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Initiating a crystallographic study of UDP-galactopyranose mutase from *Escherichia coli*

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UDP-galactopyranose mutase, the enzyme responsible for the conversion of UDP-galactopyranose to UDP-galactofuranose, has been crystallized in a form suitable for X-ray diffraction studies. UDP-galactofuranose is a key component of mycobacterial cell walls. Crystals of both the native protein and a selenomethionine variant have been grown by the vapour-diffusion method in hanging drops, and diffract to beyond 3.0 Å using synchrotron radiation. Equilibration was against a solution of 20%(*w*/*v*) polyethylene glycol (4K), 12%(*v*/*v*) 2-propanol, 0.1 *M* HEPES pH 7.6 at 293.5 K. Crystals grow as thin plates of dimensions 0.4 × 0.2 × ∼0.02 mm. They are orthorhombic, space group $P2_1$, with unit-cell dimensions $a = 71.12$, $b = 58.42$, $c = 96.38$ Å, $\beta = 96.38^\circ$. 92% (native) and 94% (selenomethionine) complete data sets have been recorded to 2.9 Å ($R_{\rm merge}$ = 5.0%) and 3.0 Å ($R_{\rm merge}$ = 6.9%), respectively. The Matthews coefficient is 2.35 Å^3 Da^{-1} for a dimer in the asymmetric unit, the solvent content being 47%. Diffraction data have also been recorded on a putative platinum derivative to 3.5 Å.

1. Introduction

The alarming increase in the incidence of tuberculosis worldwide led to the World Health Organization (WHO) declaring a 'global emergency' in 1993. It estimates that three million people die each year from tuberculosis and that this figure may rise to four million when the devastating link between tuberculosis and human immunodeficiency virus (HIV) is considered (Duncan, 1997). Being co-infected with HIV and *Mycobacterium tuberculosis* increases the risk of contracting a clinically significant disease from a 10% lifetime risk to around 10% a year (Young, 1995). Although in most cases of tuberculosis the present chemotherapy available is effective, it is apparent that more and more strains resistant to current therapeutic agents are emerging.

The cell wall of mycobacteria is essential for cell growth and survival in the host. It confers on bacteria an extraordinary resistance to drugs, the host immune system, dehydration and acid treatment (Draper, 1982). Ethambutanol, one of the most successful front-line drugs currently used to treat tuberculosis, functions by inhibiting cell-wall assembly (Winford, 1982). The structure of the mycobacterial cell wall is elaborate, consisting of several unusual carbohydrate polymers (glycans). The arabinogalactan layer covalently attaches a highly impermeable layer of mycolic acids to a peptidoglycan layer and is thus a key component of the cell wall. Arabinogalactan consists in part of alternating β-1,5 and β-1,6 galactofuranosyl residues. Galactofuranose is found only in bacteria and its synthesis is thus an intellectually appealing target for inhibition. The biosynthesis of UDP-galactofuranose (Gal_f) takes place by the

direct conversion of UDP-galactopyranose by UDP-galactopyranose mutase (mutase; Nassau *et al.*, 1995). Highly homologous enzymes (40% sequence identity) are found in both *Escherichia coli* and *Klebsiella pneumonia* (Weston *et al.*, 1998; Fig. 1). Analysis of the primary sequences identifies a consensus $\beta\alpha\beta$ (Rossmann fold) motif consistent with nucleotide sugar-binding enzymes. All three enzymes are known to incorporate flavin adenine dinucleotide (FAD), and the enzyme from *K. pneumonia* has recently been shown to require nicotinamide adenine dinucleotide (phosphate) (NADP) sugar-binding enzymes for activity (Koplin *et al.*, 1997). As the reaction carried out does not involve the net transfer of electrons, the mechanistic role of these cofactors is puzzling. In a program to study cell-wall biosynthesis, we have crystallized the native and selenomethionine variant enzyme from *E. coli*. Our experiments suggest that the *E. coli* enzyme also incorporates NAD(P).

2. Protein purification

All chemicals were purchased from Sigma (Poole, UK), except polyethylene glycol which was obtained from Fluka (Gillingham, UK). The clone for UDP-galactopyranose mutase was generously donated by Glaxo Wellcome.

5 ml of an enriched growth medium, Terrific Broth (Maniatis *et al.*, 1982), containing ampicillin (200 µg ml^{-1}) was inoculated with a single colony of BL21(DE3)(pORF6) and incubated overnight at 310 K with shaking. This was used to inoculate 500 ml of similarly treated media. Incubation continued under the same conditions until an OD_{600} of 0.6 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 m*M* to initiate protein expression. At this point the temperature was lowered to 303 K and incubation was continued for a further 3 h, after which the cells were harvested by centrifugation (30 min, 8000 rev min^{-1}, 277 K). The use of an enriched media, lower induction temperatures and lower IPTG concentrations all served to increase the amount of soluble protein obtained.

Cells were resuspended in ice-cold sonication buffer [50 m*M* tris(hydroxymethyl)aminomethane (Tris), 1 m*M* phenylmethysulfonyl fluoride (PMSF), 0.1 m*M* dithiothreitol (DTT)] at a concentration of 1.0 g cells per 1.0 ml buffer. Lysozyme was added (100 µg ml^{-1}) and the cells incubated at room temperature for 30 min. Sonication followed for 6 × 30 s intervals at 277 K. Cell debris was pelleted by centrifugation (30 min, 20000 rev min^{-1}, 277 K). Deoxyribonuclease was added (20 ng ml^{-1}) and the cell extract filtered (0.02 µm).

The selenomethionine variant of the protein was expressed by inhibition of methionine synthesis as described by Doublie (1997).

Crude extract was applied at 5 ml min^{-1} to a 7.84 ml anion-exchange column (Perseptive Biosystems, HQ) equilibrated in buffer *A* (50 m*M* Tris pH 7.6, 1 m*M* DTT) and eluted over a 0–500 m*M* NaCl gradient over 15 column volumes. Protein elution was detected on-line at 280 and 420 nm. UDP-galactopyranose mutase was easily identified by its distinct yellow colour, a function of the FAD co-factor. A_{420} peaks from subsequent runs were pooled and concentrated under nitrogen pressure in an Amicon ultrafiltration cell. Pooled fractions from the anion-exchange column were brought to 40% $NH_4(SO_4)_2$ saturation (2.4 g ml^{-1}) and applied at 5 ml min^{-1} to a 7.84 ml hydrophobic interaction chromato-

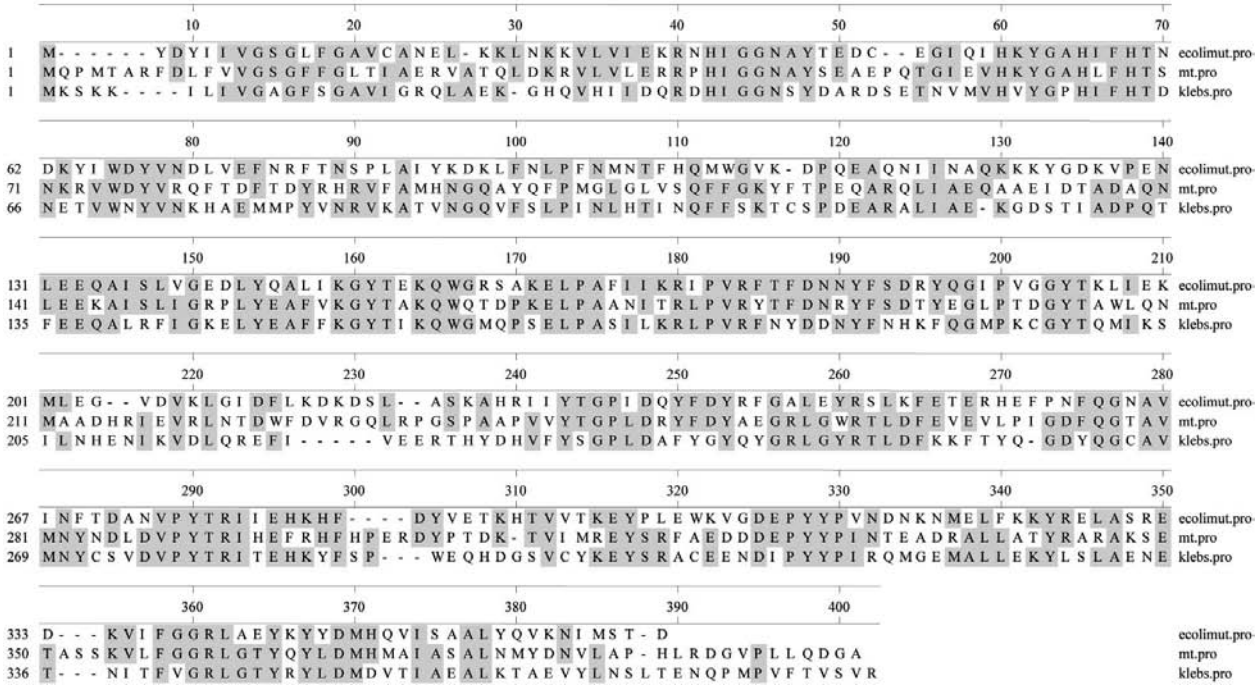

Figure 1
Alignment displaying the sequence homology between UDP-galactopyranose from *E. coli*, *M. tuberculosis* and *K. pneumonia*. The shaded area represents the amino acids common to each enzyme. Overall sequence homology is 40%.

graphy column (Perseptive Biosystems, HP) equilibrated in buffer *B* [50 m*M* Tris, 40% $NH_4(SO_4)_2$, 1 m*M* DTT]. Protein was eluted in a decreasing $NH_4(SO_4)_2$ gradient (100–0%) over 15 column volumes. A_{420} fractions were again combined and concentrated as before. Protein concentration was assayed by the method of Bradford (1976). This purification protocol yields highly homogenous protein for both the native and selenomethionine versions, as judged by sodium dodecyl sulfate and isoelectric focusing polyacrylamide gel electrophoresis. A yield of 8 mg per litre of culture was obtained. Dynamic light-scattering studies showed the protein to be monodisperse with no polydispersity value measurable and a molecular weight of 96 kDa, which is consistent with a dimer of predicted mass 86 kDa. Mass spectroscopic studies (which confirmed selenium incorporation to be greater than 85%) suggest that, as isolated, only 50% of the protein contains both cofactors and about 10% contains neither cofactor.

Crystallization conditions for the native protein were arrived at after screening using the vapour-diffusion hanging-drop methodology (Ducruix & Giegé, 1992). Two crystal forms were obtained. A single crystal of dimensions 0.4 × 0.2 × 0.05 mm was grown over one week from a solution of 10 mg ml^{-1} protein using 10%(*w*/*v*) polyethylene glycol (6K), 100 m*M* HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.0) as precipitant. The yellow crystal diffracted to 2.7 Å when fresh. The data was indexed using *DENZO* (Otwinowski, 1993) in an orthorhombic space group (*P*222, $P2_12_12_1$, $P2_12_12$ or $P222_1$) with cell dimensions a = 56, b = 94, c = 134 Å, $\alpha = \beta = \gamma = 90^\circ$. We have not been able to reproduce this form, despite extensive efforts. However, crystals grown over 5 d from a solution of 8 mg ml^{-1} protein, 100 m*M* HEPES pH 7.6, 20%(*w*/*v*) polyethylene glycol (4K), 12% 2-propanol, 0.01 *M* L-cysteine have been consistently reproduced. Crystals are yellow diamond-shaped flat plates of dimensions 0.04 × 0.02 × ∼0.002 mm. The second crystallization conditions are identical for selenomethionine protein with the addition of 1 m*M* DTT. Multiple seeding (2 d for each step, 5+ steps being typical) is required to obtain crystals of 0.5 × 0.3 × <0.06 mm (Fig. 2) which are suitable for diffraction analysis. Preincubation with both cofactors substantially decreases the crystallization time and number of seeding steps required, although it has little effect on the final crystal size. Using the in-house Nonius rotating-anode and image-plate system, diffraction to 3.5 Å is observed. Conditions for cryoprotection of the crystals have been developed (15% glycerol or 15% methane pentanediol) allowing data collection at cryogenic temperatures.

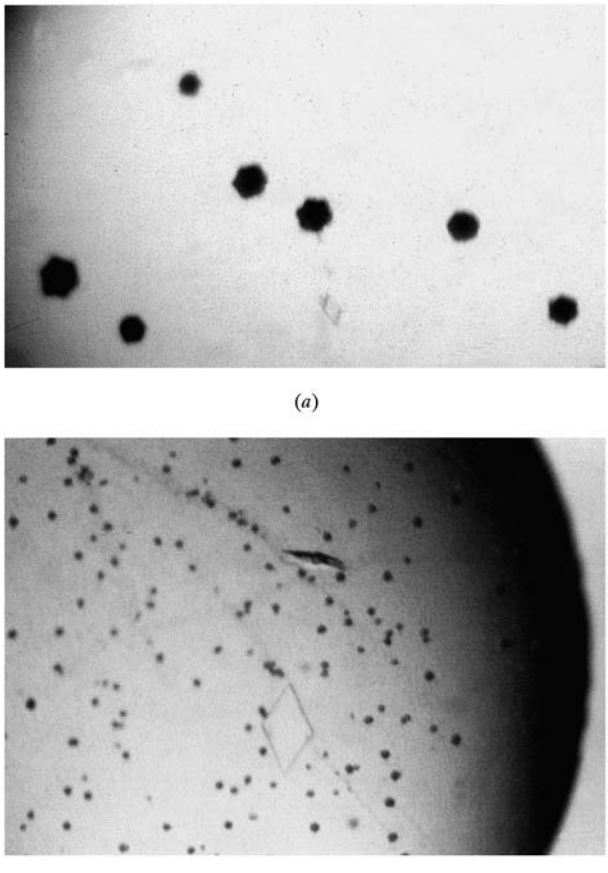

Figure 2
Crystals of UDP-galactopyranose mutase (*a*) before and (*b*) after seeding. The dimensions before seeding are 0.08 × 0.04 × ∼0.04 mm and increase to 0.5 × 0.3 × <0.06 mm.

3. Data collection

A single crystal of UDP-galactopyranose mutase with dimensions 0.8 × 0.4 × ∼0.04 mm was cryoprotected (15% glycerol in mother liquor); soaking took place for approximately 2 min. The crystal was then scooped up with a rayon loop (Hampton Research) and rapidly transferred to the cold nitrogen stream (110 K). Diffraction data were collected on

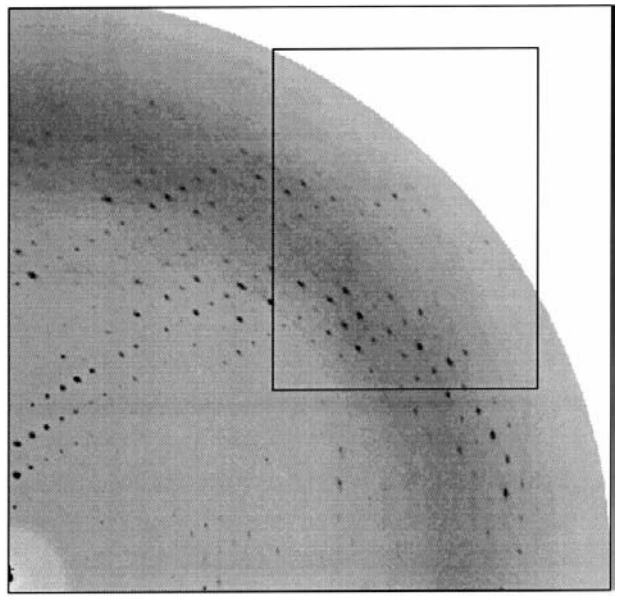

Figure 3
Section of a 1° oscillation diffraction pattern from a crystal of UDP-galactopyranose mutase taken at BM14, ESRF, Grenoble recorded with an MAR345 image plate. The resolution at the edge of the photograph corresponds to 2.8 Å.

BM14 (λ = 1.001 Å) using a MAR345, 18 cm mode image plate at the European Synchrotron Radiation Facility, Grenoble, France. Data were collected as 145 non-overlapping images in steps of 1° at a crystal-to-detector distance of 242 mm. Diffraction was visible to 2.7 Å, though weak below 2.9 Å (Fig. 3). No significant crystal decay was observed. Data were processed using *DENZO* and *SCALEPACK* (Otwinowski, 1993) yielding 17660 unique reflections. The crystal was indexed in a monoclinic space group with unit-cell dimensions a = 71.12, b = 58.42, c = 97.89 Å, $\alpha = \gamma = 90$, β = 96.38°. Analysis of diffraction data identified systematic absences consistent with space group $P2_1$. Assuming a dimer of molecular mass 43 kDa for the monomer, V_m is 2.35 Å^3 Da^{-1} which falls in the allowed range for protein crystals (Matthews, 1968) and indicates a solvent content of 47%. The data are 92% complete from 15 to 2.9 Å, with an $R_{\rm merge}$ of 5.0% and a redundancy of 2.5. For the highest resolution shell (3.0–2.9 Å), the data have a redundancy of 2.3 with an $R_{\rm merge}$ of 27%.

As no homologous structure is known, we will need to determine phases experimentally. A putative platinum-derivative data set has also been recorded. A crystal of dimensions 0.4 $\times$0.2 $\times$ $\sim$0.03 mm was soaked in 3.5 mM K_2PtCl_6 for 9.5 h and back-soaked in cryoprotectant for 2 min before being rapidly transferred to the cold nitrogen stream (100 K). Data were collected as 298 non-overlapping images at 0.5° oscillation on station ID14 EH3 at the ESRF using an MAR CCD with a crystal-to-detector distance of 148 mm. The data are 85% complete to 3.5 Å and have an $R_{\rm merge}$ of 8.1% and a redundancy of 2.8. For the highest resolution shell (3.7–3.5 Å), the data have a redundancy of 2.9 with an $R_{\rm merge}$ of 14%. We were not able to locate the heavy-atom positions with this data. However, an EXAFS experiment did confirm the presence of platinum. In addition, a data set was collected with selenomethionine-variant protein. Data was again collected at 100 K, λ = 0.926 Å, crystal-to-detector distance of 148 mm, using an MAR CCD on ID14 EH3 at the ESRF. A single crystal of dimensions 0.4 $\times$ 0.25 $\times$ $\sim$0.03 mm was used to record the entire data set in two sweeps of 25 s per 1° oscillation. The data are 94% complete (74% of the anomalous data) to 3.0 Å. $R_{\rm merge}$ is 6.9% and the redundancy is 1.8. For the highest resolution shell (3.16–3.0 Å), the data have a redundancy of 1.9 with an $R_{\rm merge}$ of 13.7%.

We expect structure determination to be aided by the presence of two molecules within the asymmetric unit, allowing non-crystallographic averaging. We have extended our studies to the enzymes from *K. pneumonia* and *M. tuberculosis*. Small crystals of the *K. pneumonia* enzyme have been obtained. We expect to solve these structures by molecular replacement based on the *E. coli* structure.

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