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Crystallization and preliminary X-ray crystallographic analysis of the Rv2002 gene product from *Mycobacterium tuberculosis*, a β-ketoacyl carrier protein reductase homologue

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A 260-residue protein (FabG3) encoded by the Rv2002 gene of *Mycobacterium tuberculosis* shows amino-acid sequence similarity to β-ketoacyl carrier protein (ACP) reductase, FabG. A soluble mutant (I6T/V47M/T69M) was produced by the green fluorescent protein-based directed-evolution method. It was crystallized at 296 K using the hanging-drop vapour-diffusion method. The diffraction quality of the crystal improved significantly after annealing/dehydration. X-ray diffraction data were collected to 1.8 Å resolution using synchrotron radiation. The crystal belongs to the space group $P3_121$ (or $P3_221$), with unit-cell parameters $a = b = 70.38$, $c = 148.93$ Å. The asymmetric unit contains two subunits, with a corresponding V_M of 1.90 Å^3 Da^{-1} and a solvent content of 35.3%.

1. Introduction

Fatty-acid biosynthesis is an essential process providing membrane components and energy reserves. Fatty-acid elongation involves repetitive cycles of condensation, keto reduction, dehydration and enoyl reduction catalyzed by a β-ketoacyl ACP synthase, a β-ketoacyl ACP reductase, a β-hydroxyacyl ACP dehydratase and an enoyl ACP reductase, respectively. Two types of fatty acid synthase systems can achieve these enzymatic cycles. Fatty acid synthase I (FAS-I), a multifunctional enzyme with all the above enzymatic activities, is generally present in most eukaryotes except plants. Alternatively, fatty acid synthase II (FAS-II) systems, which catalyze the various individual steps by discrete dissociable enzymes, are present in bacteria and plants. In both FAS-I and FAS-II systems, the substrate is continuously activated *via* thioester linkage to the prosthetic group of an acyl carrier protein (ACP) or coenzyme A (Kremer *et al.*, 2000). Mycobacteria are unique in that they possess both type I and type II systems. FAS-I is capable of *de novo* synthesis of fatty acids in mycobacteria (Brindley *et al.*, 1969; Bloch, 1975). The mycobacterial FAS-II system is analogous to other bacterial FAS-II systems except for its primer specificity. It is not capable of *de novo* synthesis but preferentially extends palmitoyl-coenzyme A to a mixture of homologous molecules ranging in size from 18 to 30 C atoms (Brindley *et al.*, 1969).

Mycolic acids, which are a homologous series of C_{60} to C_{90} α-alkyl β-hydroxy fatty acids produced by all mycobacteria, are key components of the mycobacterial cell wall and form an effective barrier to the penetration of antibiotics and chemotherapeutic agents. Evidence to date strongly suggests that normal mycolic acid metabolism is crucial to the survival of *M. tuberculosis* (Winder, 1982). The FAS-II system lies in the early stage of mycolic acid biosynthesis in mycobacteria (Takayama & Qureshi, 1984). Thus, the FAS-II system is believed to be an attractive target for developing new antimycobacterial drugs. Isoniazid, one of the most effective and widely used antimycobacterial drugs, inhibits the enoyl reductase in the FAS-II system (Banerjee *et al.*, 1994).

The Rv2002 gene product (FabG3) of *M. tuberculosis* comprises 260 amino-acid residues, with a calculated molecular mass of 27 030 Da. It shows a sequence identity of 31% with the FabG protein, the gene product of Rv1483, which was characterized as a β-ketoacyl carrier protein (ACP) reductase (Banerjee *et al.*, 1998). The crystal structure of a plant β-ketoacyl carrier protein reductase, showing a sequence identity of 34% with the Rv2002 gene product of *M. tuberculosis*, has been reported (Fisher *et al.*, 2000). The *fabG* gene was shown to be essential for the growth of *Escherichia coli* (Zhang & Cronan, 1998). In order to obtain clues to the possible biological role of the Rv2002 gene product, we have initiated its structure determination. Here, we report the overexpression, crystallization and preliminary X-ray crystallographic data of its soluble mutant (I6T/V47M/T69M).

2. Experimental

2.1. Cloning, expression and directed evolution

The genomic DNA of the *M. tuberculosis* H37Rv strain was used as the template for

polymerase chain reaction (PCR) reactions. All DNA manipulations were carried out using standard procedures (Sambrook & Russell, 2001). Wild-type and mutant Rv2002 were cloned into a C-terminal hexahistidine-tagging vector, which was constructed using the same method of green fluorescent protein (GFP) fusion vector as described previously (Waldo *et al.*, 1999), except that the green fluorescent protein (GFP) encoding gene was replaced with a gene fragment encoding the histidine tag. The primers used in cloning and directed evolution were 5′-AGATATACATATGTC-TGGACGGTTGATAGGAAAGGTCGC-3′ and 5′-AATTCGGATCCCGTCACCCAT-TCCGGCTGCGAG-3′. *E. coli* strain XL2-Blue (Stratagene) was used in cloning and DH10B (Gibco BRL) was used in mutant library transformation. BL21-CodonPlus(DE3)-RIL (Stratagene) and B834(DE3) (Novagen) were used for expression.

2.2. Protein production and purification

E. coli B834(DE3) cells harbouring the plasmid containing the Rv2002 mutant gene were grown aerobically in Luria–Bertani medium containing 30 mg ml^{-1} kanamycin at 310 K until an OD_{600} of between 0.6 and 0.8 was reached. After being cooled to 298 K, the cells were induced with 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for a further 18 h. The cells were then harvested by centrifugation at 4200*g* (6000 rev min^{-1}; Sorvall GSA rotor) for 10 min at 277 K. The cell pellet was resuspended in ice-cold buffer *A* [20 m*M* Tris–HCl pH 7.9, 500 m*M* NaCl and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)] containing 5 m*M* imidazole and disrupted by ultrasonication. The crude cell extract was centrifuged at 95 800*g* (35 000 rev min^{-1}; Beckman 45 Ti rotor) for 1 h at 277 K. The clear cell lysate was loaded onto a HiTrap chelating HP column (Amersham-Pharmacia), which was previously charged with Ni^{2+} and equilibrated with buffer *A* containing 5 m*M* imidazole. After the column was washed with buffer *A* containing 80 m*M* imidazole, the bound proteins were eluted with a 20 ml linear concentration gradient of imidazole from 300 to 500 m*M* in buffer *A*. Next, gel filtration was performed on a HiLoad XK 16 Superdex 200 prep-grade column (Amersham Pharmacia), which was previously equilibrated with buffer *B* (20 m*M* Tris–HCl pH 8.0 and 0.1 m*M* PMSF) containing 100 m*M* NaCl. Further purification was achieved using two successive ion-exchange chromatography steps with Uno Q-1 and Uno S-1 columns (Bio-Rad). The Rv2002 mutant gene product was recovered in the flowthrough using buffer *B*, whereas the contaminating proteins remained adsorbed on the two ion exchangers. Finally, the purified sample was concentrated to 14.7 mg ml^{-1} using a YM10 membrane (Amicon) and then stored at 253 K. The protein concentration was estimated by measuring the absorbance at 280 nm, employing the calculated extinction coefficient of 1.04 mg^{-1} ml cm^{-1}.

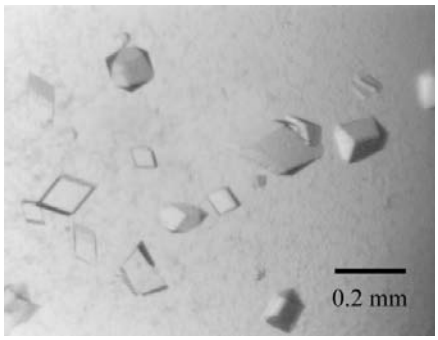

Figure 1
Crystals of a soluble mutant (I6T/V47M/T69M) of the Rv2002 gene product (FabG3) from *M. tuberculosis* in complex with β-NAD$^+$. Their approximate dimensions are 0.2 × 0.2 × 0.2 mm.

2.3. Crystallization

Crystallization was achieved by the hanging-drop vapour-diffusion method at 296 K using 24-well VDX plates (Hampton Research). Before preparing a hanging drop, 100 µl of the purified protein at 14.7 mg ml^{-1} concentration in buffer *B* was mixed with 2 µl of 100 m*M* β-NAD$^+$ solution. Each hanging drop was prepared by mixing equal volumes (2 µl each) of protein solution containing β-NAD$^+$ and the reservoir solution. Each hanging drop was placed over a 1.0 ml reservoir solution. Initial crystallization conditions were established using screening kits from Hampton Research (Crystal Screens I, II and MembFac) and from Emerald BioStructures, Inc. (Wizard I and II).

2.4. Crystal annealing/dehydration and data collection

The crystal was flash-frozen using 2-methyl-2,4-pentandiol as a cryoprotectant, which was added to the reservoir solution to a final concentration of 10%(*v*/*v*). Since the crystal gave poor spot shapes, particularly at high resolution, it was removed from the cold nitrogen-gas stream and was placed in a 100 µl drop of the cryoprotectant solution in a spot plate (Hampton Research) without sealing. The cryoprotectant solution dried out slowly and became very sticky after about 5 h, without phase separation occurring. The annealed and dehydrated crystal was flash-cooled again. X-ray diffraction data were collected at 100 K with an ADSC Quantum 4R CCD detector at the BL-18B experimental station of the Photon Factory, Tsukuba, Japan (Sakabe, 1991). The crystal was rotated through a total of 120°, with a 1.0° oscillation range per frame. The data set was processed and scaled using the programs *MOSFLM* (Leslie, 1992) and *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results

The wild-type Rv2002 gene (*fabG*3) of *M. tuberculosis* was expressed as inclusion bodies in *E. coli*. Therefore, the green fluorescent protein (GFP) based directed-evolution method (Waldo *et al.*, 1999) was applied to prepare soluble mutants. This method was developed to overcome the problem of inclusion-body formation, which is often encountered when recombinant proteins are overexpressed in *E. coli*. After three cycles of forward evolution without a backcrossing cycle, several soluble mutants with a few (three to five) point mutations were obtained. One of the soluble mutants with three point mutations, I6T/V47M/T69K, was selected for purification and crystallization. All three mutations avoid the conserved regions among the reported sequences of β-ketoacyl carrier protein (ACP) reductase and its homologues from *Mycobacterium* species.

Crystals of suitable size were obtained when the reservoir solution 20%(*w*/*v*) polyethylene glycol 3000, 200 m*M* calcium acetate and 0.1 *M* Tris–HCl pH 7.0 was used. The crystals grew to maximum dimensions of 0.2 × 0.2 × 0.2 mm within one week (Fig. 1). The flash-frozen crystal diffracted to ∼2.1 Å, but its spot shape was very poor, particularly at high resolution. We observed that the diffraction quality of the crystal dramatically improved upon annealing/dehydration, with the diffraction limit extending to ∼1.8 Å and the spots becoming sharper (Fig. 2). However, the exact mechanism of improvement is obscure.

A total of 40 259 unique reflections were measured with a redundancy of 7.0. The merged data set is 99.7% complete to 1.8 Å and gives an $R_{\rm merge}$ of 6.3%. The space group was determined to be $P3_121$ (or $P3_221$) on the basis of systematic absences and the unit-cell parameters are $a = b = 70.38$ (0.05) Å, $c = 148.93$ (0.07) Å, where estimated standard deviations are

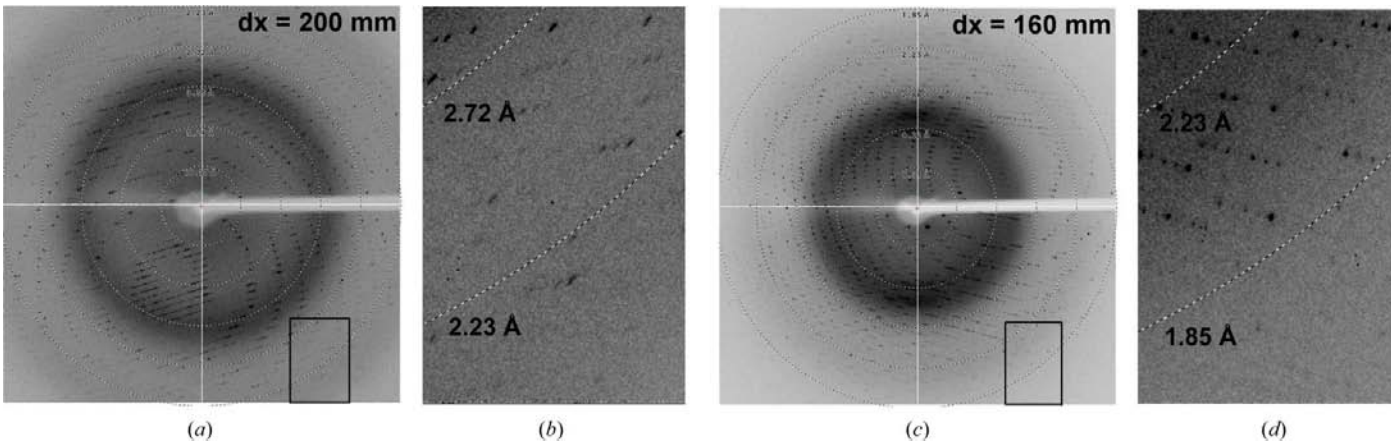

Figure 2
A comparison of X-ray diffraction images before and after crystal annealing/dehydration. (*a*) The crystal diffracted to ∼2.1 Å and gave poor-shaped spots before annealing/dehydration. The crystal-to-detector distance dx is 200 mm. (*b*) Magnified view of the boxed region in image (*a*). (*c*) The crystal diffracted to ∼1.8 Å and gave sharp spots after annealing/dehydration. The crystal-to-detector distance dx is 160 mm. (*d*) Magnified view of the boxed region in image (*c*).

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (1.90–1.80 Å).

X-ray wavelength (Å)	0.9800 (Photon Factory, BL-18B)
Space group	$P3_121$ (or $P3_221$)
Unit-cell parameters (Å,°)	$a = b = 70.38$, $c = 148.93$, $\alpha = \beta = 90$, $\gamma = 120$
Resolution (Å)	20.0–1.80
No. of measured reflections	280336
No. of unique reflections	40259
$R_{\rm merge}$† (%)	6.3 (34.5)
Data completeness (%)	99.7 (99.7)
$\langle I/\sigma(I)\rangle$	10.2 (2.2)
Redundancy	7.0 (5.8)

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

given in parentheses. The asymmetric unit is most likely to contain two subunits of the Rv2002 gene product, giving a $V_{\rm M}$ of 1.90 Å^3 Da^{-1} and a solvent content of 35.3% (Matthews, 1968). Table 1 summarizes the data-collection statistics. *In vitro* functional assay with both β-NAD$^+$ and β-NADP$^+$ is under way and the structure will be determined using multiple-wavelength anomalous diffraction (MAD) method, as molecular replacement is not possible.

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References

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., Collins, D., de Lisle, G. & Jacobs, W. R. Jr (1994). *Science*, **263**, 227–230.
Banerjee, A., Sugantino, M., Sacchettini, J. C. & Jacobs, W. R. Jr (1998). *Microbiology*, **144**, 2697–2707.
Bloch, K. (1975). *Methods Enzymol.* **35**, 84–90.
Brindley, D. N., Matsumura, S. & Bloch, K. (1969). *Nature (London)*, **224**, 666–669.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Fisher, M., Kroon, J. T. M., Martindale, W., Stuitje, A. R., Slabas, A. R. & Rafferty, J. B. (2000). *Structure*, **8**, 339–347.
Kremer, L., Baulard, A. R. & Besra, G. S. (2000). *Molecular Genetics of Mycobacteria*, edited by G. F. Hatfull & W. R. Jacobs Jr, pp. 173–190. Washington, DC: ASM Press.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACMB Newsl. Protein Crystallogr.* **26**.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Sakabe, N. (1991). *Nucl. Instrum. Methods A*, **303**, 448–463.
Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd ed. New York: Cold Spring Harbor Laboratory Press.
Takayama, K. & Qureshi, N. (1984). *The Mycobacteria: a Sourcebook*, edited by G. P. Kubica & L. G. Wayne, pp. 315–344. New York: Marcel Dekker.
Waldo, G. S., Standish, B. M., Berendzen, J. & Terwilliger, T. C. (1999). *Nature Biotechnol.* **17**, 691–695.
Winder, F. G. (1982). *The Biology of the Mycobacteria: Physiology, Identification and Classification*, Vol. 1., edited by C. Ratledge & J. Stanford, pp. 353–438. London: Academic Press.
Zhang, Y. & Cronan, J. E. Jr (1998). *J. Bacteriol.* **180**, 3295–3303.