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Expression, purification, crystallization and preliminary X-ray diffraction analysis of uracil phosphoribosyltransferase of *Toxoplasma gondii*

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Recombinant uracil phosphoribosyltransferase (UPRT) enzyme of *Toxoplasma gondii* was expressed in *Escherichia coli* and purified from the cell-free extract by a combination of chromatographic steps. The recombinant protein was enzymatically active when tested in an *in vitro* UPRT assay. The purified protein was crystallized using the hanging-drop vapor-diffusion technique with ammonium phosphate as precipitant. The crystallized protein also exhibited UPRT activity. Crystals diffract to 2.4 Å resolution and belong to space group $P3_121$ or $P3_221$ with unit-cell dimensions $a = b = 119.9$, $c = 70.8$ Å and two molecules per asymmetric unit.

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan that causes the most commonly recognized opportunistic infection, toxoplasmosis, of the central nervous system in immunocompromized individuals. *T. gondii* is capable of synthesizing pyrimidine nucleotides either *de novo* or by salvage pathways that utilize preformed pyrimidine nucleobases or nucleosides (Iltzsch & Klenk, 1993). *T. gondii* lacks any pyrimidine nucleoside kinase or phosphotransferase activity. Unlike mammalian cells, the salvage of pyrimidine nucleosides in *T. gondii* does not occur by direct phosphorylation to nucleoside 5′-monophosphates (Iltzsch, 1993). Therefore, nucleosides must be converted to the nucleobase uracil in order to be salvaged by *T. gondii*. The enzyme uracil phosphoribosyltransferase (E.C. 2.4.2.9; UPRT) catalyzes conversion of uracil to uridine 5′-monophosphate utilizing 5′-phosphoribosyl-α-1-pyrophosphate (PRPP). This enzyme is widely distributed in the microbial world (Neuhard, 1982; Martinussen & Hammer, 1994). The cDNA for *T. gondii* UPRT encodes a protein of 244 amino acids. The UPRT gene of *T. gondii* was cloned and characterized (Donald & Roos, 1995). We report here the expression of *T. gondii* UPRT in *E. coli* and purification, crystallization and preliminary X-ray diffraction analysis of the active recombinant enzyme. To our knowledge, this is the first report of the crystallization of an UPRT enzyme.

2. Materials and methods

2.1. Expression of recombinant UPRT in *E. coli*

The coding sequence for *T. gondii* UPRT was amplified using the polymerase chain reaction technique. The amplified sequence was cloned into the *Bam*HI and *Sal*I sites of pET21a vector (Novagen, Inc.) using T4 DNA ligase. This would result in an amino-terminal T7 tag in the recombinant protein. The product of the ligation reaction was transformed into *E. coli* DH5α(F′) competent cells and the transformants were selected on LB agar plates containing 50 µg ml^{-1} ampicillin. Plasmid DNA preparation from several clones were digested with *Bam*HI and *Sal*I and the reaction mixtures were examined on a 1.2% agarose gel for an insert of approximately 630 bp. Plasmid DNA (pEUPRTa) from a positive clone was sequenced by automated DNA sequencing and subsequently was transformed into *E. coli* BL21(DE3)plyS competent cells.

For expression of the recombinant UPRT (rUPRT), BL21(DE3)plysS cells carrying pEUPRTa plasmid were grown in LB medium containing ampicillin (50 µg ml^{-1}) and chloramphenicol (34 µg ml^{-1}) at 310 K. This culture was diluted 1:100 in fresh LB medium containing ampicillin, chloramphenicol and 0.2% glucose, and was grown at 310 K. Isopropylthio-β-D-galactoside (IPTG) was added to the culture to a final concentration of 1 m*M* when the absorbance of the culture reached 0.6 at 600 nm. The culture was grown for 2 h post induction and cells were harvested by centrifugation at 4000 r min^{-1} for 15 min. The pellets were stored at 193 K until use.

2.2. Purification of recombinant UPRT

The following buffers were used in the purification. Buffer *A*: 50 m*M* Tris acetate, 3 m*M* DTT, pH 7.5; buffer *B*: 25 m*M* Tris acetate, 3 m*M* DTT, pH 7.8; buffer *C*: 25 m*M* Tris acetate, 3 m*M* DTT, pH 7.0; buffer *D*: 10 m*M* MES, 3 m*M* dithiothretol (DTT), pH 6.5. Unless specifically mentioned, all experiments were performed at 277 K.

The cell pellet from 2 l of bacterial culture was thawed and suspended in 50 ml of cold buffer *A* by stirring for 30 min. The suspension

was subjected to sonication (30 s cycles twice) on ice followed by centrifugation at 30000 rev min^{-1} for 30 min. Polyethyleneimine (PEI) solution was added to the supernatant to a final concentration of 0.5%(*w*/*v*) and the mixture was stirred for 20 min followed by centrifugation at 30000 rev min^{-1} for 20 min. Solid ammonium sulfate was added to the solution with constant stirring to attain 40% saturation. The suspension was stirred for 30 min, and then centrifuged at 30000 rev min^{-1} for 20 min. The pellet was suspended in a minimal volume of buffer *B* and dialyzed against the same buffer. The dialysate was applied to a Q-Sepharose FF (Pharmacia) column (20 ml) equilibrated with buffer *B*; the column was washed with buffer *B* and the bound protein was eluted with a linear gradient of sodium chloride (concentration 0–0.4 *M*) in 200 ml buffer *B*. Fractions of 5 ml were collected and analyzed by SDS–PAGE on 12% acrylamide gel. The rUPRT eluted at about 0.15–0.2 *M* salt concentration. The protein from selected fractions was precipitated by adding ammonium sulfate to 70% saturation and collected by centrifugation at 35000 rev min^{-1} for 20 min. The pellet was suspended in buffer *C* and dialyzed thoroughly against the same buffer. The dialysate was applied on a DNA–cellulose (Pharmacia) column (12 ml) and the column was washed with 10 column volumes of buffer *C*; the bound protein was eluted with a linear gradient of sodium chloride (0–0.5 *M* in 120 ml of buffer *C*). Fractions were analyzed by SDS–PAGE. Fractions containing the rUPRT were pooled and dialyzed against buffer *D*. The dialysate was concentrated by ultrafiltration to a final concentration of 10 mg ml^{-1}.

2.3. N-terminal sequencing

The purified protein was subjected to amino-terminal sequencing for 22 cycles using automated Edman degradation on a gas-phase microsequencing system Portron Model PI2090E (Beckman Instruments, Inc.).

2.4. Enzyme assay

The purified protein was assayed *in vitro* for uracil phosphoribosyltransferase activity (Rasmussen *et al.*, 1986). The standard reaction mixture contained 50 m*M* Tris–HCl buffer pH 8.0, 5 m*M* magnesium chloride, 1 m*M* ^{14}C-uracil (0.1 mCi ml^{-1}), 1.2 m*M* PRPP, 1 m*M* guanosine 5′-triphosphate and 10 µl of 0.1 mg ml^{-1} purified protein. The reaction mixture was preheated for 2 min at 310 K, after which the reaction was started by adding the PRPP solution. The reaction mixture was incubated at 310 K for 5 min. 20 µl of the reaction mixture was spotted on DE-81 ion-exchange filter papers (Whatman). The papers were washed three times in 15 m*M* Tris–HCl pH 8.2, air dried and counted in scintillation fluid using a Beckman liquid scintillation counter.

For determination of the activity in the crystalline protein, three crystals were washed overnight in 3 ml of a washing solution containing 2.2 *M* ammonium phosphate and 0.1 *M* Tris–HCl pH 8.0 with three changes of the washing solution. After all reagents were added to the tube, the reaction was started by adding three crystals using a nylon loop. The assay was performed as described above.

2.5. Crystallization

The protein was crystallized using the hanging-drop vapor-diffusion technique (McPherson, 1990). 4 µl of concentrated protein was mixed with 16 µl of a reservoir solution containing 2.0 *M* ammonium phosphate at pH 8.0 and the mixture was equilibrated at room temperature.

2.6. Data collection and processing

Preliminary characterization of the crystals was performed using a copper rotating-anode X-ray source and an R-AXIS II image-plate detector at room temperature. The best crystals diffracted to 2.4 Å resolution and were not very stable in the X-ray beam. The crystals were soaked in a cryoprotectant solution containing 20% glycerol in an artificial mother liquor. The artificial mother liquor was prepared by mixing a saturated ammonium phosphate solution, 1.0 *M* Tris–HCl buffer pH 8.0 and deionized water in the proportion 6.5:1.0:2.5. Diffraction data from a native crystal (0.4 × 0.5 × 0.3 mm) were collected at 103 K using an X-stream low-temperature device on an R-AXIS IV generator. The crystal-to-detector distance was 140 mm and the oscillation step was 0.8° with 8 min exposure per image. The native data set was processed using the *HKL* package (Otwinowski, 1993).

3. Results and discussion

UPRT enzyme plays a critical role in nucleotide biosynthesis using the salvage pathway. The enzyme is present in many microorganisms. Some sequence homology in the putative substrate-binding site has been observed among UPRTs from various organisms (Donald & Roos, 1995). However, the three-dimensional structure of no UPRT enzyme has been elucidated. In order to determine the three-dimensional structure of this enzyme, we have expressed the recombinant UPRT of *T. gondii* in *E. coli*. The amplified coding sequence for the protein was cloned into the *Bam*HI/*Sal*I sites of the prokaryotic expression vector pET21a. The entire coding sequence was verified by automated DNA sequencing to confirm the DNA sequence of the insert. The recombinant protein was expressed in *E. coli* BL21(DE3)plysS cells carrying the plasmid pEUPRTa using IPTG as the inducer. On SDS–PAGE, the overexpressed protein migrated as a band of approximately 28 kDa. The recombinant protein was expected to contain 13 additional residues at its amino terminus arising from the expression vector. When cells were lysed in buffer *A*, rUPRT was obtained in the soluble supernatant, from which the nucleic acids were removed by precipitation with PEI. The major portion of the rUPRT was precipitated by attaining 40% ammonium sulfate saturation in the solution. The resuspended and dialyzed rUPRT was then bound to an anion-exchange column, Q-Sepharose FF, at

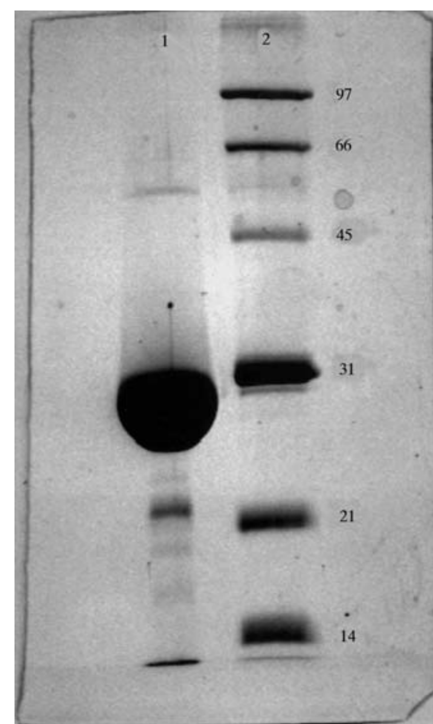

Figure 1
SDS–PAGE of fractions representing the purified UPRT protein. 10 µl of protein solution was boiled with an equal volume of 2× SDS sample-denaturing buffer. 10 µl of the boiled mixture was subjected to electrophoresis on 12% polyacrylamide gel containing 0.1% SDS. The gel was stained with Coomassie Brilliant Blue G250. Lane 1: 25 µg of purified UPRT. Lane 2: molecular-weight standard with molecular masses shown in kDa.

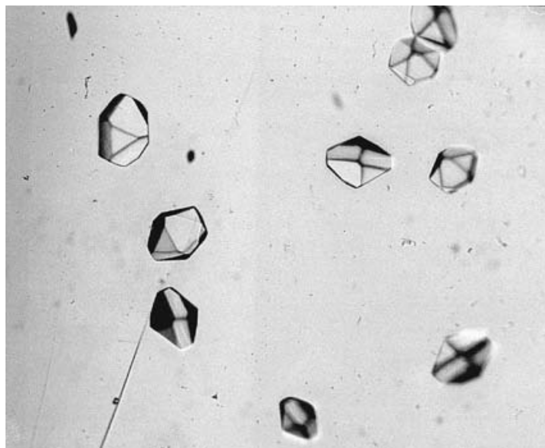

Figure 2
UPRT crystals grown from ammonium phosphate at pH 8.0. Typical crystals grow to approximately 0.25 mm in each dimension.

pH 7.5, and was eluted from the column with a linear gradient (0–0.3 *M*) of sodium chloride. On SDS–PAGE analysis, fractions eluted with 0.15–0.2 *M* salt showed a major band of molecular mass of approximately 28 kDa. The protein concentrated from these fractions was bound to a DNA cellulose column at pH 7.0 and the rUPRT was eluted as a single peak at about 0.2 *M* sodium chloride concentration. The protein in this peak was nearly homogenous, as revealed by SDS–PAGE analysis (Fig. 1). The recombinant protein was analyzed by amino-terminal sequence analysis. The sequencing was performed for 22 cycles. The first 13 residues of the recombinant protein arise from the expression vector. This sequence was followed by the sequence AQVPASGKL which was identical to the deduced amino-acid sequence of *T. gondii* UPRT (Genebank Accession number U10246).

The purified protein was assayed for UPRT activity. The assay was performed with slight modification of the method described by Rasmussen *et al.* (1986). The UPRT activity was assayed by measuring the formation of radiolabeled UMP. When the reaction mixture was spotted on anion-exchange DE81 papers (Whatman), ^{14}C UMP was bound to the paper and the unreacted uracil was removed by washing the paper with Tris buffer pH 8.0. The enzyme activity was found to be optimal in the pH range 7.5–8.0. When protein solution in the enzymatic assay was substituted with three crystals, the resulting activity was equivalent to that of 5 µg of the purified protein.

The protein crystallized at room temperature in less than 2 d. A picture of a typical hanging drop containing rUPRT crystals is shown in Fig. 2. The crystals belong to the trigonal system with unit-cell parameters $a = b = 119.9$, $c = 70.8$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. Symmetries in the diffraction intensities were consistent with space group $P3_221$ or $P3_121$. The native data collected to 2.4 Å resolution were about 99% complete with an average of seven observations per reflection and an *R* factor of 0.052. The average I/σ for the entire data is 20.5 and I/σ in the highest resolution shell is 9.8. Assuming two molecules per asymmetric unit, V_m is calculated to be 2.66 Å^3 Da^{-1} (Matthews, 1968). A self-rotation search performed using *X-PLOR* (Brünger, 1996) indicated the presence of a non-crystallographic twofold axis at $\psi = 113.4$, $\varphi = 41.5$, $\kappa = 180°$. We are currently searching for heavy-atom derivatives for structure determination.

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