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Expression, purification, crystallization and preliminary crystallographic analysis of recombinant pteridine reductase of *Trypanosoma cruzi*

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The recombinant version of *Trypanosoma cruzi* pteridine reductase was expressed in *Escherichia coli*, purified to homogeneity from the soluble fraction of bacterial extract by metal-chelate affinity chromatography and crystallized in the presence of the cofactor (NADPH) and an inhibitor (methotrexate) at 295 K using sodium acetate as precipitant. The crystals are trigonal, belonging to space group $P3_1$ (or $P3_2$), with unit-cell parameters a = 74.35, c = 179.96 Å under cryogenic conditions. The asymmetric unit contains a tetramer, with a corresponding V_M of 2.3 $Å^3$ $Da^{-1}$and a solvent content of 46%. Native data have been collected to 2.1 Å resolution using Cu $K\alpha$ X-rays.

1. Introduction

T. cruzi, a member of the family of trypanosomatid protozoan parasites, is the causative agent of Chagas' disease or American trypanosomiasis. *T. cruzi* is transmitted to humans through the feces of infected bloodsucking insects in endemic areas of Latin America or by non-vectorial mechanisms such as blood transfusion. Vector-control efforts for Chagas' disease have been quite successful, but the prevalent form of transmission is now through blood transfusion, which often leads to a chronic disease stage (Urbina, 1999). An estimated 16–18 million cases are reported each year and approximately 100 million people in 21 countries are at risk (25% of the population of Latin America). Chagas' disease is a debilitating and often fatal disease. There are no vaccines and the drug benznidazole (Roche), presently in use to control the acute stage of the disease, shows restricted applicability in chronic patients, besides presenting severe side effects (De Castro, 1993; Urbina, 1999).

Trypanosomatids lack a pathway for the biosynthesis of both pteridines and folates and salvage these chemicals 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 and the reduction of pterins (Bello *et al.*, 1994). The enzyme is also capable of catalyzing the two-step reduction of folate to dihydrofolate and subsequently to tetrahydrofolate, reactions which are also catalyzed by the dihydrofolate reductase (DHFR) component of the parasitic DHFR-thymidylate synthase bifunctional polypeptide (DHFR-TS). Since PTR1 is less sensitive to the classical antifolate drug methotrexate (MTX) than DHFR (by a factor of 200), it has the potential to provide a metabolic bypass for the MTX. When overexpressed in the cell it can mediate resistance to MTX (Bello *et al.*, 1994). Therefore, PTR1 compromises the effectiveness of any drug targeted against DHFR-TS to treat *T. cruzi* infections. Related enzymes with different substrate specificity have been reported in other microorganisms (Nare *et al.*, 1997). In order to be useful, antifolate therapy targeting DHFR-TS must be combined with inhibitors of pteridine reductase activity. The crystal structure of pteridine reductases should aid in the search for new inhibitors by providing a detailed view of the enzyme–pterin interactions and allowing the elucidation of the structural basis for catalysis and substrate recognition that allows the dual functionality of the enzyme.

PTR1, which belongs to the family of short-chain dehydrogenases, is an NADPH-dependent broad-spectrum pteridine reductase. The sequence alignment of pteridine reductase from *T. cruzi* shows 48% identity and 58% homology at the amino-acid level with PTR1 from *Leishmania major* and *L. tarentolae*, respectively (Robello *et al.*, 1997). Sequence comparisons with other short-chain dehydrogenases/reductases (SDR family) show less than 25% sequence identity. Previously, only PTR1 from *L. major* has been crystallized (Gourley *et al.*, 1999). Recently, the crystal structure of this enzyme has been reported (Gourley *et al.*, 2001), although coordinates are currently not available.

Overexpression of pteridine reductase in *T. cruzi* resulted in a fivefold increase in resistance to MTX and 10.4-fold and 5.6-fold increase in resistance to aminopterin and trimethoprim, respectively (Robello *et al.*,

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Table 1
Crystallographic parameters and data-collection statistics.

Values in parentheses refer to the highest resolution shell.

Space group	$P3_1$ or $P3_2$
Unit-cell parameters (Å)	a = 74.35
	c = 179.96
Matthews coefficient V_M (Å^3 Da^{-1})	2.3
Solvent content (%)	46.0
Resolution (Å)	25–2.10
	(2.18–2.10)
Total observations	267270
Unique reflections	55667 (4952)
Average $I/\sigma(I)$	13.9
R_{sym} (I) (%)	4.8 (27.5)
Completeness (%)	85.8 (76.3)

1997), suggesting that this enzyme could be a possible target for rational drug design. Here, we describe the expression, purification, crystallization and preliminary crystallographic analysis of recombinant pteridine reductase from *T. cruzi*.

2. Experimental

2.1. Protein expression and purification

The *T. cruzi* pteridine reductase gene (Robello *et al.*, 1997) was subcloned into the T7-promoter-based *E. coli* expression pET15b system (Novagen) and the recombinant protein was expressed in *E. coli* strain BL21(DE3)pLysS. The expression vector codes for a hexahistidine tag at the N-terminus of the gene product designed to facilitate purification of the recombinant protein using immobilized metal affinity chromatography and a thrombin cleavage site for removal of the hexahistidine tag. Bacteria were cultured in Luria–Bertani (LB) broth containing 50 µg ml^{-1}ampicillin, 34 µg ml^{-1} chloramphenicol and 0.2% glucose; expression of pteridine reductase was induced with 0.4 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) for 17 h at 298 K. Cells were lysed by thawing frozen pellets in 50 m*M* Tris–HCl pH 8.2, 150 m*M* sodium chloride, 1 m*M* benzamidine, 0.5 m*M* phenylmethylsulfonylfluoride (PMSF), 5 m*M* β-mercaptoethanol (β-ME) and the cell-free extract, prepared by centrifugation of the suspension at 20 000 rev min^{-1} for 1 h, was applied to a 10 ml Ni-NTA Superflow column (Qiagen). The unbound proteins were washed from the column with up to 40 column volumes of 50 m*M* Tris–HCl buffer pH 8.2, 5 m*M* β-ME, containing 500 m*M* NaCl and 30 m*M* imidazole. Elution of bound protein was achieved by using a linear 30–500 m*M* imidazole gradient. Pooled protein fractions were dialyzed against 20 m*M* Tris–HCl pH8.0, 20 m*M* NaCl, 5 m*M* β-ME and then concentrated to 15 mg ml^{-1} using ultrafiltration on YM10 membrane (Amicon). The typical yield of purified protein was 100 mg per litre of cell culture. Analytical size-exclusion chromatography on a Superose12 gel-filtration column (Pharmacia) indicated that the recombinant protein was tetrameric in solution. All the protein samples used for crystallization had the 20-residue affinity tag left intact.

2.2. Crystallization

A ternary complex of pteridine reductase was prepared by adding 2 ml of a binding buffer (20 m*M* Tris–HCl buffer pH 8.0, containing 1 m*M* methotrexate, 1 m*M* NADPH, 20 m*M* NaCl and 5 m*M* β-ME) to 0.2 ml of the 15 mg ml^{-1} protein solution. Following incubation on ice for 20 min, the mixture was concentrated to 0.2 ml in a centrifugal concentrator (Centricon 10; Amicon) and the concentrated sample was used for crystallization trials.

All crystallization experiments were performed at 295 K by the hanging-drop vapour-diffusion method in Linbro plates. Initial crystallization experiments were performed with Crystal Screen I kit (Hampton Research) employing the sparse-matrix method (Jancarik & Kim, 1991). The initial drop size was 2 µl, with a protein to well solution ratio of 1:1. Clusters of crystals appeared after 2 d in 0.1 *M* cacodylate buffer pH 6.5, with 1.4 *M* sodium acetate (NaOAc) as precipitant (Crystal Screen reagent #7). Crystals with a typical size of 0.4 × 0.25 × 0.15 mm could be grown overnight at 295 K.

2.3. Data collection and processing

Diffraction data were collected in a Cryostream at 103 K on a Rigaku R-AXIS IV image-plate area detector using Cu $K\alpha$ (λ = 1.54 Å) radiation from a Rigaku RU-200 rotating-anode X-ray generator. A crystal was dipped into a cryoprotectant composed of 25%(v/v) glycerol in the reservoir solution and mounted in a nylon loop before being placed into the Cryostream. Intensity data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Statistics of data collection and processing are listed in Table 1.

3. Results and discussion

Pteridine reductase is a homotetramer with a subunit mass of approximately 30 kDa. The molecular weight (163 kDa; data not shown) of the native protein determined on the basis of its elution volume from the gel-filtration column was 30% larger than the value calculated for the tetramer. The protein was enzymatically active in an *in vitro* assay as described by Nare *et al.* (1997) and Robello *et al.* (1997).

Recombinant pteridine reductase from *T. cruzi* was crystallized as a ternary complex with MTX and NADPH. Data processing and scaling (R_{merge} of 4.8%) resulted in a data set of 55 667 unique reflections (completeness 85.8% to 2.1 Å resolution; see Table 1). The systematic absences and data statistics indicated that the crystals belong to the trigonal crystal system, space group $P3_1$ (or its enantiomorph $P3_2$). Data processing and scaling in related space groups gave substantially higher values of R_{merge} (37.8% for $P6_1$, 34.2% for $P3_112$, 26.6% for $P3_121$). Assuming that the asymmetric unit contains a complete tetramer, the calculated Matthews coefficient V_M was 2.3 Å Da^{-1}, which corresponds to a solvent content of 46% (Matthews, 1968). A plot of the self-

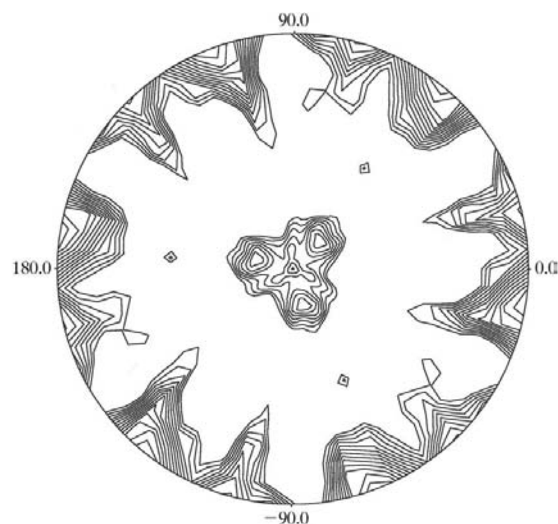

Figure 1
A self-rotation function plot with κ = 180° for dyad symmetry. The plot was generated using the program *POLARRFN* from the *CCP*4 suite. The radius and resolution range were 28 Å and 15–3 Å, respectively. The program produces sections of constant rotation angle κ for different axis directions defined by ω (angle from pole) and φ (angle around equator). The program lists the following polar rotation angles (ψ, φ, κ) for the three non-crystallographic twofolds (1, 97 90 180; 2, 8 35 180; 3, 186 55 180). The peak heights were between 3 and 4.5σ (approximately 1/3 to 1/4 of the origin peaks).

rotation function, calculated using the program *POLARRFN* (written by Dr W. Kabsch) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994), clearly reveals the presence of the three non-crystallographic twofolds indicative of the proposed 222 symmetry of the tetramer (Fig. 1).

Attempts to solve the structure by molecular replacement (MR) using rat liver dihydropteridine reductase (PDB code 1dhr) as a search model were not successful. Monomer as well as dimer search models were attempted using *CNS* (Brunger *et al.*, 1998) and *AMoRe* (Navaza, 1994). The sequence identity among members of the SDR family for which three-dimensional models are available is less than 25%. In the absence of a suitable model for structure determination by MR, a selenomethionine derivative of the protein has been prepared for multiwavelength anomalous dispersion (MAD) analysis.

The structure of *T. cruzi* pteridine reductase will provide detailed molecular framework for the rational design of inhibitors and comparative study of the enzyme from different organisms.

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