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Crystallization of a protein using dehydration without a precipitant

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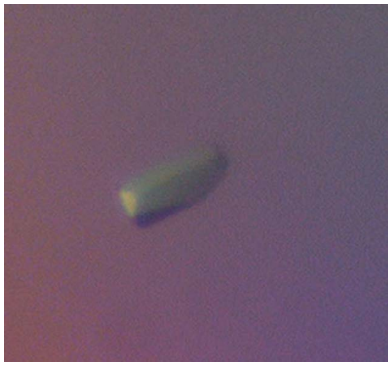

Hypoxic response protein I (HRPI) is a protein of unknown biochemical function whose expression is very strongly upregulated in response to oxygen depletion in *Mycobacterium tuberculosis*. Crystals have been grown from a solution of full-length HRPI by the unusual method of dehydration without the use of precipitants. The crystals produced diffract to a maximum resolution of 2.1 Å and belong to space group $P4_12_12$ (or $P4_32_12$), with unit-cell parameters $a = b = 79.18$, $c = 37.34$ Å.

1. Introduction

About one-third of the world's population is infected with *Mycobacterium tuberculosis*, the bacterium responsible for tuberculosis (Dye *et al.*, 1999). Central to the pathogenic success of this bacterium is its ability to persist within its host in a semi-dormant asymptomatic state, termed non-replicating persistence (NRP), for many years, sometimes decades (Arend & van Dissel, 2002; Lillebaek *et al.*, 2002). It has been proposed that the low oxygen concentration found inside the granulomas that form within the lungs of infected individuals may be one of the triggers for the bacterium to enter into NRP (Wayne & Hayes, 1996). None of the existing antibiotics used to treat tuberculosis are effective against bacteria in the NRP state and this is a major impediment to successful therapy.

A number of proteomic and genome-wide microarray analyses have been carried out using hypoxic *in vitro* models of non-replicating *M. tuberculosis* persistence (Boon *et al.*, 2001; Sherman *et al.*, 2001; Rosenkrands *et al.*, 2002; Bacon *et al.*, 2004; Muttucumaru *et al.*, 2004; Voskuil *et al.*, 2004). It is apparent from these studies that the processes involved in the adaptation of the bacterium to hypoxia are generally poorly understood, as many of the genes identified with expression significantly altered by hypoxia are unannotated. Structural and functional analyses of the proteins encoded by these genes would contribute to the understanding of the bacterial hypoxic response and may lead to the identification of targets for new drugs that act against non-replicating bacteria.

One unannotated protein, which we have termed hypoxic response protein I (HRPI), was very strongly upregulated in all the aforementioned studies of hypoxic *M. tuberculosis*. HRPI is a 143-residue protein encoded by the open reading frame Rv2626c; its expression is regulated by the DosR (dormancy survival regulator) transcription factor (Park *et al.*, 2003). As part of the *M. tuberculosis* Structural Genomics Consortium initiative (http://www.tbgenomics.org), we targeted HRPI for crystallization and structure determination in order to gain further insights into its molecular function. Here, we describe unusual crystallization in a solution of HRPI without the use of precipitating agents. Simple dehydration of concentrated protein solution in a sitting-drop experiment resulted in the production of crystals which are suitable for crystallographic analysis and which diffract X-rays to a maximum resolution of 2.1 Å.

2. Materials and methods

2.1. Protein expression and purification

PCR amplification of HRPI was carried out using Platinum *Pfx* polymerase (Invitrogen) and *M. tuberculosis* H37Rv genomic DNA

as template. The primers used were 5′-AGGAGG**GGATCCA**T-TGACCACCGCACGCGACATCAT-3′ and 5′-GTACCTCCGATG-**CTCGAG**GATAAAG-3′. The PCR product was digested with *Bam*HI and *Xho*I (restriction sites in bold) and ligated into the plasmid pProEX HTb (Invitrogen), which adds an rTEV-cleavable 28-residue N-terminal His tag to the construct.

Following sequence confirmation, the construct was transformed into BL21 (DE3) *Escherichia coli*. Expression cultures were grown in LB medium at 310 K to an OD_{600} of 0.6–0.7 and then induced with 1 m*M* IPTG for 20–23 h. Cells were harvested by centrifugation and lysed on ice by sonication in 20 m*M* HEPES pH 7.5, 150 m*M* NaCl, 10 m*M* imidazole and 1 mg RNase A, 0.2 mg DNase I, 10 mg lysozyme and one Complete mini protease-inhibitor cocktail tablet (Roche), using 16 ml buffer per litre of expression culture.

The protein lysate was centrifuged to remove the insoluble material, passed through a 0.22 µm filter and run onto a 5 ml HiTrap Chelating HP column (Amersham Biosciences) charged with Ni^{2+} ions and equilibrated in buffer containing 20 m*M* HEPES pH 7.5, 150 m*M* NaCl and 10 m*M* imidazole. The protein was eluted using a gradient from 10 to 500 m*M* imidazole in buffer over ten column volumes. Fractions collected during the course of the gradient were analyzed by SDS–PAGE and those containing recombinant HRPI were pooled. Recombinant tobacco etch virus protease (rTEV) was then added to cleave the His tag and the mixture was left to dialyze into the previous equilibration buffer overnight at room temperature. This cleavage left five extra residues (GAMGS) on the N-terminus of the expressed protein. The protein–protease mix was then run through the HiTrap column again in order to remove the rTEV and cleaved His tag. The protein was then dialyzed into buffer without imidazole (20 m*M* HEPES pH 7.5, 150 m*M* NaCl) and concentrated to 35 mg ml^{-1} in preparation for crystallization trials.

2.2. Crystallization

All crystal trials were carried out at 291 K in 96-well Intelli-Plate plates (Hampton Research) using the sitting-drop vapour-diffusion method. Crystallization experiments were carried out using the Centre for Molecular Biodiscovery Crystallization Facility. Experiments were set up using a Cartesian Honeybee robot to transfer protein solution; reservoir solutions were dispensed using a Perkin–Elmer MultiPROBE.

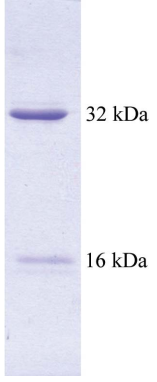

Figure 1
SDS–PAGE of HRPI demonstrating the SDS-resistant dimer (approximately 32 kDa), the monomer (16 kDa) and a small amount of degradation product immediately below the monomer.

Table 1
Data-collection statistics for HRPI.

Values in parentheses are for the outermost shell of data.

Space group	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å)	
$a = b$	79.18
c	37.34
Resolution range (Å)	50.0–2.10 (2.18–2.10)
Wavelength (Å)	1.0
No. of measured reflections	107803
No. of unique reflections	7366
Multiplicity	14.6
Completeness (%)	99.9 (100)
$\langle I\rangle/\langle\sigma(I)\rangle$	31.5 (7.1)
$R_{\rm merge}$† (%)	11.4 (46.3)

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

2.3. Data collection

Crystals were flash-frozen in the protein buffer (20 m*M* HEPES pH 7.5, 150 m*M* NaCl) supplemented with 32%(*v*/*v*) glycerol. Diffraction data were collected to a maximum resolution of 2.1 Å at beamline 5.0.2 at the Advanced Light Source (ALS, Lawrence Berkeley Laboratory, CA, USA) using an ADSC Q315 CCD detector. The oscillation range was 1.0° per image and a total of 198° of data were collected. Data were processed using the *HKL* program package (Otwinowski & Minor, 1997), producing a data set that was 99.9% complete with an overall $R_{\rm merge}$ of 11.4%. Full data-collection statistics are listed in Table 1.

3. Results and discussion

After purification and His-tag cleavage, HRPI migrated as two major bands on SDS–PAGE, with apparent molecular weights of approximately 16 and 32 kDa, corresponding to the monomer and dimer forms of the protein, respectively (Fig. 1). The presence of a dimer on denaturing gels is unusual and may represent either very tight dimerization or a strongly hydrophobic interaction. Size-exclusion chromatography revealed the dimer to be the only species present in solution (data not shown).

Initial attempts at crystallizing the protein, including screening 681 different conditions, were unsuccessful. Initial screens included Crystal Screens I and II (Hampton Research), a systematic PEG–pH screen (Kingston *et al.*, 1994), a PEG/Ion screen (Hampton Research), Footprint Screen No. 1 and the PEG Footprint Screen (Stura *et al.*, 1992). However, one additional 96-well plate was mistakenly set up with 100 nl protein drops only, with no precipitant solution either mixed with the protein or placed in the well. The plate was then sealed and left at 291 K for seven months. At this point, small cube-shaped crystals (Fig. 2) were found in seven of these protein-only drops, even though the only contents of these wells were HRPI and buffer (20 m*M* HEPES pH 7.5, 150 m*M* NaCl). The liquid surrounding the crystals had dehydrated to a very viscous consistency.

The crystals were retrieved from the surrounding viscous liquid by dropping 1 µl cryoprotectant over the drop. This immediately started to thin the liquid, freeing the crystals within, so that one could be scooped out using a nylon loop mounted on a metal pin. The crystal was then immediately frozen in liquid nitrogen. The crystals started to dissolve in the cryoprotectant if left longer than ∼1 min, so there was generally only time to retrieve one crystal from any one drop. Of the crystals collected, only one diffracted to produce a useful data set. Owing to the irreproducibility of these crystals and the difficulty in mounting them for data collection, further biochemical analysis of the crystals was not obtained.

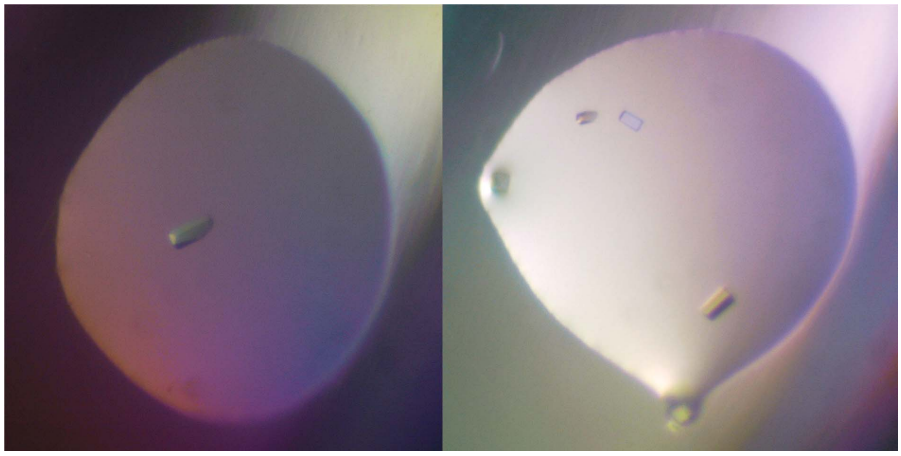

Figure 2
Crystals of HRPI grown by slow dehydration in the absence of precipitants.

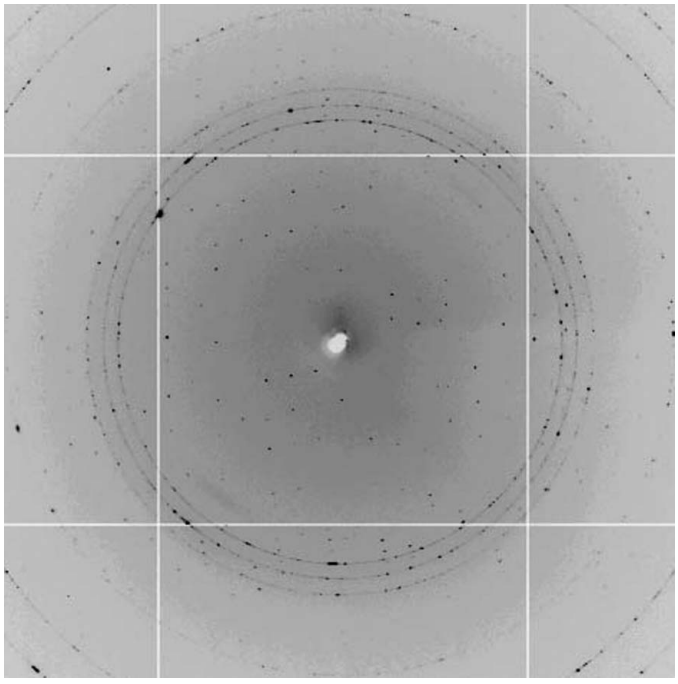

Figure 3
The diffraction pattern of the HRPI crystals. The frame edge is at 2.1 Å resolution. Data-reduction statistics were not compromised by the presence of ice-diffraction rings.

The crystal belongs to the tetragonal space group $P4_12_12$ (or $P4_32_12$), with unit-cell parameters $a = b = 79.18$, $c = 37.34$ Å (diffraction pattern in Fig. 3). A twinning test (Yeates, 1997) using *CNS* v.1.1 (Brünger *et al.*, 1998) on this data revealed no merohedral twinning. Assuming that the asymmetric unit contains one monomer of 15.921 kDa, the Matthews coefficient is 1.8 Å^3 Da^{-1} and the solvent content is 32.5%.

Protein crystallization occurs as a result of the metastable supersaturation of a protein solution (Weber, 1991), normally brought about by the addition of inert precipitants such as polyethylene glycols. However, there is no *a priori* reason why supersaturation cannot be reached by the slow dehydration of a protein solution, although this is a difficult process to perform reproducibly. Dehydration has previously been reported to increase the diffraction limit of many protein crystals, presumably by improving the packing of protein molecules in the crystal lattice. It is uncertain whether the very low Matthews coefficient of these crystals is related to their method of production.

Crystals of a truncated form of HRPI have also been grown by conventional means and the structure of the truncated protein has been solved (M. L. Sharpe, E. N. Baker and J. S. Lott, manuscript in preparation; PDB code 1xkf). Attempts were made to solve the structure of full-length HRPI by molecular replacement using several homology models based on the truncated HRPI structure and the native full-length data in different resolution ranges. The programs *AMoRe* (Navaza, 1994), *MOLREP* (Vagin & Telplyakov, 1997) and *PHASER* (Storoni *et al.*, 2004) were used and Patterson correlation refinement was also attempted in *CNS* (Brünger *et al.*, 1998), but no solution could be found.

The inability to solve the structure using the truncated model suggests that it is unlikely that it was the truncated form of HRPI that crystallized. Additionally, the unsuccessful molecular replacement is a possible indication that there are significant conformational differences between the truncated and full-length versions of the protein. This is consistent with an observed difference in dimerization behaviour after truncation (M. L. Sharpe, E. N. Baker and J. S. Lott, manuscript in preparation). Although no contaminants were detected by Coomassie stain on SDS–PAGE (Fig. 1), the slight possibility that the crystals are of a contaminating protein remains and cannot be formally ruled out because no crystals were left over after mounting and the native form was unable to be reproduced. We are currently preparing selenomethionine derivatives of HRPI for crystallization in an attempt to solve its full-length structure by single or multiple anomalous dispersion techniques.

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