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Biochemical and inhibition studies of glutamine synthetase from *Leishmania donovani*

Vinay Kumar, Shailendra Yadav, Neelagiri Soumya, Rohit Kumar, Neerupudi Kishore Babu, Sushma Singh*

Department of Biotechnology, National Institute of Pharmaceutical Education and Research, SAS Nagar, Mohali 160062, Punjab, India

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ABSTRACT

Leishmaniasis is a group of tropical diseases caused by protozoan parasites of the genus *Leishmania*. *Leishmania donovani* is a protozoan parasite that causes visceral leishmaniasis, a fatal disease if left untreated. Chemotherapy for leishmaniasis is problematic as the available drugs are toxic, costly and shows drug resistance, hence, there is a necessity to look out for the novel drug targets, chemical entities and vaccine. Glutamine synthetase (GS) catalyzes the synthesis of glutamine from glutamate and ammonia. In the present study, we have identified and characterized GS from *L. donovani*. The nucleotide sequence encoding putative glutamine synthetase like sequence from *L. donovani* (*Ld*GS, LDBPK_060370) was cloned. A 43.5 kDa protein with 6X-His tag at the C-terminal end was obtained by overexpression of *Ld*GS in *Escherichia coli* BL21 (DE3) strain. Expression of native *Ld*GS in promastigotes and recombinant *L. donovani* glutamine synthetase (r*Ld*GS) was confirmed by western blot analysis. An increase in expression of GS was observed at different phases of growth of the parasite. Expression of *Ld*GS in promastigote and amastigote was confirmed by western blot analysis. Immunofluorescence studies of both the promastigote and amastigote stages of the parasite revealed the presence of *Ld*GS in cytoplasm. GS exists as a single copy gene in parasite genome. Kinetic analysis of GS enzyme revealed K_m value of 26.3 ± 0.4 mM for L- glutamate and V_{max} value of 2.15 ± 0.07 U mg^{-1}. Present study confirms the presence of glutamine synthetase in *L. donovani* and provides comprehensive overview of *Ld*GS for further validating it as a potential drug target.

1. Introduction

Leishmaniasis is a group of vector-borne disease caused by an intracellular obligate protozoan parasite belonging to the genus *Leishmania*. Clinical forms of leishmaniasis include cutaneous, mucosal and visceral leishmaniasis. Visceral leishmaniasis (VL) also known as kala-azar is fatal in over 95% of cases if left untreated. Around half a million new cases of VL are reported worldwide each year and over 90% of new cases occur in Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan [1–3]. VL is caused by two leishmanial species, *Leishmania donovani* or *Leishmania infantum*, depending on the geographical area. *L. infantum* infects mostly children and immune suppressed individuals, whereas *L. donovani* infects all age groups [1]. Infection to the mammalian host is caused by flagellated metacyclic promastigotes that are deposited in the skin during feeding of the sand fly vector. Current treatment regimen for the disease includes pentavalent antimonials, amphotericin B, paromomycin and miltefosine [4]. Antileishmanial drug treatment is problematic as the available drugs are toxic, costly and face drug resistance particularly in India [5,6]. Increasing resistance of *Leishmania* towards pentavalent antimonials has raised serious concern. Therefore, there is an urgent need for efficacious drugs and vaccines against VL. Understanding of the various metabolic pathways and the essentiality of enzymes involved in them will provide more information regarding the potential of metabolic proteins to be used as drug target against leishmaniasis. One such enzyme under consideration is glutamine synthetase. Glutamine synthetase (GS; EC 6.3.1.2, also known as γ-glutamyl: ammonia ligase) catalyzes the ATP dependent synthesis of glutamine from glutamate and ammonia (Fig. 1) [7].

In the first step of the enzymatic reaction, glutamate is phosphorylated to yield gamma glutamyl phosphate. Ammonia then replaces the phosphate to form the amide moiety of glutamine. This catalytic mechanism is considered to be same for all the existing

* Corresponding author.
E-mail address: sushmasingh@niper.ac.in (S. Singh).

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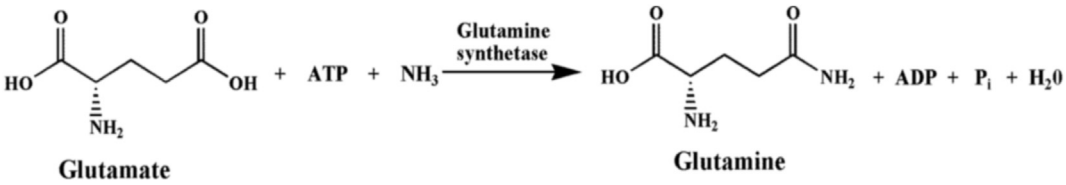

Fig. 1. Glutamine synthetase catalyzes the formation of L-glutamine from L-glutamate.

forms of the GS as is evident by the presence of highly conserved residues at the glutamate and ammonia binding sites [8,9]. In many organisms ammonia assimilation by GS leads to synthesis of glutamine from glutamate by the enzyme GS. This pathway is referred to as GS/GOGAT pathway [10]. Evolution of GS, one of the most ancient genes is considered a good molecular clock [11,12]. It is a ubiquitous enzyme, which is involved in nitrogen metabolism, recycling of neurotransmitter glutamate, and the synthesis of glutamine for the production of amino acids, sugars and glucosamine-6-phosphate [13]. Amino acids are essential components of metabolism in kinetoplastids. Some amino acids in *Leishmania* and *Trypanosoma* are reported to act as source of energy production and as triggers to differentiation process [14–16]. Amino acids can be used as carbon and energy sources and they act as crucial nutrients during the *Trypanosoma cruzi* life cycle. They also participate in several biological processes that help the parasite adjust to various environmental changes [17,18]. Among other amino acids, glutamate is required for the metacyclogenesis of *T. cruzi* [19,20]. Moreover, glutamate is converted to glutamine, the amino donor for several essential metabolic pathways in *Leishmania*, including pyrimidine and amino sugar synthesis [21,22]. L-proline is the main source of energy generation for *Leishmania* promastigotes [23]. Till date, three distinct forms of GS have been reported on the basis of their length of amino acids: GSI, consisting of 12 identical subunits (450–470 amino acids each), is present mostly in prokaryotes but, have been recently identified in mammals and plants also [24,25]. The quaternary structure of GSII enzymes has been controversial, however the octameric as well as decameric arrangement of GSII subunits (350–420 amino acids each) is primarily found in eukaryotes and some bacteria (Rhizobiaceae and Streptomycetaceae families, which also have GSI, and GSIII) [26,27]. As GSI, GSIII is composed of 12 identical subunits (but with about 700 amino acids each), and was first found in *Bacteroides fragilis* and identified later in a few more anaerobic bacteria and cyanobacteria [28–30].

In *Leishmania chagasi* GS was identified as T cell antigen [31]. It is expressed by the amastigote stage and is reported to stimulate immune T-cell proliferation and cause increase in IFN-γ levels. Thus, GS acts as a potential target for the immune response during mammalian infection [31]. It was reported in *L. mexicana*, that inhibition of either TCA cycle or GS strongly inhibits amastigote growth and viability *in vitro* and in infected macrophages [21]. There are several essential enzymes in *L. donovani* which utilize glutamine as their substrate, one of them being *L. donovani*, asparagine synthetase (AS). There are two structurally distinct types of AS: A and B. AS-A from *Trypanosoma brucei, T. cruzi* and *L. donovani* parasites were reported not only to use ammonia but also glutamine as nitrogen donor [32–34].

The internal L-glutamine pool acts as a sensor of external nitrogen. In *Salmonella enterica* serovar Typhimurium, mutation of *gln*A resulted in a marked reduction of virulence and survival ability in host cells [35,36]. *Streptococcus suis* serotype 2 (*S. suis*2) is an important pathogen, responsible for diverse diseases in swine and human [37,38]. Deletion of GS gene (Δ*gln*A mutant) had significantly reduced the virulence of *S. suis*2, suggesting that glutamine metabolism is especially important for the virulence of pathogens [39]. In *Mycobacterium tuberculosis*, the *gln*A-1 gene encodes a class I GS (GSI) enzyme that is released into the culture medium and plays a crucial role in pathogenicity [40]. Also *gln*A-1 mutant strain of *Mycobacterium bovis* infects THP-1 cells with reduced efficiency and also exhibited attenuated growth in macrophages [41]. *Bacillus subtilis* GS was reported to be moonlighting protein which, apart from its enzymatic functions, has a critical role in the control of gene expression [42].

GS inhibitors have also been reported to inhibit mycobacterial and *L. mexicana* glutamine synthetase [21]. Because of its importance, the enzyme has been projected as a target for antimicrobial drugs. GS has been identified as a potential drug target *in silico* approach in *Plasmodium falciparum* and *M. tuberculosis* [43,44]. Amino acids participate in a variety of metabolic pathways leading to the synthesis of products which are crucial for survival of parasites. Therefore, metabolic enzymes can be considered good target for drug design. GS has an important role in the metabolic process of glutamate. To our knowledge, little is known about the biological function of GS in *L. donovani*. This paper is the first to report the cloning and characterization of the glutamine synthetase from *L. donovani*. Present study would be helpful in ascertaining functional aspects of the enzyme in the parasite.

2. Materials and methods

2.1. Materials

All the restriction enzymes were obtained from Bangalore Genei Pvt. Ltd., India. L-Glutamic acid, GenElute™ plasmid miniprep kit, GenElute™ gel extraction kit, oligonucleotide primers, sodium bicarbonate, streptomycin, penicillin G, His-Select® HF nickel affinity gel and alkaline phosphatase conjugated anti-rabbit IgG were purchased from Sigma (St. Louis, USA). *Taq* DNA polymerase, dNTPs (deoxynucleotide triphosphates) were from Invitrogen (Carlsbad, USA), T_4 DNA ligase (NEB Pvt. Ltd., UK), Wizard gDNA purification kit was purchased from Promega Biotech India Pvt. Ltd. PCR DIG Probe Synthesis kit and DIG immunological detection kit (Roche), Anti-His monoclonal antibody was purchased from Calbiochem. Poly-L-lysine coated coverslips was from BD Biosciences and Polyclonal anti-rabbit GS antibody was customized from Abgenex Pvt. Ltd., Bhubaneswar, India. RPMI-1640 HEPES modified medium and foetal bovine serum were purchased from Gibco/BRL (Life Technologies Scotland, UK). Other chemicals used in this study were of analytical grade and commercially available.

2.2. Parasite and culture conditions

L. donovani (wild type, MHOM/80/IN/Dd8) promastigotes were cultured and maintained at 24 °C in RPMI-1640 HEPES modified medium supplemented with 0.2% sodium bicarbonate, 100 μg/mL penicillin G, 100 μg/mL streptomycin, 100 μg/mL gentamicin and 10% heat inactivated foetal bovine serum (FBS). Medium was maintained at pH 7.2. For the generation of axenic amastigotes, 1×10^8 cells of *L. donovani* wild type promastigotes were grown in potassium buffered RPMI-1640 base medium (pH 5.5) at 37 °C with 5% CO_2 according to the method described by Debrabant *et al.* [45] and used after two to three passages.