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Expression, purification, crystallization and preliminary X-ray analysis of cyclophilin A from the bovine parasite *Trypanosoma brucei brucei*

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Abstract

Cyclophilin A from the bovine parasite *Trypanosoma brucei brucei* has been cloned, expressed in *Escherichia coli*, purified and crystallized in the presence of cyclosporin A using ammonium sulfate as a precipitant. The crystals belong to the orthorhombic crystal system with unit-cell dimensions of $a = 118.61$, $b = 210.15$ and $c = 153.21$ Å. A data set complete to 2.7 Å has been collected using rotating-anode radiation, however the crystals diffract to at least 2.1 Å resolution using synchrotron radiation.

1. Introduction

Cyclophilins (CyP) form a growing family of homologous proteins expressed by a variety of cell types in species as diverse as those from bacteria, fungi and mammals (Handschumacher *et al.*, 1984). They exhibit peptidyl–prolyl *cis–trans* isomerase activity (Takahashi *et al.*, 1989; Fisher *et al.*, 1989) and have been shown to play a crucial role in the folding of many housekeeping proteins. In addition, cyclophilin A is the receptor for cyclosporin A (CsA), an immunosuppressive drug currently used in transplantation surgery (Kahan, 1983). In humans, the CyP/CsA complex inhibits calcineurin, a calcium- and calmodulin-dependent phosphatase which is believed to modulate the phosphorylation state of transcription factors involved in turning on genes encoding various cytokines including interleukin 2.

Unexpectedly, the immunosuppressive drug CsA was found to posses antiparasite activity in a variety of protozoal and helmintal infections (Page *et al.*, 1995). It is not known how the drug exerts these effects; however, the answer may lie in the enzymatic functions of the parasite cyclophilins.

This report describes cloning and expression in *E. coli* of the gene encoding for the bovine parasite *T. b. brucei* cyclophilin A and its subsequent crystallization in complex with CsA. The crystals are suitable for high-resolution crystallographic studies, and a data set complete to 2.7 Å has been collected. Preliminary analysis of this diffraction data is presented.

2. Materials and methods

2.1. *Gene identification and cloning*

The *T. b. brucei* cyclophilin A homologue was cloned using the RADES-PCR approach (Murphy & Pellé, 1994) in the course of identification of genes differentially expressed during trypanosome life-cycle stages. Genes identified as such were cloned in pGEM-T vector (Promega) and sequenced. Specific primers were then designed to generate the complete open reading frames of interesting clones. Among these was the gene coding for *T. b. brucei* cyclophilin A identified due to its homology with known cyclophilins (Pellé & Murphy, manuscript in preparation). The full-length clone comprises 1087 nucleotides (sequence tbbcyp; Genebank database U68270) and codes for 177 a.a. with a deduced molecular weight of 18 762. The mini-exon or spliced leader is from base 1 to 39. The ATG start codon is at position 79 whereas the stop codon is at position 610.

2.2. *Bacterial expression and protein purification*

The *T. b. brucei* cyclophilin A open reading frame was generated by PCR using forward and reverse specific primers designed to introduce the unique *Bam*HI and *Pst*I sites at the 5′ and 3′ ends of the amplified DNA fragment, respectively, and to create the deletion of the ATG initiation codon. The PCR product was purified and cloned into pGEM-T vector. The cloned fragment was excized with *Bam*HI and *Pst*I from the recombination plasmid and subcloned into pQE-30 (Qiagen) expression plasmid vector (which contains multiple cloning sites) previously digested with *Bam*HI and *Pst*I and dephosphorylated. The pQE-30 expression vector possesses an ATG start codon and six consecutive histidine residues (6xHis tag) for efficient purification of recombinant proteins. The resulting recombinant plasmid expressing the wild-type cyclophilin (termed pQE-30/TbcypA.wt) was transformed into *E. coli* JM109 for overexpression.

An overnight culture of *E. coli* strain JM101 containing the recombinant expression vector pQE-30/TbcypA.wt was diluted 100-fold in TB medium (Tartof & Hobbs, 1988). Expression of *T. b. brucei* recombinant cyclophilin A was induced after 8 h by the addition of IPTG (0.5 m*M*), and growth was continued overnight.

Cells were harvested by centrifugation and lysed by sonication in 50 m*M* Tris–HCl pH 7.5, containing 4 mg ml^{-1} lysozyme and 0.1% Tween 20. Sodium chloride (0.5 *M*) and glycerol (10%) were added to the soluble fraction. These lysates were loaded on a 5 ml HITRAP chelating column (Pharmacia), previously charged with Zn^{2+}. After extensive washing with 50 m*M* Tris–HCl pH 7.5, 0.5 *M* NaCl, 10% glycerol, and 0.1% Tween 20, proteins were eluted using a linear gradient of imidazole. Fractions containing *T. b. brucei* recombinant cyclophilin A were pooled and run on a preparative superdex 75 gel-filtration column (Pharmacia) in 50 m*M* Tris–HCl pH 7.5 and 0.05% azide. A reducing SDS–PAGE gel stained with Coomassie blue showed the resulting recombinant protein to migrate as a single band of 22 kDa due to the presence of the His-tagged residues.

2.3. *Crystallization*

The purified protein was concentrated to 15 mg ml^{-1}, by ultrafiltration with a centricon concentrator (Amicon). Cyclosporin A (Sigma) was mixed at an equimolar ratio with CyP. Crystallization trials were carried out at room temperature using the hanging-drop vapor-diffusion method (McPherson, 1982) and a sparse-matrix sampling (Jancarik & Kim, 1991). While no visible crystals resulted, precipitates in several conditions with ammonium sulfate led to a screen of pH *versus* ammonium sulfate concentration. This produced crystals in 30–40% saturation ammonium sulfate buffered with 100 m*M* HEPES or Tris–HCl (pH 7.5–8.5). Under these conditions, crystals formed from a dense precipitate. To overcome the initial precipitation, we further optimized our approach. The final conditions used a droplet of 10–5 µl containing CyP (6–7.5 mg ml^{-1}), CsA (equimolar), and 6–9% ammonium sulfate in 12.5 m*M* imidazole (pH 6.5) suspended above a reservoir of 35–40% ammonium sulfate in 100 m*M* imidazole, pH 6.5.

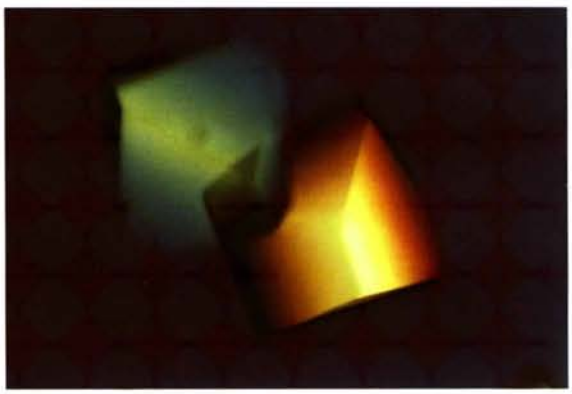

Fig. 1. Photograph (taken through polarized lenses) of two *Trypanosoma brucei brucei* cyclophilin A crystals each measuring approximately 0.4 × 0.5 × 0.5 mm.

2.4. *X-ray crystallography*

The largest crystals were mounted in a thin-walled glass capillary. X-ray data were collected at room temperature using an Enraf–Nonius FAST area detector with a rotating-anode source operated at 40 kV and 70 mA. The resulting data set was processed using the program *MADNES* (Messerscmidt & Pflugrath, 1987) and the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). Additional data were collected using a MAR Research image plate and either a Rigaku rotating-anode source operated at 40 kV and 90 mA or station 9.5 at the Synchrotron Radiation Source in Daresbury, UK. These data were processed using the *HKL* package (Otwinowski, 1993).

3. Results and discussion

The 177 amino acid parasite cyclophilin A is 72% identical in sequence to bovine cyclophilin A (which is 98% identical to that of human); however, there are important differences between the host and parasite sequences. First, the parasite CyP has an N-terminal extension of ten amino acids and a three amino-acid insert at position 98. More importantly, the parasite sequence has several amino-acid changes in the residues that comprise the CsA drug-binding site. Finally, based on the amino-acid sequence aligned with an NMR structure of the human cyclophilin A (Spitzfaden *et al.*, 1994), it appears that CyP from *T. b. brucei* may form a unique intramolecular disulfide bond between Cys50 (Ser in other species of cyclophilin A) and Cys174.

Single crystals (as large as 0.7 × 0.6 × 0.4 mm) of *T. b. brucei* cyclophilin A complexed with cyclosporin A grew within one week after setup (Fig. 1). These crystals were stable

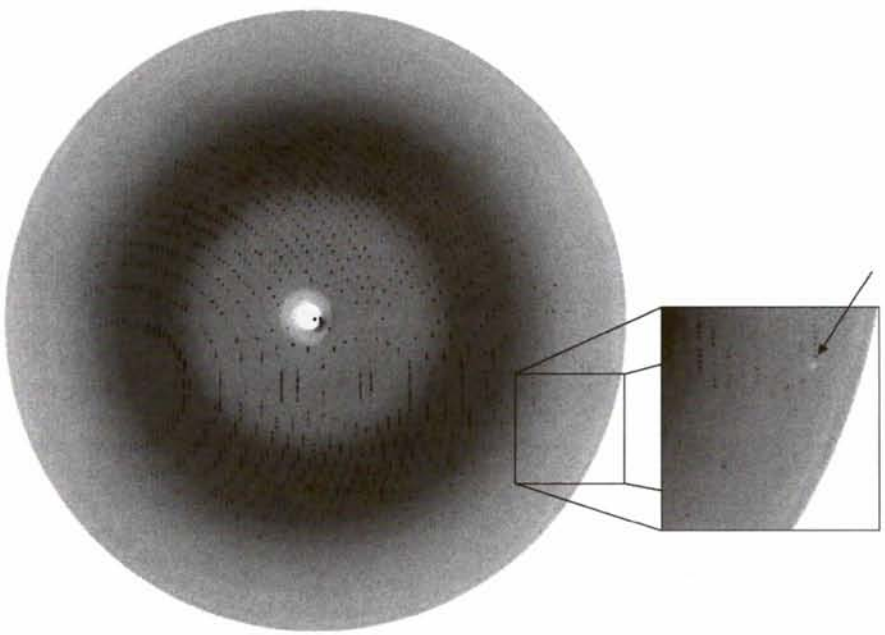

Fig. 2. A 0.5° oscillation image of a *Trypanosoma brucei brucei* cyclophilin A crystal taken using a MAR Research image plate (5 min exposure) on station 9.5 at the Synchrotron Radiation Source in Daresbury, UK. The arrow indicates a reflection at 2.1 Å resolution.

in the X-ray beam and diffracted to at least 2.5 Å resolution on a conventional rotating-anode source and to 2.1 Å using synchrotron radiation (Fig. 2). The crystals were determined to be orthorhombic with unit-cell dimensions of a = 118.61, b = 210.15 and c = 153.21 Å. Systematic absences showed that the space group is $P2_12_12$. The unit-cell volume is consistent with between 15 and 30 monomers in the asymmetric unit assuming V_M values between 3.3 and 1.6 $Å^3\ Da^{-1}$ for protein crystals (Matthews, 1968).

Using synchrotron radiation, we have collected from a single crystal a 67.8% complete data set to 2.1 Å with an R_{sym} of 7.6%. For the highest resolution shell (2.17–2.10 Å) the completeness is 71.5% and the I/σ_I is 2.2. When this data is merged with 2.7 Å data from a second crystal collected using a rotating-anode source, the result is a 96.6% complete data set with an R_{sym} of 11.2%. For the highest resolution shell, 2.8–2.7 Å, the completeness is 96.8% and the I/σ_I is 5.1.

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