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Structure and kinetics assays of recombinant *Schistosoma mansoni* dihydrofolate reductase

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ABSTRACT

The parasite *Schistosoma mansoni* possesses all pathways for pyrimidine biosynthesis, in which dihydrofolate reductase (DHFR), thymidylate cycle participants, is essential for nucleotide metabolism to obtain energy and structural nucleic acids. Thus, DHFRs have been widely suggested as therapeutic targets for the treatment of infectious diseases. In this study, we expressed recombinant *Sm*DHFR in a heterologous manner to obtain structural, biochemical and kinetic information. X-ray diffraction of recombinant *Sm*DHFR at 1.95 Å resolution showed that the structure exhibited the canonical DHFR fold. Isothermal titration calorimetry was used to determine the kinetic constants for $NADP^+$ and dihydrofolate. Moreover, inhibition assays were performed using the commercial folate analogs methotrexate and aminopterin; these analogs are recognized as folate competitors and are used as chemotherapeutic agents in cancer and autoimmune diseases. This study provides information that may prove useful for the future discovery of novel drugs and for understanding these metabolic steps from this pathway of *S. mansoni*, thus aiding in our understanding of the function of these essential pathways for parasite metabolism.

1. Introduction

Schistosomiasis is a chronic disease caused by parasitic blood flukes of the genus *Schistosoma* (Conteh et al., 2005); this disease is directly associated with poor socioeconomic conditions and is widespread in many developing countries, mainly in Africa, South and Central America, and Southeast Asia (WHO, 2015). The World Health Organization reports that more than 249 million people require preventive treatment, but only 14.4% of infected people are currently treated. The etiologic agents of this disease are flatworms of the genus *Schistosoma*, among which *S. mansoni* is the most common in Brazil. *Schistosoma* worms possess all pyrimidine pathways, including the *de novo*, salvage pathway and thymidylate cycle pathways (Senft et al., 1972; Senft and Crabtree, 1983); however, for purine metabolism, only the purine salvage pathway is present (Dovey et al., 1984; Senft et al., 1973).

Dihydrofolate reductase (DHFR, EC 1.5.1.3), an enzyme found in all organisms, is a well-characterized anti-parasitic drug target (Jaffe et al., 1972; Schweitzer et al., 1990; Sharma and Chauhan, 2012). This enzyme catalyzes the reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$) and converts 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF), which is an intermediate in the synthesis of purines, thymidylic acid, and several amino acids (*e.g.*, methionine). DHFR is present in organisms from bacteria to humans and is a drug target in the pyrimethamine treatment of malaria and of human tumors because rapidly growing cells require folate to produce thymine (Jaffe et al., 1972; Schweitzer et al., 1990; Sharma and Chauhan, 2012).

The structures of DHFR homologs from different organisms reveal a highly conserved fold, a central eight-stranded beta-pleated sheet comprising seven parallel strands and a peripheral

Abbreviations: DHFR, dihydrofolate reductase; r*Sm*DHFR, recombinant *Schistosoma mansoni* DHFR; NADPH and $NADP^+$, nicotinamide adenine dinucleotide phosphate; DHF, 7,8-dihydrofolate; THF, 5,6,7,8-tetrahydrofolate; dTMP, deoxythymidine monophosphate; bp, base pairs; cDNA, complementary DNA; PCR, polymerase chain reaction; LB media, Luria broth media; IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MTX, methotrexate; SEC, analytical size exclusion chromatography; FBS, folate-binding site; r*Mt*DHFR, recombinant *Mycobacterium tuberculosis* DHFR.

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antiparallel strand. These beta-strands are connected by four alpha helices, resulting in an alpha-beta-alpha sandwich architecture. A conserved region between residues 9 and 24 is termed "Met20" or "loop 1" and, together with other loops, constitutes part of the major subdomain that surrounds the active site that is common to members of the DHFR family (Sawaya and Kraut, 1997; Venkitakrishnan et al., 2004).

DHFR plays a central role in THF homeostasis, which is essential for purine and thymidylate synthesis for cellular growth and expansion. The inhibition of DHFR leads to dTMP deficiency and inhibits cellular growth. The properties and different enzymatic mechanisms of this enzyme have been extensively investigated (Schnell et al., 2004). Nevertheless, it is crucial to gain full knowledge of the energetic metabolism of this enzyme because the importance of DHFR in chemotherapy is a direct result of its key role in thymidylate synthesis and the effectiveness of anti-folate drugs in treating certain types of cancers (Dovey et al., 1984). DHFR is the sole source of THF, and as such represents the Achilles' heel of rapidly proliferating cells; thus, this enzyme is the target of several anticancer and antibiotic drugs (Schnell et al., 2004).

A genome analysis of *Schistosoma mansoni* revealed one gene for *Smdhfr* that encodes one protein (187 amino acids; *Sm*DHFR) (Berriman et al., 2009). Crude extracts of *S. mansoni* yield a specific DHFR activity of 0.6 U per milligram of protein (Weinstein and Jaffe, 1987). Under the same conditions, strong preferences for DHF over folate and for NADPH over NAD as cofactors were demonstrated, and the K_M values for DHF and NADPH were determined as approximately 7 μM and 28 μM, respectively (Weinstein and Jaffe, 1987).

Recently, Bilsland et al. (2011) used DHFR homologs from several parasites (*Plasmodium falciparum*, *Plasmodium vivax*, *Schistosoma mansoni*, *Leishmania major*, *Trypanosoma cruzi* and *Trypanosoma brucei*) and humans in a yeast complementation assay to rapidly identify new therapeutic targets. The authors also tested these DHFRs against pyrimethamine and methotrexate (MTX), and the results revealed that recombinant *S. mansoni* DHFR (r*Sm*DHFR) is sensitive to both of these compounds, demonstrating that *Schistosoma* DHFR is a promising drug target (Bilsland et al., 2011); indeed, DHFR is classified as a drug target by WHO – TDR in their TDR target list. These compounds are known folate competitors and are used as chemotherapeutic agents to treat cancer and autoimmune diseases.

To better characterize the structure and kinetics of recombinant *Sm*DHFR, we expressed r*Sm*DHFR in a heterologous manner and solved its structure using X-ray crystallography; kinetic parameters were obtained using isothermal titration calorimetry (ITC). Moreover, inhibition assays were performed using the folate competitors MTX and aminopterin. These data can aid in determining whether r*Sm*DHFR is suitable for entry into drug discovery pipelines.

2. Materials and methods

2.1. Recombinant SmDHFR expression and purification

The 561-bp sequence of *Smdhfr* (Smp_175230) was amplified from an enriched cDNA library by PCR using the primers *5' ACTGTATCATATGATGCGTCTAAATGTTGTTGTGGCAG 3'* (forward) and *5' ACTGTATCATATGATGCGTCTAAATGTTGTTGTGGCAG 3'* (reverse). After cloning into the propagation vector pTZ-57R/T, the fragment was inserted at the *NdeI*/*XhoI* site of the pET28-a(+) expression vector (Novagen, USA). The *Smdhfr* gene sequence was confirmed by DNA sequencing.

To express r*Sm*DHFR, *Escherichia coli* BL21(λDE3) cells from a single colony transformed with the *Smdhfr*-pET28-a(+) vector were grown in 10 mL of LB media overnight at 37 °C and then inoculated into 1 L of 2XYT media containing 30 μg/mL kanamycin. The culture was then cultured to reach an OD_{600} of 0.6. Expression was induced by adding 0.1 mM IPTG, and the culture was continued for a further 4 h at 37 °C. The cells were then harvested by centrifugation (13,000 rpm) at 4 °C. The pelleted cells were then resuspended in 50 mL of lysis buffer (50 mM sodium di-hydrogen phosphate pH 7.8, 200 mM sodium chloride, 10 mM imidazole and 5 mM 2-mercaptoethanol). The cells were lysed by the addition of lysozyme (0.420 mg/mL, 30 min incubation on ice) followed by sonication at 4 °C, and the cell lysate was clarified by centrifugation (12,000 rpm) for 45 min at 4 °C.

The protein was purified using an affinity column that was packed with 3 mL of Talon resin that had been pre-equilibrated with lysis buffer. The column was washed with 30 ml of wash buffer (50 mM sodium phosphate pH 7.8, 200 mM sodium chloride, 20 mM imidazole and 5 mM 2-mercaptoethanol), and the protein was then eluted using 20 mL of elution buffer (50 mM sodium phosphate pH 7.8, 200 mM sodium chloride, 200 mM imidazole and 5 mM 2-mercaptoethanol). To confirm the efficiency of recovery, the fractions recovered in all purification steps were visualized using 15% SDS-PAGE. The yield obtained was 60 mg of recombinant *Sm*DHFR per liter of 2XYT medium.

The purified recombinant *Sm*DHFR sample was dialyzed against 100 volumes of 20 mM Tris–HCl pH 7.8, 200 mM sodium chloride and 5 mM 2-mercaptoethanol and was then concentrated to 8.0 mg/mL for the structural studies and kinetic experiments.

2.2. rSmDHFR crystallization data collection and structure resolution

The recombinant *Sm*DHFR sample was used in robotic crystallization trials using a Honeybee 963 robot (Genomic Solutions) at the Physics Institute of Sao Carlos (IFSC-USP). Commercial crystallization kits using sitting drop plates were employed; the drops contained 1 μL of 8.0 mg/mL r*Sm*DHFR and 1 μL of well solution. The plates were incubated at 18 °C. r*Sm*DHFR crystals were obtained after two weeks under condition H4 of the Ammonium Sulfate Kit (Qiagen); the condition was 100 mM Tris–HCl pH 8.0 and 2.0 M ammonium sulfate. The protein crystals were protected using a cryoprotective solution (mother liquor plus 20% glycerol) and were cooled in cryo-loops that were placed directly in liquid nitrogen for data collection.

X-ray diffraction data were collected at 100 K on beamline ID29 at ESRF (Grenoble – France) using a Pilatus 6 M detector (λ = 0.9762 Å). The diffraction data were indexed, integrated and scaled using the XDS package (Kabsch, 2010).

The structure was solved by molecular replacement using Phaser (McCoy et al., 2007); the structure of chicken liver DHFR (PDB ID: 8DFR) was used as the search model after modification using Chainsaw (Stein, 2008). The structure was refined using Phenix (Adams et al., 2010) and COOT (Emsley and Cowtan, 2004) for model building into σ_a-weighted $2F_o - F_c$ and $F_o - F_c$ electron density maps. Sulfate and water molecules were identified and positioned using a combination of COOT and Phenix. The structure obtained was the apo-form, all attempts to obtain structures of the complexes with $NADP^+$, DHF, methotrexate and aminopterin were unsuccessful.

R and R_{free} were monitored to evaluate the validity of the refinement protocol, and the stereochemistry of the model was assessed using Molprobity (Chen et al., 2010). The coordinates and structure factors have been deposited in Protein Data Bank (PDB ID: 3VCO).

2.3. Kinetic assays using isothermal titration calorimetry

To determine the kinetic parameters and enzymatic activity of recombinant *Sm*DHFR, isothermal titration calorimetry (ITC) was