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Molecular cloning and biochemical characterization of *Leishmania donovani* serine hydroxymethyltransferase

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Abstract

Serine hydroxymethyltransferase (SHMT) catalyzes the inter conversion of serine and tetrahydrofolate (H_4-folate) to form glycine and 5,10-methylene H_4-folate and generates one-carbon fragments for the synthesis of nucleotides, methionine, thymidylate, choline, etc. In spite of being an indispensable enzyme of the thymidylate cycle, SHMT in *Leishmania donovani* remains uncharacterized. The study of *L. donovani* SHMT (*ld*SHMT) becomes important as this gene is preferentially expressed in the amastigote stage of parasite, which resides in human macrophages. Here we report cloning, expression and purification of a catalytically active *ld*SHMT. The homogeneity of recombinant protein was analyzed by denaturing gel electrophoresis and protein was found to be 95% pure having yield of 1 mg/l. The recombinant protein is a tetramer of 216 kDa as evidenced by gel filtration chromatography and uses serine and tetrahydrofolate as substrates with Km of 1.6 and 2.4 mM, respectively. Further biochemical studies revealed that pH optimum of *ld*SHMT is 7.8 and enzyme is thermally stable up to 45 °C. *ld*SHMT was found sensitive towards denaturants as manifested by loss of enzyme activity at the concentration of 1 M urea or 0.25 M guanidine hydrochloride. This is the first report of purification and characterization of recombinant SHMT from any protozoan source. Studies on recombinant *ld*SHMT will help in evaluating this enzyme as potential drug target.

Keywords: Serine hydroxymethyltransferase; *Leishmania donovani*; Folate; Nucleotide biosynthesis

The leishmaniasis comprises a group of diseases with extensive morbidity and mortality in most developing countries. *Leishmania*, causative agent of this parasitic disease is distributed worldwide, being endemic in 88 countries. According to the World Health Organization, leishmaniasis currently affects nearly 12 million people and there are 2 million new cases per year with growing tendency [1]. Moreover, it is estimated that approximately 350 million people live at the risk of infection with *Leishmania* parasites [2]. *Leishmania*/HIV co-infections have increased in Mediterranean countries, where up to 70% of potentially fatal visceral leishmaniasis (VL)[1] cases are associated with HIV infection, and up to 9% of AIDS cases suffer from newly acquired or reactivated VL [3]. No effective vaccines are available against *Leishmania* infections as yet and treatment relies solely on chemotherapy with pentavalent antimonials as first-line drugs and amphotericin B and pentamidine as second-line agents [4–6]. Resistance to antimony is now observed in several parts of the world [7,8] most notably in the state of Bihar, India, where more than 50% of the patients are unresponsive to treatment, or relapse after conventional chemotherapy [9,10]. Ketoconazole, allopurinol, and miltefosine are been used as drug against *Leishmania* parasite as the biochemical pathways such as the ergosterol biosynthetic pathway, the exquisite requirements for purine salvage and high levels of ether lipids differs from its mammalian host. With the exception of miltefosine [11], these drugs are not effective against visceral leishmaniasis. There are reports of development of resistance against miltefosine in *Leishmania*. [12]. Recent

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[1] *Abbreviations used:* VL, visceral leishmaniasis; DHFR, Dihydrofolate reductase; TS, thymidylate synthase ; SHMT, Serine hydroxymethyltransferase; PLP, pyridoxal-5′-phosphate; DAB, Diamino benzidine.

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advancements of genome sequence of *Leishmania major* have provided new insight to discover new drug targets with a view to develop novel antileishmanial drug in near future [13].

Dihydrofolate reductase (DHFR) and thymidylate synthase (TS) together with serine hydroxymethyltransferase (SHMT) comprise the three enzymes of the thymidylate cycle essential for replication in virtually all organisms. Differences in amino acids at catalytic site of these enzymes in the host and target organism have been capitalized to develop antibacterial and antiprotozoal agents [14,15]. With such considerable efforts being put into designing drugs against these enzymes, it is surprising that very little attention has been paid to the inhibition of SHMT. Of the trio of enzymes involved in the thymidylate synthase cycle, SHMT is the only enzyme yet to be studied clinically as a target for leishmanial chemotherapy.

Serine hydroxymethyltransferase (SHMT), a member of the alpha class of the pyridoxal-5′-phosphate (PLP) dependent enzymes, is ubiquitous for generating one-carbon fragments for the synthesis of nucleotides, methionine, thymidylate, choline, etc. SHMT catalyses the inter conversion of serine and glycine with 5,6,7,8-tetra hydrofolate (THF) serving as the one carbon acceptor. The 5,10-methylene-tetrahydrofolate produced by SHMT participates directly in pyrimidine biosynthesis by donating a methyl group used for methylating 2′-deoxyuridine monophosphate (dUMP) to 2′-deoxythymidine monophosphate (dTMP). Overexpression and purification of soluble active SHMT has been reported from many sources like mammals (human, rabbit, sheep), plants (*Arabidopsis*, Pea) and prokaryotes (*Escherichia coli*) but purification of recombinant SHMT has not been reported from any protozoan sources. The SHMT activity has been described in a number of trypanosomatid parasites which cause the deadly diseases like Chagas disease, Sleeping sickness, Leishmaniasis, etc. [16,17] but overexpression and purification of recombinant protein in trypanosomatids has not been reported. Recent reports showed that *Leishmania* contains two SHMT isoforms, one present in cytosol and another present in mitochondria which are preferentially expressed in the amastigote stage of parasite. Studies suggest that the folate metabolism in *Leishmania* is compartmentalized. The mitochondrion may have an important regulatory role in folate metabolism in *Leishmania* and this pathway differs from other kinetoplast parasites [18]. The recombinant *ld*SHMT protein will certainly help for further characterization.

In the present study, we cloned the *ld*SHMT gene by PCR amplification from genomic DNA of *Leishmania donovani* and constructed a recombinant expression vector pQE60-*ld*SHMT. This His-tag-fused *ld*SHMT protein was then expressed in *E. coli*, purified through a single step Ni–NTA affinity chromatography and finally validated by western blot. Oligomeric structure, pH optimum, thermal stability and effect of denaturants on *ld*SHMT are reported here.

Materials and methods

Chemicals and reagents

THF, L-serine, 2-mercaptoethanol, PLP, and phenylmethanesulfonyl fluoride were obtained from Sigma Aldrich. Restriction enzymes used for cloning were obtained from MBI, Fermentas. Monoclonal His-antibody used for western blotting was obtained from Santa Cruz Biotechnology. pGEM-T Easy cloning vector was purchased from Promega. Expression vector pQE60 and Ni–NTA superflow were obtained from Qiagen, [3-^{14}C] serine was obtained from Amersham. All other biochemicals were of the highest grade available.

Leishmania donovani culture

The WHO reference strain of *L. donovani* (MHOM/IN/80/Dd8) was obtained from Imperial College London (UK) and maintained *in vitro* as promastigotes in high glucose RPMI 1640, supplemented with 10% heat inactivated fetal bovine serum containing 40 μg/ml gentamycin at 25 °C.

Bacterial strains and plasmid vectors

TA cloning vector pGEMT Easy was used to clone PCR product (*ld*SHMT) and pQE60 vector having C terminal His_6-tag was used for expression of the recombinant protein. The recombinant plasmids were transformed in *E. coli* M15 cells for expression.

Construction of expression vector pQE60-ldSHMT

PCR primers 5′-CGGGATCCATGATCTGTCGTG CCTCGATG-3′ (forward) and 5′- CGGGATCCTTGA ATGGGGTAGGCGCTCTCCAG-3′ (reverse) with *Bam*HI restriction sites shown as underlined were designed based on *L. major* SHMT gene (**LmjF28.2370**) sequence. Amplification of SHMT gene was carried out using genomic DNA of *L. donovani* as template using the following reaction cycles: 1 cycle for 3 min at 95 °C, 30 cycles for 1 min at 95 °C, 1 min 30 s at 58 °C, 2 min at 72 °C and 1 cycle for 10 min at 72 °C. The amplified product after gel purification was cloned in pGEMT-Easy vector (named as pGEMT-*ld*SHMT) transformed in *E. coli* DH5α competent cells. Nucleotide sequencing of pGEMT-*ld*SHMT clone was done in both directions to confirm the sequence of the amplicon and sequence submitted to GenBank. The SHMT gene in pGEMT was digested with restriction endonuclease *Bam*HI and subcloned into the prokaryotic expression vector pQE60 to produce the recombinant plasmid pQE60-*ld*SHMT that contains C-terminal His_6-tag for purification purpose. The SHMT gene was cloned in pQE60 vector and the positive clones were screened for the right orientation of the gene by restriction digestion. The *Xho*I site is present