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Trypanothione biosynthesis in *Leishmania major*☆

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Abstract

Trypanothione plays a crucial role in regulation of intracellular thiol redox balance and in defence against chemical and oxidant stress. *Crithidia fasciculata* requires two enzymes for the formation of trypanothione, namely glutathionylspermidine synthetase (GspS; EC 6.3.1.8) and a glutathionylspermidine-dependent trypanothione synthetase (TryS; EC 6.3.1.9), whereas *Trypanosoma cruzi* and *Trypanosoma brucei* use a broad-specificity trypanothione synthetase to make trypanothione from glutathione (GSH) and spermidine. Here, we report the identification of two genes in *Leishmania major* with similarity to previously identified *GSPS* and *TRYS*. *GSPS* is an apparent pseudogene containing two frame shift mutations and two stop codons, whereas *TRYS* is in a single open-reading frame. The enzyme encoded by *TRYS* was expressed and found to catalyse formation of trypanothione with GSH and either spermidine or glutathionylspermidine. When GSH is varied as substrate the enzyme displays substrate inhibition (apparent $K_m = 89\,\mu M$, $K_i^s = 1\,mM$, $k_{cat} = 2\,s^{-1}$). At a fixed GSH concentration, the enzyme obeys simple hyperbolic kinetics with the other substrates with apparent K_m values for spermidine, glutathionylspermidine and MgATP of 940, 40 and 63 μM, respectively. Immunofluorescence and sub-cellular fractionation studies indicate that TryS localises to the cytosol of *L. major* promastigotes. Phylogenetic analysis of the GspS and TryS amino acid sequences suggest that in the trypanosomatids, TryS has evolved to replace the GspS/TryS complex in *C. fasciculata*. It also appears that the *L. major* still harbours a redundant *GSPS* pseudogene that may be currently in the process of being lost from its genome.
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1. Introduction

In the search for novel drug therapies against diseases caused by *Leishmania* and *Trypanosoma* spp., we have identified trypanothione [N^1,N^8-bis(glutathionyl)spermidine] as a unique and common metabolite as a potential drug target in the Trypanosomatidae [1]. This thiol plays a pivotal role in a number of processes such as, regulation of intracellular thiol redox balance, synthesis of deoxyribonucleotides, drug resistance and in defence against chemical and oxidant stress [2–5]. In *Leishmania*, trypanothione has also been implicated in the mode of action of antimonials [6] and in resistance to trivalent antimony in laboratory derived resistant strains [7,8].

Two enzymes are involved in the stepwise biosynthesis of trypanothione in *Crithidia fasciculata*, namely glutathionylspermidine synthetase (GspS) and trypanothione synthetase (TryS) [9–11]. These proteins co-purify as a heterodimeric complex and rapidly lose activity when separated [9]. However, in both *Trypanosoma cruzi* and *Trypanosoma brucei*, a broad-specificity monomeric trypanothione synthetase is capable of synthesising trypanothione from spermidine and glutathione (GSH) [12–14]. To date there is no evidence of a corresponding *GSPS* in the *T. cruzi* or *T. brucei* databases and our attempts to amplify the gene from genomic

Abbreviations: GSH, glutathione; GspS, glutathionylspermidine synthetase; TryS, trypanothione synthetase; TCEPtris(2-carboxyethyl)phosphine

☆ *Note:* The nucleotide sequence data reported here are available in the EMBL, GenBank and DDBJ databases under the GenBank accession numbers AJ311570 (*LmTRYS*) and AJ748279 (*LmGSPS*).

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DNA have been unsuccessful. Disruption of TryS by RNAi in *T. brucei* confirms that GspS is absent and that TryS is essential for viability [15], (M.R. Ariyanayagam, S.L. Oza, A.H. Fairlamb, unpublished data). All of these proteins have also been shown to have weak amidase activity and are capable of hydrolysing glutathionylspermidine and trypanothione back to GSH and spermidine. This amidase activity, which is located at the *N*-terminus of the protein, is particularly active in GspS from *C. fasciculata* [11]. All GspS and TryS sequenced so far possess a conserved cysteine and histidine residue in the amidase domain and are members of the CHAP (cysteine, histidine-dependent amidohydrolase/peptidase) superfamily of amidohydrolases [16].

Our current studies examine whether *Leishmania major* has two independent enzymes for trypanothione biosynthesis (like *C. fasciculata*) or only one (like *T. cruzi* and *T. brucei*). This is of particular importance when trying to identify potential drug targets that may be aimed at all members of this family. Here, we report on the identification of a *GSPS* pseudogene and a functional *TRYS* gene in *L. major*. We have determined some kinetic properties of TryS as well as its subcellular localisation in *L. major*. We also discuss the implications of the evolution of a single enzyme to biosynthesise trypanothione in the trypanosomatids.

2. Materials and methods

2.1. Organisms and reagents

An *L. major* Friedlin cosmid library was kindly provided by Vanessa Leech (Cambridge University Department of Pathology). Routine manipulations were performed in *Escherichia coli* strain JM109 and overexpression in strain BL21 Star (DE3)pLysS (Invitrogen). All chemicals were of the highest grade available from Sigma, BDH and Molecular Probes. Restriction enzymes and DNA-modifying enzymes were from Promega or Roche.

2.2. Isolation of TRYS and GSPS probes from L. major genomic DNA

Two degenerate oligonucleotides were designed based on an alignment of *Ec*GspS [17], *Cf*GspS and *Cf*TryS [10] (KYQCVEF, DTC5: 5′-AAR TAY CAR TGY GTN GAR TT-3′ and EPLWK, DTC2: 5′-YTT CCA NAD NGG YTC-3′). PCR was performed in a 50 μl volume containing 0.4 mM each dNTP, 1 μg each primer, 1 μg of *L. major* (CC1) genomic DNA and 2.5 units *Taq* DNA polymerase (Promega) with buffer (100 mM Tris–HCl (pH 8), 500 mM KCl, 0.8% (v/v) Nonidet P40) and 20 mM $MgCl_2$. After a hot start at 95 °C for 10 min, *Taq* was added and the mixture was subjected to the following for 30 cycles: denaturation, 10 s, 95 °C; annealing, 30 s, 50 °C and elongation, 30 s, 72 °C. A final 10 min extension at 72 °C was also included. A 1.35-kb PCR product was identified following agarose gel electrophoresis and cloned into the *Sma*I site of pUC18 (SureClone kit, Pharmacia) giving the plasmid pUC18-*Lm*TryS. This method yielded a putative *TRYS*, but no corresponding *GSPS*. A subsequent BLAST search identified a partial sequence for a putative *GSPS* in *L. major* (accession number AQ849191). The following oligonucleotides were designed based on its sequence (LmG_F: 5′-GGC TCC TTC TTG GGG GCC AAG-3′ and LmG_R: 5′-CGG TCA GGA GCA GAG CAG CGC-3′) and used to amplify a 490 bp product using similar PCR conditions outlined above (a higher annealing temperature of 65 °C gave greater primer specificity).

2.3. Southern blot analysis and RT-PCR

The *L. major* Friedlin cosmid library consists of 9216 clones constructed in the shuttle vector cLHYG that has been gridded onto nylon membrane [18]. Probes for Southern analysis were generated by PCR using primers based on the partially sequenced *TRYS* from *L. major* (LF: 5′-AAG TGC CAG TGT GTT GAA TTT GCG-3′ and LR: 5′-CGG CTC GAA GTA GAG AAT TTC CCA-3′). Blots were hybridised at 60 °C with the corresponding LF/LR PCR probe labelled with fluorescein-11-dUTP and detected by anti-fluorescein alkaline phosphatase conjugate (Gene Images, Amersham Biosciences).

Restriction digest analysis of *L. major* Friedlin genomic DNA using the *GSPS* probe (corresponding to AQ849191) identified a *Sac*I fragment of approximately 4 kb, which was subsequently inserted into pUC18 and sequenced.

Total RNA was isolated from *L. major* Friedlin promastigotes using RNeasy (Qiagen) and treated with DNA-free (Ambion) to ensure that contaminating DNA was removed. RT-PCR was performed using Superscript III one-step RT-PCR system with platinum *Taq* DNA polymerase (Invitrogen). Primers designed against the *L. major* spliced leader sequence (SL: 5′-TAA CGC TAT ATA AGT ATC AGT TTC-3′) were used with gene specific primers against *GSPS* (G1: 5′-AGC GCT GGG CAC GTC ACA CTC-3′, G2: 5′-CTT ATT TGG CGG CAT CAT CTC-3′, G3: 5′-TGA TAG ACG ACG GCG GAG TCG-3′ and G4: 5′-CTA CCG AGC GAG ACG TTT TCG-3′) and *TRYS* (T1: 5′-GCA GGT GAA ACT CGT GAT TCG-3′ and T2:5′-GCG CTG TCA GCA TTG TAC TCG-3′) as reverse primers. The PCR amplified regions of interest were cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced.

2.4. Expression and purification of LmTryS in E. coli

The complete open reading frame of *LmTRYS* was amplified by PCR from cosmid 16A7 using an *Nde*I containing forward primer (LmTF: 5′-CAT **ATG** TCA TCT CTG CAG CGC GCG TCT GTG-3′) and a *Bam*HI containing reverse primer (LmTR: 5′-GGA TCC **TTA** CTC GTC CTC GGC CAT CTT GTC-3′), the initiator and terminator codons are in bold, restriction sites are underlined. The PCR product of ∼1.95 kb was then cloned (via TOPO cloning vector) into

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