTCAGTGGTGGTGGT-GGTGGTGTCGTTGTGCTCCTATCAAT-3' were designed to contain a start-site codon and codons for six histidines, respectively. The PCR product was ligated into the pET23a (Novagene) expression vector using NdeI and HindIII restriction-endonuclease sites. The cloned gene sequence was confirmed by automated DNA sequencing. The plasmid harbouring the mycobacterial trxR gene was transformed into the Escherichia coli BL21 (DE3) expression system and the recombinant protein was purified using Ni2+-nitrilotriacetate (NTA) chromatography. Briefly, cells were grown in Terrific broth at 310 K until they reached an  $OD_{600}$  of 0.6 and were then induced with 1 mM isopropyl  $\beta$ -D-thiogalactoside. The cells were lysed by sonication and centrifuged at  $13\ 000\ \text{rev}\ \text{min}^{-1}$  for 20 min at 277 K. The supernatant was loaded onto an Ni2+-NTA

 ${\rm \@C}$  2004 International Union of Crystallography Printed in Denmark – all rights reserved

column. The column was washed with 50 mM phosphate buffer pH 8 containing 300 mM NaCl, 5% glycerol and 20 mM imidazole. The protein was eluted with the same buffer supplemented with 250 mM imidazole. The purity was checked on Coomassie-stained SDS-PAGE, where it appeared as a single band (Fig. 1).

### 2.2. Crystallization

The purified protein was dialyzed against 10 mM Tris-HCl pH 8.0 buffer and then concentrated to  $30 \text{ mg ml}^{-1}$ . Before crystallization, 10 mM reduced DTT was added to the protein solution and the solution was incubated for 30 min on ice. Initial crystallization trials were performed using a random screening of chemical conditions in our laboratory. The screening was conducted using the hanging-drop vapour-diffusion technique in 24-well plates. A 2 µl droplet of protein mixed in a 1:1 ratio with reservoir solution was equilibrated against 500 µl reservoir solution at room temperature. Crystals suitable for X-ray diffraction (Fig. 2) were obtained using 0.1 M sodium phosphate-citrate buffer pH 5.0 and 15% PEG 3350 at room temperature. Crystals grew within 36 h to dimensions of 0.5  $\times$  0.5  $\times$ 0.3 mm.

#### 2.3. X-ray data collection and processing

Crystals were soaked in cryoprotectant solution for a few seconds prior to freezing in a nitrogen cold stream. The cryoprotectant solution contained 11% ethylene glycol, 20% PEG 3350 and 0.1 *M* sodium phosphate–citrate buffer pH 5.0. X-ray diffraction data were collected at 100 K on beamline XRD-1 (wavelength = 0.927 Å) at the ELETTRA synchrotron facility, Trieste,

#### Figure 1

10% SDS–PAGE gel showing protein purification. Lane 1, cell supernatant after sonication; lane 2, flowthrough; lanes 3, 4 and 5, washthrough from  $Ni^{2+}$ –NTA column; lane 6, molecular-weight markers (kDa); lane 7, purified TrxR.

Italy using a MAR CCD 165 detector. The crystal-to-detector distance was maintained at 200 mm with an oscillation range per image of 1°, covering a total oscillation range of 100° (Fig. 3). Determination of the unit-cell parameters and integration of reflections were performed using the programs *DENZO/SCALEPACK* (Otwinowski & Minor, 1997). The intensities were then converted to the corresponding structure-factor amplitudes using the *TRUNCATE* program from the *CCP4* suite (Collaborative Computational Project, Number 4, 1994).

Molecular replacement was attempted using the AMoRe program (Navaza, 1994) from the CCP4 suite. The homologous E. coli TrxR (dimeric) crystal structure (PDB code 1tde), which has 46% sequence identity (Waksman et al., 1994), was used as the search model.

#### 3. Results and discussion

The trxR gene encoding a polypeptide of 335 amino acids was cloned and expressed in E. coli and the protein was purified to homogeneity. The protein purity was observed to be better than 98% as shown in Fig. 1. On gel-filtration chromatography, the purified TrxR protein eluted in a dimeric form, which is consistent with the physiological quaternary state of the E. coli TrxR (Moore et al., 1964; Russel & Model, 1988) (data not shown). Crystallization trials using the mycobacterial TrxR yielded bright yellow coloured crystals (Fig. 2) of dimensions  $\sim 0.5 \times 0.5 \times 0.3$  mm. These crystals were not stable when data collection was attempted at room temperature. However, the crystals appeared to be comparatively stable under cryoconditions. Diffraction data were collected at the ELETTRA synchrotron source, Italy in the presence of the cryoprotectant ethylene glycol. The completeness of the data set was found to be



#### Figure 2

A crystal of reduced TrxR grown using 15% PEG 3350 and 0.1 *M* sodium-phosphate citrate pH 5.0. The size of one of the crystals shown is approximately  $0.5 \times 0.5 \times 0.3$  mm.

#### Table 1

Data-collection statistics.

Values in parentheses refer to the last resolution shell (3.11–3.0 Å).

Wavelength (Å)	0.927
Space group	P41212
Unit-cell parameters (Å)	a = 107.4, c = 118.2
Resolution limits (Å)	50.0-3.0
Unique reflections	14186 (1368)
$I/\sigma(I)$	16.2 (4.1)
$R_{\text{merge}}$ † (%)	8.1 (36.9)
Completeness (%)	97.8 (97.9)
Probable solvent content (%)	51.4
No. molecules in AU	2
Matthews coefficient ( $Å^3 Da^{-1}$ )	2.5

 $\dagger R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$ , where  $I_i(hkl)$  are the intensities of symmetry-redundant reflections and  $\langle I(hkl) \rangle$  is the average intensity over all observations.

97.8% with acceptable statistical parameters. The 3 Å resolution data were processed using the HKL2000 program and the space group was determined to be  $P4_x2_x2$ , with unit-cell parameters a = 107.4, c = 118.2 Å. Analysis of the systematic absences was not conclusive because only a few reflections of the relevant form were present. The  $R_{\text{merge}}$ values increased rapidly beyond 3 Å resolution and hence data were only processed to 3 Å resolution. Assuming the presence of two monomers in the asymmetric unit, a value for the Matthews coefficient of  $2.5 \text{ \AA}^3 \text{Da}^{-1}$  was obtained, which corresponds to a solvent content of 51.4% (Matthews, 1968). Data-collection and resolution statistics are shown in Table 1. The self-rotation function plot did not show any peaks for the expected twofold noncrystallographic symmetry. The Patterson calculated using native intensities also did not show any significant peak apart from the origin, ruling out the possibility of pseudotranslation. The N(Z) test and the  $\langle I^2 \rangle / \langle I \rangle^2$ 



**Figure 3** A 1° oscillation image collected at the ELETTRA synchrotron at 100 K temperature from a native TrxR crystal. The inset clearly shows the presence of reflections beyond the solvent ring.

electronic reprint

distribution clearly indicated that the intensity data were not twinned.

The AMoRe (Navaza, 1994) program was used for molecular-replacement calculations using E. coli dimeric TrxR (PDB code 1tde) as the search model. The screw axes of the space group (which could not be identified earlier) were determined during translation searches in AMoRe. The best molecularreplacement solution was obtained in space group  $P4_12_12$ . The correctly oriented and positioned molecule of TrxR showed a correlation coefficient of 64.2 and an R factor of 47.5%. In comparison, the next best solution showed a correlation coefficient of 47.1 and an R factor of 55.1%. The placement of the model using the molecularreplacement solution did not show any unfavourable overlaps between symmetryrelated molecules.

Currently, refinement of the molecularreplacement solution is in progress. The crystal structure of *M. tuberculosis* TrxR will contribute to the rational design of inhibitors. The structure will also increase the understanding of the catalytic mechanisms of Trx/TrxR in *M. tuberculosis*.

We thank Stewart Cole and colleagues for providing a cosmid DNA library of M. tuberculosis H37Rv. We also thank the staff of the XRD1 beamline at the ELETTRA synchrotron, Trieste, Italy for assistance during data collection and ICTP for financial assistance in performing experiments at ELETTRA. We thank Howard Einspahr for useful suggestions. This work is supported by grants from the Department of Biotechnology and the Council of Scientific and Industrial Research (CSIR), New Delhi, India. MA gratefully acknowledges financial support from the CSIR for a Junior Research Fellowship and RC and SCM that from the Wellcome Trust, UK.

## References

- Chae, H. Z., Kang, S. W. & Rhee, S. G. (1999). *Methods Enzymol.* 300, 219–226.
- Cole, S. T. et al. (1998). Nature (London), **393**, 537–544.
- Collaborative Computational Project, Number 4 (1994). Acta Cryst. D**50**, 760–763.
- Dolin, P. J., Raviglion, M. C. & Kohi, A. (1994). Bull. World Health Org. 72, 213–220.
- Farr, S. B. & Kogoma, T. (1991). Microbiol. Rev. 55, 561–585.
- Holmgren, A. (1989). J. Biol. Chem. 264, 13963– 13966.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497. Moore, E. C., Reichard, P. & Thelander, L. (1964).
- J. Biol. Chem. 239, 3445–3452.
- Navaza, J. (1994). Acta Cryst. A50, 157-163.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Russel, M. & Model, P. (1988). J. Biol. Chem. 263, 9015–9019.
- Schorey, J. S., Carroll, M. C. & Brown, E. J. (1997). Science, 277, 1091–1093.
- Takemoto, T., Zhang, Q. M. & Yonei, S. (1998). Free Radic. Biol. Med. 24, 556–562.
- Waksman, G., Krishna, T. S. R., Williams, C. H. Jr & Kuriyan, J. (1994). J. Mol. Biol. 236, 800–816.
- Williams, C. H. Jr (1995). FASEB J. 9, 1267– 1276.