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Crystallization and preliminary crystallographic analysis of a novel cytochrome P450 from *Mycobacterium tuberculosis*

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The product of the Rv2276 gene of *Mycobacterium tuberculosis* is a cytochrome P450 (P450 MT2, CYP121) which has been shown to bind tightly to a range of azole-based antifungal drugs (e.g. miconazole, clotrimazole). These drugs are potent inhibitors of mycobacterial growth, suggesting that P450 MT2 (CYP121) may be a potential drug target. The enzyme has been overexpressed in *Escherichia coli* and crystallized by the hanging-drop method. Crystals of P450 MT2 (CYP121) belong to the hexagonal space group $P6_122$ or $P6_522$, with unit-cell parameters $a = b = 78.3$, $c = 265.6$ Å. Native data have been collected to 1.6 Å resolution and Hg-derivative data to 2.5 Å resolution using a synchrotron-radiation source.

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1. Introduction

Tuberculosis (TB) is a debilitating and potentially fatal disease which has made a dramatic resurgence in the last 20 years and the prevalence of strains of *Mycobacterium tuberculosis* that have become resistant to traditional drugs (including rifampicin, ethionamide, isoniazid and ethambutol) highlights the requirement for new drugs and treatment strategies (World Health Organization, 1999). The recent determination of the genome sequence of *M. tuberculosis* strain H37Rv has enhanced our understanding of the biology of this pathogen (Cole *et al.*, 1998). In particular, it is clear that a large proportion of the genome encodes enzymes involved in the synthesis and inter-conversion of lipids (which form part of the complex *M. tuberculosis* cell envelope) and also encodes an extraordinarily large number of cytochromes P450 (P450s). The P450s are haem-containing oxygenase enzymes found in large numbers in mammals and plants, where they carry out roles in biosynthesis and detoxification, but they are relatively rare in prokaryotes, where they generally participate in the degradation of organic compounds such as camphor (Miles *et al.*, 2000). The abundance of P450s in *M. tuberculosis* would therefore point towards functions of greater importance in this organism and indeed one of the P450s present, CYP51 (P450 MT1), which is encoded by the Rv0764c gene, has been characterized and shown to be a sterol demethylase (Podust *et al.*, 2001). It is now thought that sterols (as well as polyketides and complex cross-linked fatty acids) are present in the membrane of *M. tuberculosis*. Sequence comparison of *M. tuberculosis* P450s with P450s of known function, such as P450eryF from *Saccharo-*

polyspora erythraea which is involved in erythromycin synthesis (Cupp-Vickery & Poulos, 1995), indicates that many of these enzymes have roles to play in polyketide and lipid metabolism. The presence of such enzymes raises the issue of unusual metabolic pathways in *M. tuberculosis* and also the possibility that these enzymes could be useful as novel anti-TB drug targets. In particular, the fact that azole-based P450 inhibitors such as ketoconazole, fluconazole and clotrimazole that target sterol-metabolizing P450s are effective antifungal agents demonstrates the applicability of this approach. Recent studies have demonstrated that selected azole drugs are very potent anti-mycobacterials, with inhibitory concentrations in the nanomolar range. Our recent work has demonstrated that these drugs have high affinity not only for the sterol demethylase from *M. tuberculosis* (Guardiola-Diaz *et al.*, 2001), but also for P450 MT2 (CYP121). Preliminary studies on the cytochrome P450 which is the product of the gene Rv2276 (P450 MT2, CYP121) indicate tight binding of azole drugs with K_d values in line with the apparent potency of the compounds as anti-mycobacterials, suggesting that this enzyme may in fact be a target in *M. tuberculosis* (Souter *et al.*, 2000). We have been attempting to determine the crystal structure of the enzyme as a means of providing a basis upon which to perform a full investigation of the catalytic and kinetic features of the P450, to analyse the means by which existing azole drugs bind to the enzyme and to allow the design of new drugs with even greater anti-mycobacterial potency. In this paper, we report the crystallization and preliminary X-ray crystallographic analysis of the cytochrome P450 product of the Rv2276 gene of *M. tuberculosis*.



Figure 1
Native crystals of P450 MT2 (CYP121) grown by the hanging-drop method.

2. Materials and methods

2.1. Crystallization

The Rv2276 gene was cloned by PCR from a cosmid library of *M. tuberculosis* DNA (supplied by Professor Stewart Cole, Pasteur Institute). The gene was expressed in *Escherichia coli* in a pET11 vector under the control of an inducible T7 promoter and the P450 MT2 (CYP121) encoded was purified by column chromatography on DEAE Sephacel and hydroxyapatite, and FPLC on Q-Sepharose. The purified enzyme has a molecular weight of 43.2 kDa and contains a single cysteine-ligated *b*-type haem per molecule. The enzyme was crystallized using the hanging-drop vapour-diffusion method at 277 K with a well solution consisting of 0.1 M Na MES buffer pH 5.0–6.0 with 1.75–2.50 M ammonium sulfate as precipitant. The protein concentration was 10 mg ml⁻¹ (in 50 mM Tris-HCl buffer pH 7.4) and hanging drops were prepared using 2 µl of the enzyme solution and 2 µl of the well solution. Dark red hexagonal bipyramidal crystals were observed to form after 3–5 d, the largest of which had dimensions of approximately 1.5 × 0.3 × 0.3 mm after two weeks growth (Fig. 1).

2.2. Preparation of the Hg derivative

Preparation of a Hg derivative of P450 MT2 (CYP121) was achieved by soaking crystals in a solution of mother liquor containing 10 mM mercury nitrate for 8 h at 277 K. Unbound Hg was removed by soaking the crystals in mother liquor for 30 min prior to data collection.

2.3. Data collection

Prior to data collection, crystals were briefly immersed in a cryoprotectant solu-

tion comprising 30% glycerol in mother liquor and flash-cooled by plunging into liquid nitrogen. All data were collected at 100 K. The native crystal data set was collected at station 9.6 at Daresbury Synchrotron Radiation Source using an ADSC Quantum 4 CCD detector and a wavelength of 0.870 Å, while the Hg-soaked crystal data set was collected at station 14.1 at Daresbury Synchrotron Radiation Source using an ADSC Quantum 4 CCD detector and a wavelength of 1.488 Å. The statistics for each data set are summarized in Table 1. Indexing and scaling of the data were carried out using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997).

3. Results

Crystals of both native P450 MT2 (CYP121) and the Hg derivative were found to belong to either space group *P*₆122 or *P*₆522. The unit-cell parameters of the native crystal are *a* = *b* = 78.3, *c* = 265.6 Å and those of the Hg derivative are *a* = *b* = 77.7, *c* = 263.8 Å. The native crystal was found to diffract to 1.6 Å resolution, while the derivatized crystal data were collected to a resolution of 2.5 Å. A Patterson difference map was calculated from the two data sets, the *w* = 2/3 Harker plane of which indicated the clear presence of one Hg site per protein molecule (Fig. 2).

4. Conclusions

Although belonging to a family of enzymes for which there are seven structures publicly available, all attempts to solve the structure of P450 MT2 (CYP121) by molecular replacement have proved unsuccessful. The closest sequence homology is with P450eryF (PDB code 1oxa), with 28.1% identity. We are therefore using the MIR approach to solve the structure of this important enzyme. Having already collected a data set for a single-site Hg derivative, we are currently screening for other derivatives. Preliminary work indicates that azole derivatives may prove an effective new class of drugs against the multidrug-resistant strains of the tuberculosis pathogen and that P450 MT2 (CYP121) may be the key P450 inhibited by these drugs. The recent structure of a polyketide-metabolizing P450 (P450eryF) in complex with one of these drugs (ketoconazole) reinforces the fact that it is not only sterol-metabolizing P450s that bind these

Table 1
Data-collection statistics.

| Crystal | Native | Hg derivative |
|---|----------------------|----------------------|
| Resolution (Å) | 20.0–1.6 (1.66–1.60) | 20.0–2.5 (2.59–2.50) |
| No. of observations | 548981 | 182241 |
| No. of unique reflections | 63580 (5489) | 14688 (786) |
| $\langle I \rangle / \langle \sigma(I) \rangle$ | 17.5 (3.0) | 20.6 (8.0) |
| Completeness (%) | 98.1 (86.5) | 85.2 (47.0) |
| <i>R</i> _{merge} (%) | 10.1 (24.7) | 11.0 (11.4) |

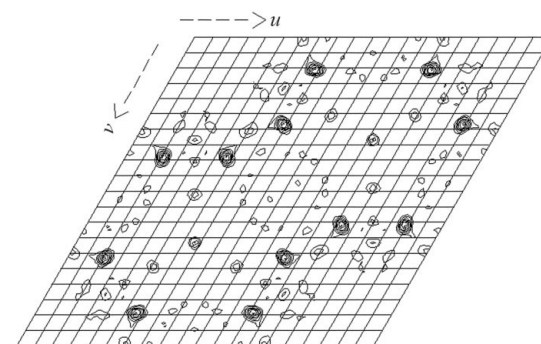


Figure 2
The difference Patterson Harker plane *w* = 2/3. The peak pattern corresponds to a single Hg site per protein molecule.

drugs efficiently (Cupp-Vickery *et al.*, 2001). Thus, we are also attempting to co-crystallize P450 MT2 (CYP121) with a variety of these azoles in order to establish the structural basis for their tight binding to this important pathogen P450.

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