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Crystallization of recombinant *Leishmania major* pteridine reductase 1 (PTR1)

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The enzyme pteridine reductase (PTR1) has recently been discovered in the protozoan parasite *Leishmania* and validated as a target for therapeutic intervention. PTR1 is responsible for the salvage of pteridines and also contributes to antifolate drug resistance. Structural analysis, in combination with ongoing biochemical characterization will assist the elucidation of the structure–activity relationships of this important enzyme and support a structure-based approach to discover novel inhibitors. Recombinant *L. major* PTR1 has been purified from an *Escherichia coli* expression system and used in crystallization experiments. Orthorhombic crystals have been obtained and data to 2.8 Å has been measured. The space group is $P2_12_12$ or $P2_12_12_1$ with unit-cell dimensions of a = 103.9, b = 134.7, c = 96.2 Å. One homotetramer, of molecular mass approximately 120 kDa, probably constitutes the asymmetric unit and gives a Matthews coefficient, V_m, of 2.8 Å^3 Da^{-1} and 56% solvent volume. Self-rotation function calculations show a single well defined non-crystallographic twofold axis with features that might represent additional elements of non-crystallographic symmetry. The detail of exactly what constitutes the asymmetric unit will be resolved by structure determination.

1. Introduction

Leishmania, a genus of trypanosomatid protozoan parasites, infects millions of people worldwide causing a spectrum of tropical diseases collectively termed leishmaniasis (World Health Organization, 1984). The severity of these diseases varies from minor cutaneous lesions to a visceral form which, if left untreated, is fatal. Despite their considerable promise there is as yet no suitable vaccine for leishmaniasis and the current therapies are inadequate due to toxicity and increasingly prevalent drug-resistant forms of the parasites. One way forward in the search for improved drugs against protozoan parasites involves a multi-disciplinary approach to identify and validate suitable drug targets, which are most often enzymes, to characterize the structure–reactivity relationships of the targets and to use this information to guide the search for suitable inhibitors with the desired therapeutic properties. Folate/pterin metabolism of *Leishmania* and, in particular, the recently discovered enzyme pteridine reductase is one area that might be exploited in this manner.

Trypanosomatids lack a pathway for the biosynthesis of both pteridines and folates but instead must salvage them from their hosts. In *Leishmania*, pteridine reductase 1 (PTR1) has been shown to be the primary enzyme involved in the essential salvage of unconjugated pterins (Bello *et al.,* 1994*)*. PTR1 is an NADPH-dependent broad-spectrum pteridine reductase which catalyzes the reduction of biopterin to dihydrobiopterin and subsequently to tetrahydrobiopterin (Nare, Hardy *et al.,* 1997; Nare, Luba *et al.*, 1997).

PTR1 is a homotetramer with a subunit molecular mass of approximately 30 kDa. Sequence comparisons have revealed about 25% homology with short-chain dehydrogenase/reductase (SDR family) and aldo/keto reductases (Callahan & Beverley, 1992; Papadopoulou *et al.*, 1992). Stereochemical studies have confirmed PTR1 to be a B-side dehydrogenase and a member of the SDR superfamily (Luba *et al.*, 1998). This family of enzymes comprises at least 60 distinct members which regulate diverse metabolic processes. They are functional as either homodimers or tetramers and display an enormous spread of substrate specificity (Jornvall *et al.,* 1995; Duax *et al.,* 1996). The enzymes function as dehydrogenases as well as reductases, in the latter case they reduce both C═O and C═C bonds. Crystal structures of several members of the family are available and include 3α/20β-hydroxysteroid dehy-

drogenase (Ghosh *et al.,* 1994), 7-α-hydroxysteroid dehydrogenase (Tanaka *et al.,* 1996*a*), mammalian dihydropteridine reductase (DHPR; Varughese *et al.,* 1992), 1,3,8-trihydroxy-napthalene reductase (Andersson *et al.*, 1996), carbonyl reductase (Tanaka *et al.,* 1996*b*), sepiapterin reductase (Auerbach *et al.,* 1997) and β-hydroxysteroid dehydrogenase (Mazza *et al.,* 1998). These proteins show a similar tertiary structure comprising a single α/β domain carrying a dinucleotide binding Rossmann fold. The single domain structure is distinct from the two domain structures of mammalian alcohol, lactate, malate or glyceraldehyde 3-phosphate dehydrogenases. The SDR's for which structures are available all have a conserved catalytic triad comprising a tyrosine, a lysine and serine, however, given the diversity of function and sequence it is not surprising that different structural features regulate very distinctive active sites (Jornvall *et al.,* 1995).

PTR1 is also able to catalyze the two-step reduction of folate to H_2folate and subsequently to H_4folate. This ability to reduce folate is the same reaction catalyzed by the dihydrofolate reductase (DHFR) component of the DHFR-thymidylate synthase bifunctional polypeptide (DHFR-TS) which is the presumptive primary cell target for methotrexate (Beverley, 1991; Beverley *et al.,* 1984, 1986). PTR1 is less sensitive to methotrexate than DHFR at physiological pH with IC_{50} values of 1.1 μM for PTR1, 0.005 and 0.04 μM for *L. major* and human DHFR, respectively. Therefore, PTR1 has the potential to provide a metabolic by-pass of DHFR-TS inhibited by methotrexate. Even though in the normal situation in the cell PTR1 is expressed at low levels (0.01% total cell protein) it can mediate methotrexate resistance when overexpressed (Callahan & Beverley, 1992; Papadopoulou *et al.*, 1992).

PTR1, therefore, compromises any drug targeted against DHFR-TS and provides an explanation of why antifolate therapies have failed with respect to the treatment of *Leishmania* infections. In order to be useful, antifolate therapy targeting DHFR-TS must also inhibit PTR1. PTR1 has itself been validated as a drug target since a knockout is not viable unless provided with a source of reduced pterins (Bello *et al.*, 1994; Nare, Hardy *et al.*, 1997). The crystal structure of PTR1 is sought to aid the search for new antifolate inhibitors, to allow the investigation of the enzyme pterin interactions and to elucidate the structural basis for catalysis and substrate recognition that allows the dual functionality of PTR1.

2. Methods and results

2.1. Preparation of recombinant PTR1

The *L. major* PTR1 gene (Callahan & Beverley, 1992; Bello *et al.,* 1994) was subcloned into the T7-promoter based *E. coli* expression pET15b system (Studier *et al.*, 1990; Novagen) to give the plasmid designated pET-*Lm*PTR1H. This plasmid codes for a hexa-histidine tag on the N-terminus of the gene product and allows the use of metal chelate affinity chromatography in the purification of the enzyme. The *E. coli* strain BL21(DE3) was heat-shock transformed with pET-*Lm*PTR1H and selected on Luria–Bertani (LB) agar plates containing 100 $\mu g\ ml^{-1}$ of ampicillin. Bacteria were cultured in LB broth with 50 $\mu g\ ml^{-1}$ ampicillin to mid-log phase at which point expression of PTR1 was induced with 0.4 m*M* isopropyl-β-D-thiogalactopyranoside and cell growth continued with vigorous aeration for 16 h at 301 K. Cells were harvested by centrifugation (2500*g*) at 277 K then resuspended in 50 m*M* Tris–HCl pH 7.6, 250 m*M* sodium chloride, 5 m*M* benzamidine and stored frozen in liquid nitrogen. The cells were thawed on ice and broken by passage through a French press then the insoluble cell debris was pelleted by centrifugation at 277 K (18 000*g*) for 15 min. The cell extract was filtered and then applied to a 5 ml metal chelate affinity column (HiTrap; Pharmacia) charged with nickel. The unbound proteins were washed from the column with 50 m*M* Tris–HCl, pH 7.6, containing 250 m*M* sodium chloride and 5 m*M* benzamidine. The his-tagged protein was eluted using a 0–500 m*M* imidazole gradient in the same buffer. The eluted protein was then incubated with 50 units of thrombin (Pharmacia) for 12 h at 293 K to remove the histidine tag. PTR1 was separated from the thrombin, uncleaved fusion protein and N-terminal peptide by strong anion-exchange chromatography using a RESOURCE Q (Pharmacia) column on a BioCAD 700E workstation. Pooled fractions were dialyzed overnight in 20 m*M* sodium acetate buffer, pH 5.3, concentrated to approximately 20 $mg\ ml^{-1}$ and SDS–PAGE electrophoresis together with MALDI-TOF mass spectrometry (PerSeptive Biosystems, Voyager-DE STR) were used to check the purity of the sample. The yield of purified protein is around 15 $mg\ l^{-1}$ of bacterial culture. A ternary complex of PTR1 with methotrexate and NADPH was prepared by adding 2 ml of 20 m*M* Tris–HCl buffer, pH 7.0 containing 1 m*M* methotrexate, 1 m*M* NADPH and 20 m*M* dithiothreitol to 0.2 ml of the 20 $mg\ ml^{-1}$ PTR1 solution. The mixture was incubated on ice for 20 min, then the volume reduced to 0.2 ml with centrifugal concentrators (Centricon 10; Amicon) and this sample was then used for crystallization trials.

2.2. Crystallization

Initial crystallization experiments were based around the sparse-matrix sampling approach (Jancarik & Kim, 1991) using Crystal Screens I and II purchased from Hampton Research (USA). Both hanging- and sitting-drop methods were used and crystallization trials were duplicated at 277 and 293 K. Promising crystalline precipitates and micro-crystals were obtained under a wide range of conditions some of which are reported by Luba (1997). Further experiments produced crystals displaying well defined morphology, of a size suitable for diffraction studies (Fig. 1). Such crystals were grown from 3 µl of ternary complex mixed with 3 µl of reservoir solution on the cover slip and then sealed over the reservoir using vacuum grease. The reservoirs used were 400 µl of 11–14% PEG 5000, 100 m*M* sodium acetate buffer pH 5.5, and 100 m*M* calcium acetate. The best crystals grow in 12 h at 293 K to a maximum size of 0.4 × 0.3 × 0.3 mm.

2.3. X-ray diffraction and unit-cell characterization

A resolution test was carried out on one of the first small crystals that were obtained. A sample of dimensions 0.05 × 0.05 × 0.25 mm was mounted and sealed in a glass capillary then exposed to X-rays (λ = 1.00 Å) at room temperature using beamline BM14 at the European Synchrotron Radiation Facility (Grenoble, France). Diffraction was observed to Bragg spacings of 2.3 Å. However, the diffraction pattern indicated both a high degree of mosaicity and

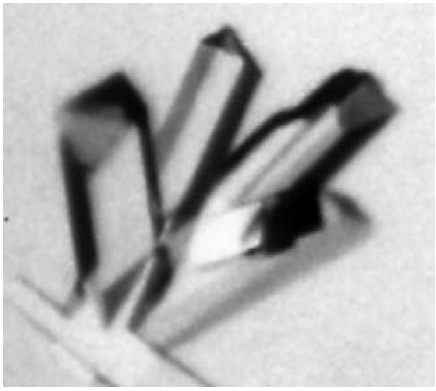

Figure 1
A cluster of orthorhombic crystals of *L. major* PTR1.

mechanical twinning hence the crystal was deemed unsuitable for data collection. This test indicated that medium-resolution data could be obtained and encouraged further experiments to optimize the quality of the crystals. We can now reproducably grow crystals of good size and appearance. One of these, with the largest dimension of 0.4 mm, was flash frozen at 100 K in a nylon loop using glycerol as a cryo-protectant, and exposed to X-rays (λ = 1.20 Å) on beamline PX9.5 at the Synchrotron Radiation Source (Daresbury, UK) using a 30 cm image-plate detector (MAR Research). We were constrained by time limitations and technical problems on the station which resulted in a greatly reduced X-ray intensity. The *HKL* suite of programs was used for autoindexing and data processing (Otwinowski & Minor, 1997). The orthorhombic crystals displayed Laue group *mmm*, with unit-cell dimensions of a = 103.9, b = 134.7 and c = 96.2 Å. We obtained 29 731 unique reflections (86.3% complete) from 160 618 measurements to 2.8 Å resolution with an $R_{\rm merge}$ of 8.5% overall and 17.7% in the highest resolution bin, 2.9–2.8 Å. The overall $I/\sigma(I)$ is 7.3 with a value of 4.2 in the highest resolution bin. Systematic absences confirm the presence of 2_1 axes along a and b but it is not yet clear whether a 2_1 is present along c. This means that the space group is identified as either $P2_12_12_1$ or $P2_12_12$.

In solution, PTR1 is functional as a homotetramer with a subunit of 288 amino acids, molecular mass approximately 30 kDa. A Matthews coefficient, V_m (Matthews, 1968), of 5.7 Å^3 Da^{-1} is calculated assuming a dimer of approximately 60 kDa in the asymmetric unit with 78% solvent volume. Alternatively, V_m is 2.8 Å^3 Da^{-1} assuming a homotetramer in the asymmetric unit with about 56% solvent volume. The range of typical V_m values observed for protein crystals is 1.68–3.53 Å^3 Da^{-1} and 27–65% solvent volume (Matthews, 1968) although there are exceptions. So, whilst a homotetramer in the asymmetric unit appears most likely we cannot exclude the possibility that only a homodimer constitutes the asymmetric unit. Self-rotation functions were calculated using the program *POLARRFN* written by Dr W. Kabsch and distributed by the Collaborative Computational Project, Number 4 (1994). The resolution limits and the volume of integration for the Patterson function were varied with one example given in Fig. 2. A single non-crystallographic twofold axis, at almost a third the peak height of the proper crystallographic axes, was identified at φ = 90, ψ = 67, κ = 180° and suggests that a dimer occupies the asymmetric unit. This detail will be resolved by structure determination.

As discussed earlier, there are a number of crystal structures of members of the SDR family of enzymes that provide models to attempt structure solution using molecular-replacement methods. However, this approach has so far proven unsuccessful. The SDR structures, for which three-dimensional models are available, share less than 24% sequence identity with PTR1. In addition, although the SDRs have similar tertiary structures, the quaternary structures vary with the enzymes being functional as either homodimers or like PTR1, homotetramers. The problem of attempting to solve an SDR family member by molecular replacement is briefly discussed by Auerbach *et al.* (1997) in their study of mouse sepiapterin reductase. In that case, alignments of 3.0 Å between 122 Cα atoms of mouse sepiapterin reductase and dihydropteridine reductase are reported and like Auerbach *et al.*, we will now seek experimental phases to facilitate structure determination.

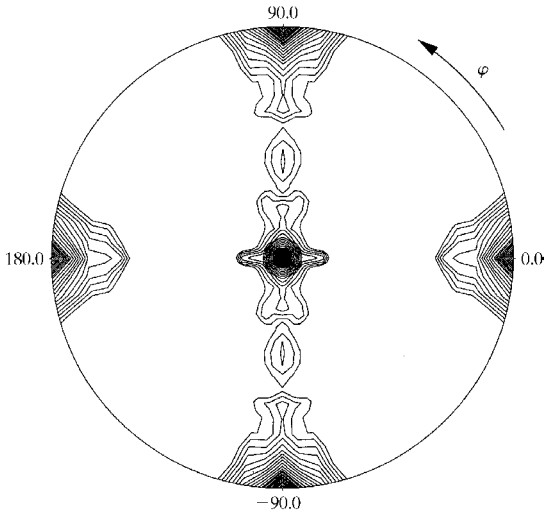

Figure 2
A self-rotation function, κ = 180° section. This has been calculated using all data in the range 15–2.8 Å with a radius of integration in the Patterson function of 30 Å. The view is down the c axis; $\psi = 90°$, $\varphi = 0$ corresponds to the direction of the a axis, and $\psi = 0$ to the direction of the c axis.

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