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Toxoplasma gondii adenosine kinase: expression, purification, characterization, crystallization and preliminary crystallographic analysis

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The obligate intracellular protozoan parasite *Toxoplasma gondii* depends on the purine-salvage pathway for its purine supply. Unlike its mammalian hosts, *T. gondii* salvages purine precursors predominantly *via* adenosine kinase, the enzyme that phosphorylates adenosine to adenosine monophosphate (AMP). The cDNA encoding *T. gondii* adenosine kinase was subcloned and expressed in *Escherichia coli*. The recombinant protein was active in an *in vitro* enzyme assay over a broad pH range. It required a divalent cation for activity. The enzyme was inactivated by the addition of 1 µ*M* mercuric chloride. The inactivation could be reversed by a reducing agent. The active recombinant protein was crystallized using sodium sulfate as precipitant at pH 8.0. The crystals diffract to 1.8 Å and belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 47.5$, $b = 68.9$, $c = 57.0$ Å, $\beta = 100.3^\circ$. The calculated V_m based on one molecule per asymmetric unit is 2.38 Å^3 Da^{-1}.

1. Introduction

T. gondii is an obligate intracellular protozoan parasite. Human infection with *T. gondii* is an important cause of morbidity and mortality (Nash *et al.*, 1998). In hosts with intact immunity, infection is usually benign (McCabe & Remington, 1981). However, persons with defective cell-mediated immunity, including recipients of organ allografts, persons undergoing cytotoxic chemotherapy, neonates with perinatally acquired diseases and persons infected with human immunodeficiency virus (HIV), are susceptible to severe and potentially fatal infections (Britt *et al.*, 1981; Grant *et al.*, 1990; Luft & Remington, 1988). There is a continuing need to define novel chemotherapeutic targets and develop new chemotherapeutic agents for treatment of *Toxoplasma* infection.

One feature of the metabolic pathways of *T. gondii* that distinguishes it from its human host is its inability to synthesize purines *de novo* (Krug *et al.*, 1989; Schwartzman & Pfefferkorn, 1982). The parasite, therefore, depends on the purine-salvage pathway for its purine requirement. The parasite is also incapable of converting guanine nucleotides to adenine. Thus, only adenine, adenosine, guanine, hypoxanthine and inosine can be metabolized by the parasite. Adenosine is incorporated into the nucleotide pools of *T. gondii* at a much faster rate than any other nucleobase or nucleoside (Schwartzman & Pfefferkorn, 1982). In *T. gondii*, adenosine is metabolized mainly *via* phosphorylation to adenosine monophosphate, from which all other purine nucleotides can be synthesized. The enzyme adenosine kinase (AK) catalyzes the phosphorylation of adenosine to AMP. Therefore, AK plays a key role in the purine salvage mechanism in *T. gondii*. The enzymatic and biochemical properties of AK from a variety of mammalian cells have been extensively studied (Mimouni *et al.*, 1994; Miller *et al.*, 1979; Pallela *et al.*, 1980; Chang *et al.*, 1983). In contrast, very little is known about AK from any parasite (Dutta *et al.*, 1987). The major impediment to conducting detailed analysis of the parasitic enzyme is the difficulty in purifying large quantities of active enzyme from the parasite. Recently, the crystal structure of human adenosine kinase was determined (Mathews *et al.*, 1998). This is the only known structure of an adenosine kinase. Here, we report the overexpression, purification, characterization, crystallization and preliminary crystallographic analysis of an active recombinant AK from *T. gondii*.

2. Materials and methods

2.1. Cloning and expression

The coding sequence for *Toxoplasma* adenosine kinase was amplified using PCR and engineered into *E. coli* expression vector pET21a (Novagen Inc.). The entire coding sequence was verified by automated DNA sequencing. The resulting plasmid pETgaK was transformed into competent cells of *E. coli* strain BL21(DE3)plysS and transformants

Table 1
Crystallographic data for the free enzyme and the putative adenosine complex.

	Free enzyme	Putative substrate complex
Total number of reflections	29958	317522
Number of unique reflections	14900	33084
Overall completeness (%)	91.7	98.6
Completeness in the highest resolution cell (%)	84.8 (2.38–2.3 Å)	89.3 (1.86–1.8 Å)
Overall $I/\sigma(I)$	10.4	13.2
Reflections with $I/\sigma(I) > 3$ in the highest resolution shell (%)	64	55
R_{merge} (%)	7.0	4.3
R_{merge} (highest resolution shell) (%)	19.7	21.0
Space group	$P2_1$	$P2_1$
Unit-cell parameters		
a (Å)	47.5	47.5
b (Å)	68.6	68.9
c (Å)	58.8	57.0
β (°)	100.3	100.3

were selected on LB agar plates containing 50 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol. For expression, cells were grown at 310 K in LB medium containing 50 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol for 6 h. The culture was diluted in LB medium containing the same antibiotics and 0.2% glucose and grown at 310 K with constant shaking. When the absorbance of the culture reached 0.8 at 600 nm, isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 1 m*M* and the culture was grown for an additional 3 h at 310 K.

2.2. Purification of recombinant protein

The bacterial cell pellet was extracted in a buffer (buffer *A*) containing 50 m*M* Tris acetate pH 8.3, 1 m*M* $MgCl_2$, 1 m*M* benzamidine hydrochloride, 10 m*M* β-mercaptoethanol (βME), 0.1 m*M* phenylmethyl-sulfonyl fluoride (PMSF) and the lysate after sonication was subjected to centrifugation at 30 000 rev min^{-1} for 30 min. The cell-free extract containing the soluble supernatant was applied to an anion-exchange column (20 ml QAE-Sepharose FF, Pharmacia), equilibrated with buffer *A* and the bound protein was eluted using a linear gradient of 0–0.15 *M* NaCl in 400 ml buffer *A*. Fractions containing active AK enzyme were pooled and dialyzed against 10 m*M* Tris acetate pH 8.0, 5 m*M* $MgCl_2$, 10 m*M* βME (buffer *B*). Further purification was achieved by chromatography on a strong anion-exchange column (MonoQ 10/10, Pharmacia) equilibrated with buffer *B*. Recombinant AK was eluted from the column using a linear gradient of NaCl (0–0.1 *M*) in 160 ml of buffer *B*. Fractions were analyzed by SDS–PAGE and an *in vitro* enzyme-activity assay.

2.3. Characterization of recombinant AK

The molecular weight of the native protein was determined by size-exclusion chromatography on a Superdex 75 (Pharmacia) column in buffer *B*. Amino-terminal sequence analysis was performed for ten cycles using automated Edman degradation on a gas-phase micro-sequencing system (Porton Model PI2090E; Beckman Instruments, Inc.).

2.4. *In vitro* enzymatic assay

In vitro adenosine kinase activity was assayed with a slight modification of the method described by Pallela *et al.* (1980). A standard reaction mixture (100 µl) contained 50 m*M* Tris–HCl pH 8.0, 10 m*M* dithiothretol (DTT), 2.5 m*M* $MgCl_2$, 2.5 m*M* adenosine triphosphate (ATP) and 100 µ*M* ^{14}C-adenosine. The reaction was started by adding 50–100 ng of protein to the reaction mixture. The reaction was conducted at 310 K. An aliquot of 25 µl of reaction mixture was spotted on DE81 ion-exchange papers (Whatman). The filter papers were washed three times in cold 15 m*M* Tris–HCl pH 8.5 followed by one wash with ethanol and dried in air. The radioactivity bound to the papers was counted using a liquid scintillation counter. The counts for each paper were subtracted from those of the control. The control reaction contained the same amount of all components but no enzyme.

2.5. Crystallization

For crystallization, the purified protein was concentrated to 40 mg ml^{-1} by ultrafiltration. Crystals of active recombinant AK were grown by the hanging-drop vapor-diffusion technique. 2 µl of protein solution was mixed with an equal volume of reservoir solution containing 1.0 *M* sodium sulfate in 0.1 *M* Tris–HCl buffer pH 8.5 and the mixture was equilibrated against 1 ml of reservoir solution at room temperature. In an attempt to crystallize the enzyme–substrate complex, concentrated protein was incubated with 20 µ*M* adenosine and 2 m*M* magnesium chloride for 1 h on ice prior to setting up crystallization.

2.6. Data collection

X-ray diffraction data were collected on an R-AXIS IV image plate at 103 K after soaking the crystal momentarily in a cryoprotectant solution containing 1.0 *M* sodium sulfate, 0.1 *M* Tris–HCl pH 8.5 and 25% glycerol. A total of 180° of data were measured using the oscillation method. Individual frames consisted of a 0.5° oscillation angle measured for 20 min at a crystal-to-detector distance of 80 mm. Intensity data were processed using the *HKL* package (Otwinowski & Minor, 1997).

Crystals of putative adenosine complex were also flash frozen using the cryo-preservative mentioned earlier. These crystals

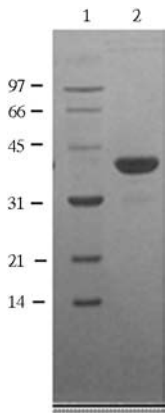

Figure 1
SDS–PAGE pattern showing the migration of purified recombinant AK in lane 2. Lane 1, molecular-weight markers (from top): 97, 67, 45, 31, 21 and 14 kDa.

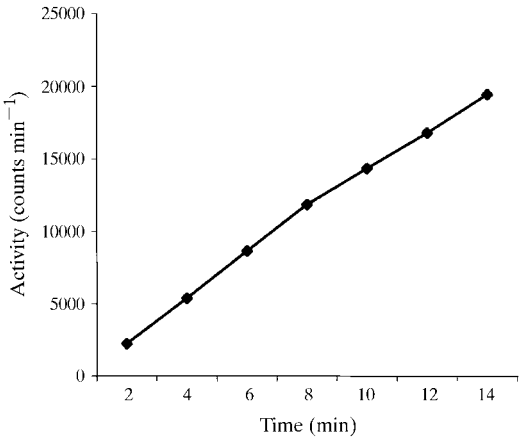

Figure 2
Adenosine kinase activity assay was performed as described in §2. Counts per minute (counts min^{-1}) representing AK activity are plotted at various time points using a final concentration of 55 µ*M* adenosine.

diffracted to 1.8 Å. Intensity data at 103 K were collected on an R-AXIS IV image plate using the oscillation method. An oscillation angle of 0.5° per frame was used with a crystal-to-film distance of 80 mm; each frame was exposed for 20 min.

3. Results and discussions

Owing to *T. gondii*'s total dependence on the salvage pathway for its purine requirement, adenosine kinase is an important enzyme for studying the purine-salvage pathway in this species. *In vitro*, adenosine kinase is at least ten times more active than any enzyme involved in purine metabolism in the parasite. The structure of any adenosine kinase other than the human enzyme is not known (Mathews *et al.*, 1998).

The recombinant adenosine kinase of *T. gondii* was overexpressed in *E. coli* and purified from the soluble fraction of the bacterial cell lysate by the combination of chromatographic steps mentioned above. On SDS–PAGE, the purified protein migrated as a single band of about 40 kDa (Fig. 1). The molecular weight of 38 200 Da, as determined by native gel-filtration chromatography, was in excellent agreement with the predicted value of 38 356 Da. Amino-terminal sequence analysis was performed to verify the coding sequence for the first ten amino acids. Thus, the amino-terminal sequence, AVDSSNSATG, was identical to that deduced from the cDNA sequence, except for the missing methionine coded by the initiation codon 'ATG'. We assume that the amino-terminal methionine was cleaved during purification of the protein. The purified protein was analyzed in an *in vitro* enzymatic assay as described in the previous section. The activity was linear to about 15 min at 310 K (Fig. 2). The enzyme was active in the pH range 5.0–9.0 (data not shown). The enzyme activity was not influenced by the presence of various concentrations of the reducing agent DTT. The enzyme was completely inactivated by 1 μM $HgCl_2$. The activity can be fully recovered in the presence of excess reducing agent. This indicates the presence of a thiol group at or near the active site of the enzyme. The enzyme can use Mg^{2+}, Mn^{2+} or Ca^{2+} to fulfil its divalent cation requirement and was completely inactivated by 100 μM EDTA. The enzyme showed no activity with deoxyadenosine or deoxyguanosine as a substrate. However, both ATP and GTP can be used as a phosphate donor, although GTP is less efficient.

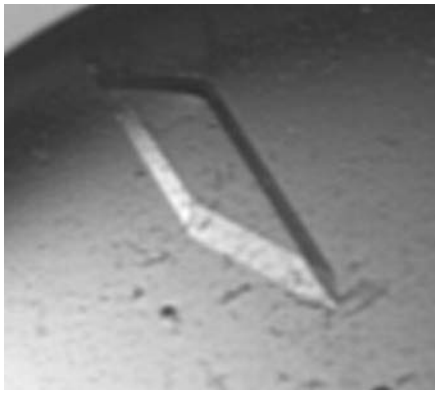

Figure 3
Crystal grown using the hanging-drop vapor-diffusion technique.

The purified protein was concentrated for crystallization. The initial crystallization conditions were found using the Crystal Screen I kit (Hampton Research). Clusters of very thin plates grew on top of each other from solution No. 4 (2.0 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.5). Attempts to obtain better crystals by refining the concentration of precipitant, the buffer pH and varying the protein concentration and temperature failed. Addition of 2–3% octyl-β-glucoside seemed to be helpful in reducing the clustering of crystals. Crystals suitable for X-ray diffraction were obtained using 0.7–1.0 *M* sodium sulfate in 0.1 *M* Tris–HCl in the pH range 8–8.5. Addition of 1–3% glycerol to the reservoir solution reduced the amount of nucleation (Fig. 3). Crystals grew to approximate dimensions of 0.5 × 0.2 × 0.2 mm within a week. Intensity data 91% complete to 2.3 Å resolution were collected. The total number of unique reflections was 15 140. $R_{\rm merge}$ and $I/\sigma(I)$ for this data set were 6% and 8.7, respectively. Details of the data-collection statistics are presented in Table 1.

Crystals of the putative substrate complex diffracted to about 1.8 Å. A total of 368 data frames spanning a total of 184° oscillation angle were collected. The overall completeness of the data to 1.8 was 98.6%. The average χ^2 for all data was 1.1. Based on the systematic absences, the space group was determined to be $P2_1$. The unit-cell parameters were a = 47.5, b = 68.9, c = 57.0 Å, β = 100.3°. Assuming one protein molecule (molecular mass 38 355 Da) in the asymmetric unit, the packing volume V_m was calculated to be 2.38 $Å^3$ Da^{-1}, giving an estimated solvent content of 49%. These values of V_m and solvent content are typical for protein crystals (Matthews, 1968).

T. gondii AK shares about 42% homology in its primary sequence with the human analog. The three-dimensional structure of human adenosine kinase showed remarkable similarities to the structure of *E. coli* ribokinase (Sigrell *et al.*, 1998), although the sequence identity was only 21%. Attempts to solve the structure of *T. gondii* AK using *E. coli* ribokinase as a search model were not successful. The structure determination of the *T. gondii* enzyme using the isomorphous replacement method is in progress. Availability of the active recombinant enzyme will facilitate biochemical studies and inhibitor screening efforts.

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