Leishmania donovani Encodes a Functional Selenocysteinyl-tRNA Synthase*

Received for publication, September 27, 2015, and in revised form, November 2, 2015  Published, JBC Papers in Press, November 19, 2015, DOI 10.1074/jbc.M115.695007

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The synthesis of selenocysteine, the 21st amino acid, occurs on its transfer RNA (tRNA), tRNASec. tRNASec is initially aminocylated with serine by seryl-tRNA synthetase and the resulting seryl moiety is converted to phosphoserine by O-phosphoseryl-tRNA kinase (PSTK) in eukaryotes. The selenium donor, selenophosphate is synthesized from selenide and ATP by selenophosphate synthetase. Selenocysteinyl-tRNA synthase (SepSecS) then uses the O-phosphoseryl-tRNA Sec and selenophosphate to form Sec-tRNA Sec in eukaryotes. Here, we report the characterization of selenocysteinyl-tRNA synthase from Leishmania donovani. Kinetoplastid SepSecS enzymes are phylogenetically closer to worm SepSecS. LdSepSecS was found to exist as a tetramer. Leishmania SepSecS enzyme was found to be active and able to complement the ΔselA deletion in Escherichia coli JS1 strain only in the presence of archaeal PSTK, indicating the conserved nature of the PSTK-SepSecS pathway. LdSepSecS was found to localize in the cytoplasm of the parasite. Gene deletion studies indicate that Leishmania SepSecS is dispensable for the parasite survival. The parasite was found to encode three selenoproteins, which were only expressed in the presence of SepSecS. Selenoproteins of L. donovani are not required for the growth of the promastigotes. Auranofin, a known inhibitor of selenoprotein synthesis showed the same sensitivity toward the wild-type and null mutants suggesting its effect is not through binding to selenoproteins. The three-dimensional structural comparison indicates that human and Leishmania homologs are structurally highly similar but their association modes leading to tetramerization seem different.

Selenocysteine (Sec)4 is the 21st amino acid in the genetic code and contains selenium. It is co-translationally inserted into selenoproteins (selenium-containing proteins) in response to the UGA codon in mRNA (1). Selenoproteins are found in each of the three domains of life: prokaryotes, archaea, and eukaryotes but are absent in yeast and higher plants. Selenoproteins have been reported to exist in kinetoplastids, apicomplexan parasites, insects, nematodes etc. (2). The human genome codes for 25 selenoproteins. Selenoproteins such as glutathione peroxidases and thioredoxin reductases have biological functions in oxidoreductions, redox signaling, and antioxidant defense (3).

Selenoprotein biosynthesis requires two specialized metabolic pathways: one that incorporates Sec into proteins and the other that synthesizes Sec. The Sec insertion machinery has the capability to distinguish between the UGA for amino acid incorporation or termination. This is due to the presence of a stem-loop structure in the 3′-untranslated region of selenoprotein mRNA in eukaryotes known as the Sec insertion sequence (SE CIS) element. The distance between the Sec UGA codon and the SECIS element plays an essential role in this distinction. SECIS binding protein 2 (SBP2) binds to the SECIS element and forms a complex with the specific elongation factor, EFSec for incorporation of Sec into the selenoprotein (1, 4). UGA recoding in higher organisms requires additional factors like SECp43, which was found to associate with the Sec-tRNA Sec-EFSec complex in vitro and also enhance the interaction between EFSec and SBP2 in vivo (5).

Sec synthesis occurs on its transfer RNA (tRNA), designated tRNA Sec. The bacterial Sec synthesis system is different from that of archaea and eukaryotes (1, 4). In bacteria following the serylation of tRNA Sec, its direct transformation to Sec-tRNA Sec occurs by SelA (Fig. 1). However, archaea and eukaryotes have an additional step to form Sec-tRNA Sec. tRNA Sec is initially aminocylated with serine by seryl-tRNA synthetase and the resulting seryl moiety is converted to phosphoserine by O-phosphoseryl-tRNA kinase (PSTK) in eukaryotes. This phosphorylation step is absent in bacteria. The selenium donor, selenophosphate is synthesized from selenide and ATP by selenophosphate synthetase in eukaryotes and SelD in bacteria (1, 4). Selenocysteinyl-tRNA synthase (SepSecS) then uses the O-phosphoseryl-tRNA Sec and selenophosphate to form Sec-tRNA Sec in eukaryotes (Fig. 1).

SepSecS in mammals was originally identified as the soluble liver antigen (SLA), the target of autoantibodies from patients with a severe form of autoimmune chronic hepatitis. SLA was found to interact with SECp43 and tRNA Sec (4). Concrete evidence that SLA is eukaryotic SepSecS emerged when it was found to convert O-phosphoseryl-tRNA Sec into Sec-tRNA Sec (6, 7). SepSecS is a member of the Fold Type 1 PLP enzyme family (8). Two SepSecS monomers interact to build together two identical active sites around PLP in a Schiff-base linkage.

*The work was supported by grants from the Department of Biotechnology, Government of India, and a Department of Science and Technology-Promotion, University Research and Scientific Excellence grant (to R. Madhubala). The authors declare that they have no conflicts of interest with the contents of this article.

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4 The abbreviations used are: Sec, selenocysteine; SepSecS, selenocysteinyl-tRNA synthase; LeSepSecS, L. donovani selenocysteinyl-tRNA synthase; LdSepSecS, L. donovani selenocysteinyl-tRNA synthase; MjSepSecS, M. jannaschii selenocysteinyl-tRNA synthase; PSTK, O-phosphoseryl-tRNA kinase; PLR, (5-hydroxy-4,6-dimethyl-3-pyridinyl)methyl dihydrogen phosphate; PLP, pyridoxal 5′-phosphoplatase; SLA, soluble liver antigen; FDHts, formate dehydrogenase H.
with lysine 284. The N terminus mediates tetramer formation such that two SepSecS dimers further associate to form a homo-tetramer (4). The structural and mechanistic insights of SepSecS action have been deciphered in eukaryotes (4, 8, 9). Two mechanisms have been proposed that ensure that SepSecS does not act on free $\text{O}^{-}\text{phospho-L-serine}$. tRNA binding has been reported to induce a conformational change in the active site of the enzyme that does not allow reaction with free phosphoserine (9) and an active site insertion is observed to contribute to PLP binding and positions a glutamate next to PLP where it repels free $\text{O}^{-}\text{phospho-L-serine}$ (4).

The presence of selenoprotein and selenocysteine biosynthetic pathways has been reported in trypanosomes (10–13). A survey of the *Leishmania* genome using the known selenoproteins as a search tool led to the identification of genes for glutathione peroxidase, selenoprotein R, and a selenoprotein SPS2 homolog (11). It was further reported that these selenoprotein homologs in *Leishmania* possess a Cys (UGU or UGC) residue at the equivalent position of Sec (UGA) (11). It was postulated that these homologs in *Leishmania* have evolved into nonsele-nium-containing proteins consistent with the loss of an identifiable SECIS element in their sequence (11). However, in another study, *in silico* analysis of the genomes of *Trypanosoma* and *Leishmania* for the occurrence of homologs of known selenoprotein genes, led to the identification of SelK and SelT, as well as a novel multidomain selenoprotein designated SelTryp (12). In *silico* subcellular localization prediction tools suggested a mitochondrial localization for SelTryp and SelT was found to have a potential export signal (12). Kinetoplastida SelK was found to have a predicted transmembrane motif (12).

In mammals, SelK associates with proteins involved in the elimination of misfolded proteins from the endoplasmic reticulum (14). SelK has been found to promote effective $\text{Ca}^{2+}$ flux during immune cell activation. It is also a target of calpain proteases that are involved in regulation of inflammation and immune response (14). Selenoprotein T in mammals is involved in the regulation of $\text{Ca}^{2+}$ homeostasis and neuroendocrine secretion in response to a cAMP-stimulating trophic factor (15). It also plays a role in cell adhesion (16). The functions of SelK and SelT in kinetoplastids need to be experimentally deciphered. SelTryp ORF has Sec present in the conserved C-terminal peptide region, $\text{Si(V)I(V)-CI(V)SUPR}$ (U is Sec). This C-terminal location is common to eukaryotic selenoproteins (e.g. thioredoxin reductase, SelK, SelS, and SelO) (12). In SelTryp, Sec is present within a $\text{CXXU}$ motif, which is often found in selenoproteins that carry out redox function through reversible formation of a selenenylsulfide bond. This observation suggests a redox function for the $\text{CXXU}$ motif in SelTryp (12).

Our earlier comprehensive bioinformatics analysis of the *Leishmania* genome led to the identification of an enzyme with SepSecS-like domains (17). *In silico* analysis showed that kinetoplastid SepSecS enzymes are phylogenetically closer to worm SepSecS, whereas the metazoan enzymes are closer to fly and viridiplantae/algae SepSecS enzymes. In the present study, we for the first time report that *Leishmania* encodes an active selenocysteinyl-tRNA synthase, which is dependent on the action of PSTK enzyme in the Sec insertion pathway. Gene deletion studies indicate that *Leishmania* SepSecS is dispensable for the survival of the parasite. The parasite was found to encode three selenoproteins, which were expressed only in the presence of SepSecS enzyme. Auranofin, a known inhibitor of selenoprotein synthesis showed the same sensitivity toward the

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**FIGURE 1. Biosynthesis of Sec on its tRNA.** A, archaeal and eukaryotic pathway. B, bacterial pathway of Sec synthesis (see text for details).
Selenocysteinyl-tRNA Synthase of L. donovani

Expression and Purification of Recombinant LdSepSecS Protein—The gene for LdSepSecS (LdBPK_091000.1) was amplified by PCR using forward primer with a flanking BamHI site (5’T-TTTGGATCCATGGATGATCGCTCGT-AAAATG-3’) and reverse primer with a flanking HindIII site (5’T-TTTTACGCTTTATCAGCCGAGCGCACAGG-3’) from L. donovani genomic DNA. The 1788-bp amplification product encompassing the entire LdSepSecS open reading frame (ORF) was cloned into pET30a (Novagen) using BamHI and HindIII restriction sites. This construct containing a His6 tag at the N terminus was transformed into the E. coli BL21(DE3) strain (Novagen). Protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside at 25 °C for 6 h. Bacteria were then harvested by centrifugation at 5000 × g for 10 min and the cell pellet was suspended in lysis buffer (50 mM Tris-Cl, pH 7.4, 10 mM imidazole, 300 mM sodium chloride, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture). Protein was purified using Ni2+-nitrilotriacetic acid-agarose resin (Qiagen) by eluting with increasing concentrations of imidazole. Purified protein was found to be >95% pure as judged by SDS-PAGE.

Antibody Production and Western Blot Analysis—Purified recombinant LdSepSecS protein (50 μg) was subcutaneously injected in mice using Freund’s complete adjuvant (Sigma), followed by three booster doses of the recombinant protein (20 μg) in Freund’s incomplete adjuvant (Sigma), at 2-week intervals. The mice were sacrificed after the last booster and serum was collected for Western blot analysis. Early log phase wild-type and genetically manipulated promastigotes were harvested and the resultant cell pellets re-suspended in lysis buffer (10 mM Tris-Cl, pH 8.0, 5 mM DTT, 10 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.5% Triton X-100, 0.3 mM phenylmethylsulfonyl fluoride (PMSF). The cell pellets were lysed by freeze-thaw cycles and sonication on ice followed by centrifugation at 20,000 × g. For recombinant LdSepSecS protein, 30 μg of the total soluble promastigote cell extracts were fractionated on a 10% SDS-PAGE gel and blotted onto nitrocellulose membrane using electrophoretic transfer cell (Bio-Rad). After blocking with 5% skimmed milk, the membrane was incubated for 2 h at room temperature with anti-LdSepSecS antibody (1:1000) generated in mice as described above. In the case of only recombinant protein, incubation was done with anti-His tag antibody (Cell Signaling Technology number 2365) (1:3000). The membrane was then washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Cell Signaling Technology number 7076S) (1:5000). The blot was developed using 3,3’-diaminobenzidine (DAB) tablets (Sigma) or ECL® kit (Amersham Biosciences) according to the manufacturer’s protocol.

Glutaraldehyde Cross-linking Studies—50 μg of rLdSepSecS in 50 mM phosphate buffer (pH 7.6) was treated with 2 μl of 5% freshly prepared glutaraldehyde solution for 30 s, 2 min, and 10 min, respectively, at 37 °C. The reaction was terminated by addition of 10 μl of 1 mM Tris-HCl (pH 8.0). Cross-linked protein samples were mixed with loading buffer containing 100 mM Tris-HCl (pH 6.8), 0.4% SDS, 20% glycerol, 0.001% bromophenol wild-type and null mutant suggesting its effect in Leishmania donovani is not through binding to selenoproteins.

Experimental Procedures

Materials—All restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs. Paromomycin, hygromycin, and zeocin were obtained from Sigma. Plasmid pET-30a was obtained from Novagen. Escherichia coli DH10β and BL21(DE3) were used as the host for plasmid cloning and protein expression, respectively. E. coli ΔselA deletion strain, plasmids harboring Methanocaldococcus jannaschii SepSecS, and PSTK genes were kind gifts from Dr. Dieter Söll (Yale University, New Haven, CT). Chromosomal DNA from L. donovani (Bob) was used to amplify the SepSecS DNA sequence. Nickel-nitrilotriacetic acid-agarose was purchased from Qiagen. DNA and protein markers were acquired from New England Biolabs. Aurafinon was obtained from Sigma. Selenium 75 was obtained from the Board of Radiation and Isotope Technology (BRIT), Department of Atomic Energy, India. Other materials used in this study were of analytical grade and were commercially available.

Strains and Culture Conditions—L. donovani Bob (LdBob strain/MHOM/SD/62/1SCL2D) was originally obtained from Dr. Stephen Beverley (Washington University, St. Louis, MO). Wild-type promastigotes were cultured at 22 °C in M199 supplemented with 100 units/ml of penicillin and 10% heat-inactivated fetal bovine serum (Gibco). Wild-type (WT) parasites were routinely cultured in media with no drug supplementations, whereas the genetically manipulated SepSecS heterozygotes (SepSecS/HYG, SepSecS/NEO) and null mutants (ΔSepSecS) were maintained in either 200 μg/ml of hygromycin or 300 μg/ml of paromomycin or both, respectively. The “add-back” line ΔSepSecS/+ was grown in 800 μg/ml of zeocin, 200 μg/ml of hygromycin, and 300 μg/ml of paromomycin. For characterizing the mutant parasites phenotypically, cells were subcultured without selection marker prior to experiments.

The mouse monocyte macrophage-like cell line J774.A1 obtained from ATCC was cultured in RPMI 1640 medium (Sigma) supplemented with 100 units/ml of penicillin, 10% FBS and 100 units/ml of penicillin and 100 units/ml of streptomycin at 37 °C with 5% CO2.

Sequence and Phylogenetic Analysis—SepSecS sequences retrieved from trirypDB (18), Swissprot/UniprotKB (19, 20), and PlasmoDB (21) were used for multiple sequence alignment. Multiple sequence alignment of these sequences was generated using CLUSTALW (22) using default parameters and used as seed alignment for phylogenetic tree generation using the Jones-Taylor-Thornton model (23). MEGA version 5.0 (24) was used for both analysis and visualization of phylogenetic tree.

Model Building and Validation—Comparative structural model of Leishmania infantum SepSecS was built using MODELLER version 9.0 (25). The model was energy minimized using AMBER96 force field in GROMACS (26). Stereochemical quality of the model was verified using PROCHECK in the PDBePDB web resource at EBI (27). Structural comparison of the model with human homolog and structural mapping of the active site residues was performed using PyMOL (28).
blue, 10% β-mercaptoethanol and subsequently analyzed on a 6% SDS-PAGE gel.

In Vivo SepSecS Assay—LdSepSecS and M. jannaschii SepSecS (MjSepSecS) genes were individually transformed into E. coli ΔselA deletion strain JS1, with or without the M. jannaschii PSTK gene. Aerobic overnight cultures were streaked on LB agar plates supplemented with 0.5% glucose and 0.01 mM isopropyl β-D-thiogalactopyranoside. The transformants were then grown anaerobically at 37 °C. After growth, the plates were removed from the anaerobic jar and immediately overlaid with 0.75% top agar (containing 1 mg/ml of benzylo viologen, 0.25 M sodium formate, and 25 mM KH$_2$PO$_4$, pH 7.0). The appearance of the blue/purple color is an indication of active FDH$_H$ (10).

Immunofluorescence Microscopy—L. donovani promastigotes were incubated with 1 nM MitoTracker Red CMXRos CNeo and DNeo. The blue/purple color is an indication of active FDH$_H$ (10). The mutants were confirmed by Southern analysis using standard protocols (31). The add-back line ΔSepSecS/+ was created by transfecting the ΔSepSecS null mutant parasites with the pSP72α-zeo-α-SepSecS episome and its generation was confirmed by PCR (data not shown) and Western blot analysis.

Growth and Infection Assay—Growth rate experiments were conducted by inoculating stationary phase parasites at a density of 1 × 10$^6$ cells/ml in M199 medium with 5% FBS in 25-cm$^2$ flasks without the respective selection drug at 22 °C. Growth rate of each culture was determined at 24-h intervals using a Neubauer hemocytometer. Growth studies with individual cell lines were done at least three times and similar results were consistently obtained.

J774.A1 murine macrophage cell line was plated at a density of 5 × 10$^5$ cells/well in a 6-well flat bottom plate. The adherent cells were infected with stationary-phase promastigotes, at a ratio of 20:1 for 5 h. Excess non-adherent promastigotes were removed by incubation of the cells for 30 s in PBS. These were subsequently maintained in RPMI1640 containing 10% FBS at

### Table 1: Primers used for generation of hygromycin (HYG) and neomycin (NEO)-specific linear replacement cassette fragments

| L. donovani primers | Sequence |
|---------------------|----------|
| A                   | 5′-GAGACCTGATCCTTTTGAATCCTGAC-3′ |
| BHYG                | 5′-GCTGAGCTGATTCCTTTTGAATCCTGAC-3′ |
| CNEO                | 5′-GGCTGAGCTGATTCCTTTTGAATCCTGAC-3′ |
| DNEO                | 5′-GGCTGAGCTGATTCCTTTTGAATCCTGAC-3′ |
| EHYG                | 5′-GGCTGAGCTGATTCCTTTTGAATCCTGAC-3′ |
| ENEO                | 5′-GGCTGAGCTGATTCCTTTTGAATCCTGAC-3′ |
| F                   | 5′-GGCTGAGCTGATTCCTTTTGAATCCTGAC-3′ |
| G                   | 5′-GGCTGAGCTGATTCCTTTTGAATCCTGAC-3′ |
| H                   | 5′-GGCTGAGCTGATTCCTTTTGAATCCTGAC-3′ |

### Table 2: Primers used for the molecular characterization of the genetically manipulated parasites by PCR-based analysis

| L. donovani primers | Sequence |
|---------------------|----------|
| Primer 1            | 5′-TGTAAGATATGCGCATATGCGA-3′ |
| Primer 2            | 5′-AGGCTGATTCCTTTTGAATCCTGAC-3′ |
| Primer 3            | 5′-GAGACCTGATCCTTTTGAATCCTGAC-3′ |
| Primer 4            | 5′-TTTGGCCTGATTCCTTTTGAATCCTGAC-3′ |
| Primer 5            | 5′-ATAGGCTGATTCCTTTTGAATCCTGAC-3′ |
| Primer 6            | 5′-AACGCGCGCTGATATGCGA-3′ |
| Primer 7            | 5′-TTTGGCCTGATTCCTTTTGAATCCTGAC-3′ |
| Primer 8            | 5′-AGGCTGATTCCTTTTGAATCCTGAC-3′ |

with primers C$_{HYG}$ and D$_{HYG}$ (Table 1). The 3′-UTR (644 bp) of the LdSepSecS gene was obtained from L. donovani wild-type genomic DNA by PCR amplification using primers E$_{HYG}$/F$_{NEO}$ and reverse primer F (Table 1). The 5′-UTR of the L. donovani SepSecS gene was then ligated to either of the antibiotic resistance marker genes by PCR using primers A and D$_{HYG}$ or A and D$_{NEO}$. This fragment (5′-UTR marker gene) was then fused with 3′-UTR using primers A and F, yielding fragments, 5′-UTR-Hyg-3′UTR or 5′-UTR-Neo-3′-UTR.

To generate episomal complementation construct, the full-length SepSecS coding sequence was amplified with a forward primer harboring the XbaI site (primer G) and reverse primer with the HindIII site (primer H) (Table 1). This amplified product was then cloned in pSp72α-zeo-α vector to get the pSp72α-zeo-α-SepSecS complementation construct. All fragments and constructs were sequenced for confirmation.

### Generation of Genetically Manipulated Parasites—After PCR amplification and purification, ~2 µg of linear replacement cassette fragments 5′-UTR-Hyg-3′UTR or 5′-UTR-Neo-3′UTR were individually transfected by electroporation in wild-type L. donovani promastigotes (30). Depending on the marker gene, transfectants were subjected to antibiotic selection. Cells resistant to antibiotic selection were subjected to a PCR-based analysis to check for the correct integration of the replacement cassettes using the primers shown in Table 2. Therefore, a second round of transfection was initiated to knock out the other copy of the SepSecS gene. The genotypes of the mutants were confirmed by Southern analysis using standard protocols (31). The add-back line ΔSepSecS/+ was created by transfecting the ΔSepSecS null mutant parasites with the pSP72α-zeo-α-SepSecS episome and its generation was confirmed by PCR (data not shown) and Western blot analysis.

### Molecular Constructs for the Replacement of SepSecS Alleles—For inactivation of the LdSepSecS gene, a targeted gene replacement strategy based on PCR fusion was employed (29). Briefly, SepSecS gene flanking regions were amplified and fused using PCR to the hygromycin phosphotransferase gene (HYG) or neomycin phosphotransferase gene (NEO). The 5′-UTR (628 bp) of the LdSepSecS gene was obtained by PCR amplification with primers A and B$_{HYG}$ or primers A and B$_{NEO}$ (Table 1). The NEO gene was amplified from pX63-NEO with primers C$_{NEO}$ and D$_{NEO}$. The HYG gene was amplified from pX63-HYG primer harboring the XbaI site (primer G) and reverse primer with the HindIII site (primer H) (Table 1). This amplified product was then cloned in pSp72α-zeo-α vector to get the pSp72α-zeo-α-SepSecS complementation construct. All fragments and constructs were sequenced for confirmation.

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J774.A1 murine macrophage cell line was plated at a density of 5 × 10$^5$ cells/well in a 6-well flat bottom plate. The adherent cells were infected with stationary-phase promastigotes, at a ratio of 20:1 for 5 h. Excess non-adherent promastigotes were removed by incubation of the cells for 30 s in PBS. These were subsequently maintained in RPMI1640 containing 10% FBS at
37 °C with 5% CO2. Giemsa staining was performed to visualize intracellular parasite load.

Auranofin Inhibition Studies—To determine the auranofin susceptibility profile of *L. donovani* wild-type and mutant promastigotes, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) assay was performed as described previously (32). Briefly, log-phase promastigotes (5 × 10⁴ cells/well) were seeded in a 96-well flat-bottomed plate (Nunc) and incubated with different drug concentrations at 22 °C. Because auranofin was dissolved in dimethyl sulfoxide, a sample without auranofin but with an identical volume of dimethyl sulfoxide served as an additional control. After 72 h of incubation, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml) was added to each well and the plates were incubated at 37 °C for 3 h. The reaction was stopped by the addition of 50 µl of 50% isopropl alcohol and 20% SDS followed by gentle shaking at 37 °C for 30 min to 1 h. Absorbance was measured at 570 nm in a microplate reader (SpectraMax M2 from Molecular Devices). The susceptibility of wild-type and mutant promastigotes to different concentrations of H₂O₂ was also determined after 24 h of exposure as described above. The susceptibility of wild-type and mutant amastigotes to auranofin was determined by visualization of intracellular parasite load using Giemsa staining of the infected J774.A1 murine macrophages, 24 h after treatment with different concentrations of the drug.

²⁷Selenium Labeling—A total of 5 × 10⁹ promastigote cells from each cell line were resuspended in 0.5 ml of M199 media supplemented with FBS. The cultures were labeled with 7 µCi of Hepes-neutralized [²⁷Se]selenite in the presence of 100 µg/ml of cysteine at 22 °C for 12 h. After labeling, the cultures were pelleted and washed with PBS. The resulting pellet was resuspended in sample buffer and heated to 100 °C for 10 min. Finally, the labeled proteins (~10⁹ cell eq) were analyzed on 12% SDS-PAGE gel and visualized with the help of a Phosphor-Imager (Fujifilm).

Results

Sequence Analysis and Phylogeny—Multiple sequence alignment of the kinetoplastid SepSecS homologs with representative sequences from other eukaryotes (such as human, mouse, and plasmodium) and archaea (Fig. 2) suggests the complete conservation of all the three active site arginines and Schiff-base forming lysine. Other PLP binding residues are all conserved or conservatively substituted. Like human and mouse SepSecS sequences, *Trypanosoma cruzi* SepSecS also contains an autoantigenic insertion at its C terminus. The catalytic and PLP binding residues were mapped based on the structural comparison study of eukaryotic and archaeal SepSecS (8). Interestingly, *Leishmania braziliensis* SepSecS contains a long N-terminal insertion absent in all other kinetoplastid and eukaryotic sequences. Subcellular localization prediction using MitoProtII (33), WoLF PSORT (34), suggest a clear mitochondrial targeting signal peptide in *L. braziliensis* (Fig. 2). Eukaryote-specific insertion sequence (INS2 in Fig. 2) is also highly conserved within the *Leishmania* species. This insertion is longer in all *Plasmodium* species. Within the kinetoplastid sequences, *Leishmania* species has certain insertion regions (INS1 in Fig. 2) that are absent in Trypanosomes. These *Leishmania*-specific sequence insertions are closer to the PLP binding residues. However, it would be interesting to see whether they are structurally any closer to the PLP binding site and the enzyme active site. Among the PLP binding residues other than the Schiff-base lysine, H166A and N247A mutations (archaea) have been shown to yield partially active enzymes (8). Although Asn²⁴⁷ (archaea) is strictly conserved in *Leishmania*; Asn²³⁹, His²¹⁶ is substituted as Gln²¹₂. Based on the structural comparison of archaean, eukaryotic SepSecS with other Fold type I PLP binding enzymes, it was argued that a strictly conserved archaean Pro²⁴⁹, which is substituted by Cys²¹⁵ (human), is an important site for mutagenesis to verify the participation of Cys²¹⁵ in the formation of perselenide intermediate (8) in eukaryotes. Although, in archaean SepSecS a conserved cysteine is in the active site, in human Cys²¹⁵, which is conserved within eukaryotes and is within 6-Å radius closer to the PLP binding site was thought to be a probable site for mutation to verify its participation in active site formation (8). However, in kinetoplastids, this residue is mutated to Ala²²⁶ by natural selection to result in an active enzyme debarring the participation of Cys²¹⁵ in active site formation in human. It can be argued to look for the next sequentially closer cysteine in active site formation. In *Ld*SepSecS, Cys²²⁹ (which is a Ser⁷⁷ in human), which is sequentially and structurally closer to Ala²²⁶ is 14-Å distance from the active site lysine. This further confirms the fact that unlike archaean SepSecS enzymes, which contain one active site cysteine to employ a perselenide formation mechanism, eukaryotes do not employ perselenide mechanism.

A comprehensive multiple sequence alignment of homologs from all domains of life (metazoa, amoebozoa, kinetoplastida, plasmodia, archaea, viridiplantae, algae, and worm) is used for phylogenetic analysis. MEGA v5 (24) is used for generation and analysis of the phylogenetic tree. A neighbor joining bootstrap tree of the SepSecS sequences (Fig. 3) clearly shows that the kinetoplastid SepSecS enzymes are phylogenetically closer to worm SepSecS, whereas the metazoan enzymes are closer to fly and plant SepSecS enzymes. The *Plasmodium* and *L. braziliensis* SepSecS could not be retrieved by a simple BLAST search in the EupathDB database (35, 36) using the human SepSecS query sequence. Although, *Plasmodium* homologs are retrieved by a specific BLAST search in PlasmoDB database, the *L. braziliensis* SepSecS is retrieved by orthology to other *Leishmania* species. Hence, the fast evolving *Plasmodium* SepSecS enzymes have a longer branch length than their eukaryotic counterparts (Fig. 3).

Structural Modeling and Comparison—To date crystal structures of SepSecS from human, mouse, and archaea are known. Among the three, the crystal structure of human SepSecS is closer to *Ld*SepSecS. Hence, a structural model of *Ld*SepSecS was built using human SepSecS (Protein Data Bank code 3HL2) as the template using MODELLER version 9.0 (25). The model was energy minimized using the AMBER96 force field in GROMACS (26). The quality of the model was then verified using PROCHECK at the PDBSUM web server (27). Structural comparison of the *Ld*SepSecS model with the human homolog using DALI (37) resulted in a best structural superposition with a Z-score of 49.8 and root mean square deviation of 0.6 Å.
structure-based sequence identity for the superposition of 43 amino acids was 43% between \( Ld \) and the human homolog. The three-dimensional structural comparison of the human and \( Ld \) enzymes. tRNAsec is anchored by a key interaction between the discriminator base and Arg398 in human SepSecS. A mutation of this Arg398 to alanine or glutamate has been shown to make the enzyme inactive (4). This catalytically important arginine is conserved as Arg527 in \( Ld \) SepSecS. To further investigate the catalytic mechanisms of \( Ld \) SepSecS, multiple sequence alignments and structural superposition were performed using CLUSTALW (22). The PLP binding lysine and active site arginine residues are highlighted in pink and green, respectively. Other PLP binding residues are highlighted in blue. The mitochondrial signal peptide of \( L. braziliensis \) SepSecS predicted using MITOPROT II (33) is highlighted in a gray background. The autoimmune antigenic region of human SepSecS (47) and a eukaryote-specific insertion “INS2” are highlighted in yellow.

The three-dimensional structural comparison of the human and \( Ld \) SepSecS monomers with the tRNA (Fig. 4A) shows the conservation of the active site arginines (\( Ld \) SepSecS: Arg75, Arg97, and Arg400) and the PLP binding Schiff-base forming Lys371. The phosphoserine binding P-loop is also structurally conserved between the human and \( Ld \) enzymes. tRNAsec is anchored by a key interaction between the discriminator base and Arg398 in human SepSecS. A mutation of this Arg398 to alanine or glutamate has been shown to make the enzyme inactive (4). This catalytically important arginine is conserved as Arg527 in \( Ld \) SepSecS. To further investigate the catalytic mechanisms of \( Ld \) SepSecS, multiple sequence alignments and structural superposition were performed using CLUSTALW (22). The PLP binding lysine and active site arginine residues are highlighted in pink and green, respectively. Other PLP binding residues are highlighted in blue. The mitochondrial signal peptide of \( L. braziliensis \) SepSecS predicted using MITOPROT II (33) is highlighted in a gray background. The autoimmune antigenic region of human SepSecS (47) and a eukaryote-specific insertion “INS2” are highlighted in yellow.

![Figure 2. Multiple sequence alignment of representative SepSecS sequences from kinetoplastids, metazoan, archaeal, and Plasmodium species generated using CLUSTALW (22). The PLP binding lysine and active site arginine residues are highlighted in pink and green, respectively. Other PLP binding residues are highlighted in blue. The mitochondrial signal peptide of \( L. braziliensis \) SepSecS predicted using MITOPROT II (33) is highlighted in a gray background. The autoimmune antigenic region of human SepSecS (47) and a eukaryote-specific insertion “INS2” are highlighted in yellow.](image-url)
mode of multimer formation, the monomeric LdSepSecS model was submitted to the SymmDock server (38) for tetramer generation. The LdSepSecS tetramer (Fig. 4B) was then compared with the human SepSecS tetramer (Fig. 4C) structure (Protein Data Bank code 3HL2) bound to tRNAsec. Although, the binding of tRNAsec induces a conformational change at the active site of the human enzyme, the dimer interface of the human and Leishmania enzymes seem quite different (Fig. 4B and C). Although in human, two active sites are formed at the dimer interface (Fig. 4C) by the interaction of two monomer units, in case of Leishmania, the interaction of monomer units are predicted to result in a single active site at the dimer interface (Fig. 4B). The tetramer interface in the human enzyme is formed by interactions between the N-terminal...
α1-loop-α2 motif (8). However, in *Leishmania*, there is an N-terminal extension that protrudes away from the monomer structure and does not participate essentially in tetramer formation. Thus, although the human and the *Leishmania* homologs are structurally highly similar but their monomer association modes leading to tetramerization seem different. Although the huge insertions in the *Leishmania* homolog might account for this varied association modes of the monomers, it would be worth investigating if tRNAsec binding brings these conformational changes to the *Leishmania* tetramer.

Overexpression and Purification of Leishmania Selenocysteinyl-tRNA Synthase—The *LdSepSecS* gene coding sequence was cloned into the bacterial expression vector pET30a. Histidine-tagged fusion protein with an estimated molecular mass of 70 kDa was induced; the size was correlated with the amino acid composition of the *LdSepSecS* protein (64 kDa) with a His6 tag (6 kDa) at the N terminus (Fig. 5). Purification of *LdSepSecS* by metal affinity chromatography yielded 1 mg of purified protein/liter of bacterial culture. As expected, the recombinant protein (r*LdSepSecS*) was recognized by anti-His tag.
monoclonal antibody (Fig. 5B). To further characterize rLdSepSecS, the purified protein was analyzed by MALDI-TOF/TOF mass spectroscopy (data not shown). The spectrum of the protein analyzed by BioTools version 2.2 showed intensity coverage of 60% for the SLA/LP autoantigen-like protein (L. infantum JPCM5). A sequence search analysis by a Mascot search revealed a top score of 217 with SLA/LP autoantigen-like protein of Leishmania spp. with accession number gi 146078497 (data not shown).

Human and archaeal SepSecS homologs are known to exist as tetramers. It has been reported that two homo-dimers associate into a tetramer through interactions between the N-terminal α-loop-α2 motifs (4, 9). Glutaraldehyde cross-linking studies demonstrated that the rLdSepSecS protein exists as a tetramer in solution as a band of ~280 kDa appeared on resolving the cross-linked protein on a SDS-PAGE gel (Fig. 5C). As reported in the case of human and archaeal homologs, LdSepSecS was observed to associate as a dimer at 140 kDa, which was...
further associated to form a tetramer at 280 kDa (Fig. 5C, lane 2).

**LdSepSecS** **Rescues Selenoprotein Biosynthesis in an E. coli ΔselA Deletion Strain**—E. coli produces the selenium-dependent formate dehydrogenase H (FDH₄) enzyme when grown under anaerobic conditions. Active FDH₄ can use formate to reduce benzyl viologen, which results in blue/purple colored colonies (7). Selenoprotein production is abolished in the E. coli/H9004 selA deletion strain JS1. Complementation of the JS1 strain with archaeal SepSecS and PSTK genes from M. jannaschii (MJ0610) has been reported to restore selenoprotein biosynthesis, as detected by FDHH activity (10). Transformants grown anaerobically were layered with top agar containing formate and benzyl viologen. Complementation of the E. coli/H9004 selA deletion was observed when strain JS1 was either co-transformed with the MjPSTK and MjSepSecS genes or with MjPSTK and LdSepSecS genes (Fig. 5D). Transformation of the LdSepSecS gene alone did not restore FDHH activity in the E. coli JS1 strain (Fig. 5D). This data implies that MjPSTK phosphorylates the endogenous seryl-tRNA⁰ to O-phosphoseryl-tRNA⁰ and then this tRNA species is converted to Sec-tRNA⁰ by LdSepSecS. Thus, the Leishmania SepSecS enzyme was found to be active in E. coli.

**Subcellular Localization of LdSepSecS**—To ascertain the localization of SepSecS in L. donovani, immunofluorescence analysis was carried out with log phase promastigotes stained with anti-LdSepSecS antibody, MitoTracker Red, and DAPI. *In silico* analysis led to the detection of a mitochondrial targeting signal peptide only in L. braziliensis. Hence, the LhrSepSecS appears to be a mitochondrial targeting protein. Although all other Leishmania species (including L. donovani) do not carry a detectable signal peptide. Analysis by confocal microscopy revealed that LdSepSecS is predominantly localized in the cytoplasm of the parasite (Fig. 6). No substantial co-localization was found with MitoTracker Red, which specifically stains the mitochondria. Controls performed with mouse preimmune sera, non-permeabilized cells, and secondary antibody alone showed no detectable signal (data not shown).

**Gene Deletion Studies of Selenocysteinyl-tRNA Synthase in L. donovani**—To determine the essentiality and function of LdSepSecS in the life cycle of the parasite, targeted gene replacement strategy was used to replace both alleles of this gene with cassettes harboring drug-resistance marker genes. This was achieved by generation of inactivation cassettes having hygromycin phosphotransferase (HYG) or neomycin phosphotransferase (NEO) as selection markers along with 5'-UTR and 3'-UTR.
The neighbor joining Bootstrap tree was constructed using MEGA v5. Bootstrap values >90 are shown in the phylogenetic tree. For analysis, we used SPCS_DANRE, SPCS_XENTR, SPCS_DICDI, SPCS_PONAB, SPCS_HUMAN, SPCS_MOUSE, SPCS_CAEBR, SPCS_CAEL, SPCS_METKA, SPCS_METJA, SPCS_METMP, Sj_0020410.1; Smp_164050.1; LdBPK_091000.1; LinJ.09.1000; LmjF.09.0950; LmxM.09.0950; LtaP09.0960; LbrM.09.1000; TcIL3000_0_57180; B3P2G3_DROER; B4JST2_DROGR.

To test whether selenium is incorporated into Leishmania proteins—to test whether selenium is incorporated into Leishmania proteins by selenocysteinyl-tRNA synthase, promastigotes were subjected to 3'-UTR of the SepSecS gene, as described under “Experimental Procedures.” Linear replacement cassettes made by PCR-based fusion were transfected into wild-type L. donovani promastigotes, leading to generation of heterozygous parasites (SepSecS/HYG and SepSecS/NEO). Replacement of a single copy of the LD-SepSecS gene was confirmed by PCR-based analysis by using primers external to the transfected inactivation cassette (data not shown). The heterozygous parasites were subsequently transfected with the other cassette to replace the second copy of the gene. PCR analysis demonstrated that cells selected on the double antibiotic medium showed 1.0- and 1.2-kb bands in the case of the HYG cassette, 1.0- and 1.3-kb bands in the case of NEO cassette (Fig. 7, A and B). No bands corresponding to the WT gene (1.38- and 1.34-kb) were obtained in this case (Fig. 7, A and B). This confirmed that both alleles of the WT LD-SepSecS gene had been replaced in these homozygous null mutant parasites (∆SepSecS).

The Western blot analysis with polyclonal antiserum to rLD-SepSecS recognized a single specific band of 64 kDa in L. donovani whole cell lysate (Fig. 7D, lane 3). Western blot analysis also confirmed generation of the LD-SepSecS mutant parasites. A band corresponding to the LD-SepSecS protein at ~64 kDa was obtained in case of WT and add-back line ∆SepSecS/+ whereas no band could be detected in the ∆SepSecS parasites with anti-LD-SepSecS polyclonal antibody (Fig. 7D). The band corresponding to the recombinant LD-SepSecS protein (~70 kDa) was used as a control (Fig. 7D).

To analyze the growth pattern of genetically manipulated promastigote cell lines, cell counting was performed using a hemocytometer for a period of 7 days after an interval of 24 h. Absence of SepSecS in the parasite marginally and insignificantly impaired ∆SepSecS promastigote growth rate compared with the WT parasites (Fig. 7E). The heterozygous null mutant parasites proliferated at a similar rate as that of the wild-type promastigotes (data not shown). To ascertain whether a genetic deficiency of SepSecS in L. donovani may have an impact on its ability to infect host cells, the infectivity assay with stationary-phase promastigotes was performed in J774.A1 murine macrophages. ∆SepSecS parasites were observed to be capable of infecting and sustaining robust infection in murine macrophages at levels comparable with those obtained with wild-type parasites (Fig. 7F).

**FIGURE 7.** Selenium Incorporation into Leishmania Proteins—To test whether selenium is incorporated into Leishmania proteins by selenocysteinyl-tRNA synthase, promastigotes were subjected to growth and infectivity assays with stationary-phase promastigotes. Arial font size 10.

**FIGURE 3.** Sequence-based phylogeny of SepSecS homologs from kinetoplastids, algae, viridiplantae, metazoa, worm, fly, Plasmodium, and archaea. The neighbor joining Bootstrap tree was constructed using MEGA v5. Bootstrap values >90 are shown in the phylogenetic tree. For analysis, we used SPCS_DANRE, SPCS_XENTR, SPCS_DICDI, SPCS_PONAB, SPCS_HUMAN, SPCS_MOUSE, SPCS_CAEBR, SPCS_CAEL, SPCS_METKA, SPCS_METJA, SPCS_METMP, Sj_0020410.1; Smp_164050.1; LdBPK_091000.1; LinJ.09.1000; LmjF.09.0950; LmxM.09.0950; LtaP09.0960; LbrM.09.1000; TcIL3000_0_57180; B3P2G3_DROER; B4JST2_DROGR.

FIGURE 5. A, purification of recombinant \( Ld \)SepSecS protein on \( \text{Ni}^{2+} \)-nitrilotriacetic acid affinity resin. \( M \), molecular weight marker; \( \text{Lane 1} \), uninduced cell lysate; \( \text{lane 2} \), induced cell lysate; \( \text{lane 3} \), eluted fraction with 300 mM imidazole showing purified \( Ld \)SepSecS. B, Western blot analysis of recombinant \( Ld \)SepSecS protein with anti-His tag mouse antibody (1:3000). \( M \), molecular weight marker. \( \text{Lanes 1}–\text{4} \), decreasing concentration of purified \( rLd \)SepSecS. C, SDS-PAGE analysis of glutaraldehyde cross-linked recombinant \( Ld \)SepSecS protein. \( M \) represents molecular weight marker. \( \text{Lane 1} \), sample with no glutaraldehyde (represents \( Ld \)SepSecS monomer); \( \text{lanes 2}–\text{4} \) represent incubation times of 30 s, 2 min, and 10 min, respectively, with 2 \( \mu \)l of 5% freshly prepared glutaraldehyde solution. D, \( E. \text{coli} \) \( \Delta \text{selA} \) deletion strain JS1 complemented with the \( Ld \)SepSecS gene with or without the \( M. \text{jannaschii} \) \( \text{PSTK} \) (\( Mj \) PSTK) gene. Complementation together with \( M. \text{jannaschii} \) \( \text{SepSecS} \) and \( \text{PSTK} \) genes was used as a positive control. The transformants were grown anaerobically and the plates overlaid with the top agar containing benzyl viologen. Appearance of the blue/purple color indicates active FDH\(_\text{a} \).

FIGURE 6. Localization of \( Ld \)SepSecS in \( L. \text{donovani} \). Immunofluorescence analysis by confocal micrograph of wild-type log phase promastigotes stained with DAPI (panel 1), anti-\( Ld \)SepSecS antibody detected using Alexa 488 (green)-conjugated secondary antibody (panel 2), and MitoTracker Red CMXRos (panel 4). Panels 3 and 5, merged micrographs, and panel 6, phase-contrast image. \( k \) and \( n \) indicate kinetoplastid and nuclear DNA, respectively. The scale bar represents 10 \( \mu \)m.
to metabolic labeling with $^{75}$Se. Subsequently, the cell extracts from various cell lines were separated on a polyacrylamide gel and the $^{75}$Se profile was visualized using a PhosphorImager. A major $^{75}$Se-containing low molecular weight band was detected at the bottom of the gel (Fig. 8). This band corresponded to the predicted molecular mass of SelK ($^{9.5}$ kDa) (Fig. 8). A second band detected around $^{25}$ kDa appeared to represent SelT (Fig. 8A). However, very long exposure (20 days) to a PhosphorImager screen revealed a faint high molecular mass band that migrated in accordance with the predicted molecular mass of SelTryp, $^{89}$ kDa (12) (Fig. 8B). $^{75}$Se-containing bands corresponding to the three selenoproteins were absent in the cell lysate of $^{SepSecS}$ null mutant parasites but were identified in the case of the add-back line $^{SepSecS/+}$. Metabolic labeling of $Leishmania$ cells with $^{75}$Se revealed specific incorporation of this radioisotope into a defined set of proteins.

**Auranofin Treatment of Wild-type and $^{SepSecS}$ Null Mutants**—Auranofin has been reported to interfere with selenium metabolism and inhibit selenoprotein synthesis by formation of an auranofin-selenide adduct (39, 40). $Trypanosoma brucei$ cells have been found to be sensitive to nanomolar concentrations of auranofin (12). Based on these observations, it was proposed that selenoprotein synthesis might be a novel target for development of an anti-parasitic drug in trypanosomes (12). However, auranofin is known to interact with both selenol and thiol groups (41). To test this conjecture and elucidate its action, the effect of auranofin was determined in wild-type and $^{SepSecS}$ null mutant promastigotes. Auranofin was
found to cause growth inhibition of both the wild-type and null mutant promastigotes (Fig. 9A); thereby suggesting its effect here is not through binding to selenoproteins. Effect of auranofin on amastigotes of wild-type and ΔSepSecS null mutant was also determined and no difference in susceptibility of these cell lines to auranofin was observed (Fig. 9C). Taken together, this data indicates that auranofin does not inhibit selenoproteins in *L. donovani*. Similar results have been reported for *T. brucei*, wherein the same sensitivity toward auranofin was observed in both the WT and SepSecS null mutant procyclic and bloodstream forms (42).

To verify the potential redox functions of selenoproteins, the effect of H₂O₂-induced oxidative stress conditions were assessed on *L. donovani* WT and mutant promastigotes. Increasing the concentration of H₂O₂ had an inhibitory effect on the survival of WT as well as null mutant promastigotes at levels that were comparable (Fig. 9B). This result indicates that selenoproteins do not confer protection against added H₂O₂-induced oxidative stress conditions.

**Discussion**

The presence of the machinery required for selenocysteine incorporation into proteins has been reported in Trypanosomes (10–13). Selenocysteine synthase or O-phosphoseryl-tRNA:SepSecS in eukaryotes and archaea is the terminal enzyme of this pathway that converts O-phosphoseryl-tRNASec into Sec-tRNASec using selenophosphate as the selenium donor compound (8).

In the present study, we for the first time report the characterization of selenocysteinyl-tRNA synthase from *L. donovani*. The ORF of *L. donovani* selenocysteinyl-tRNA synthase (*LdSepSecS*) encodes a polypeptide of 595 amino acids. As is the case in mammals and archaea (4, 8, 9), *LdSepSecS* was also found to exist as a tetramer. Leishmania SepSecS enzyme was found to be active and able to complement ΔselA deletion in the *E. coli* JS1 strain. However, this complementation could only be achieved in the presence of an archaeal PSTK, indicating the conserved nature of the PSTK-SepSecS pathway of Sec-tRNA²Sec formation in archaea and eukaryotes. *LdSepSecS* was found to localize in the cytoplasm of the parasite. Expression of
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LdSepSecS was found to be higher in early log phase promastigotes and the protein could not be detected in stationary phase parasites (data not shown). The absence of the protein in the stationary phase may be attributed to the process of metacyclogenesis, during which a lower amount of total protein content associated with a decrease in protein synthesis has been previously reported (43).

An attempt to disrupt LdSepSecS from the genome of the parasite was successful and did not lead to any significant difference in the phenotype of the mutants when compared with the wild-type parasites. Similar results have been previously reported in case of T. brucei, where the pathway for selenocysteine insertion was found to be dispensable (10, 42).

Our 75Se labeling study demonstrates the presence of three selenoproteins corresponding to the kinetoplastid homologs of SelK (9.5 kDa), SelT (28.8 kDa), and SelTryp (89 kDa), respectively (Fig. 8). 75Se labeling studies carried out with wild-type and LdSepSecS null mutants helped in ascertaining a role for LdSepSecS in selenoprotein synthesis in Leishmania as no selenoproteins could be detected in the lysate of the null mutant parasites. This is consistent with earlier studies in Trypanosoma where the presence of the three selenoproteins corresponding to the predicted molecular masses of the kinetoplastid homologs, SelK (9.5 kDa), SelT (28.8 kDa), and SelTryp (89 kDa) have been reported (10, 12). Cassago et al. (11) had earlier reported that the genes for glutathione peroxidase, selenoprotein R and selenoprotein SPS2 homologues in Leishmania have evolved into nonselenium-containing proteins.

The three selenoproteins have been reported to contain putative redox centers (CXXU in the case of SelT, SelTryp, and CXXXU in SelK) and it was suggested that impairment of their function in maintaining redox balance may influence kinetoplastid infections (12). However, comparison of the infectivity profile of the WT and LdSepSecS null mutant parasites and their susceptibility to H2O2 established that there is no such significant contribution of these selenoproteins to redox homeostasis having a plausible consequence in influencing infection in J774.A1 macrophages. These results are similar to those obtained in T. brucei, where both the WT and SepSecS knock-out cell line had similar sensitivity to H2O2-induced oxidative stress (42). Function of these three selenoproteins in Leishmania needs to be experimentally deciphered.

Auranofin is a FDA approved anti-rheumatic drug found to be effective against Entamoeba histolytica, Mycobacterium tuberculosis, and many Gram-positive pathogens (44, 45). To unveil the mechanism of action of auranofin in Leishmania, its sensitivity was assessed in both WT and SepSecS null mutants. No difference was observed in sensitivity toward auranofin in both WT and SepSecS null mutants. This suggests that the leishmanicidal action of auranofin is not due to inactivation of selenoproteins. Auranofin has been reported to interact with both selenol and thiol groups (41). Earlier reports on the x-ray crystal structure of the auranofin-trypathione reductase-NADPH complex shows that auranofin appears to inhibit L. infantum trypanothione reductase with a dual mechanism by blocking both binding and reduction of oxidized trypanothione (46).

Our results indicate that Leishmania utilizes the SepSecS enzyme to make three selenoproteins. However, absence of this enzyme in the parasite does not confer it with any altered phenotype as assessed in vitro under standard laboratory conditions. The selenocysteine insertion mechanism appears to be dispensable for the parasite but this raises the question that if it has not been lost during the course of evolution, then it definitely is required at some juncture in the life cycle of the parasite. Selenoproteins are probably required at specific time points and perform functions that are still ambiguous. The manifestation of these functions in the amastigote stage also cannot be concluded as the parasites lacking SepSecS were able to competently infect and survive in host macrophages akin to their wild-type counterparts.

In conclusion, Leishmania encodes an active selenocysteinyl-tRNA synthase that helps in inserting selenocysteine into at least three selenoproteins. The selenocysteine incorporation pathway in Leishmania appears to be conserved and requires PSTK enzyme as in the case of other eukaryotes and archaean. Although selenoproteins are dispensable for the survival of the parasite under standard growth conditions, their presence and the conservation of the enzyme involved in selenocysteine incorporation during evolution point to their specific roles that await experimental studies and validation.

Author Contributions—R. M. conducted most of the experiments. V. S. G. conducted the in silico analysis. R. M. B. designed the study, supervised the experiments, and edited the manuscript with contributions from all other authors. All authors analyzed the results and approved the final version of the manuscript.

Acknowledgments—We are grateful to Dr. Dieter Söll for the E. coli strain and plasmids. We thank the Central Instrumentation Facility at School of Life Sciences, Jawaharlal Nehru University, for MALDI-TOF analysis and for providing imaging facility. We also thank Dr. Marc Ouellette (University of Laval, Quebec, Canada) for providing Leishmania shuttle vector.

References

1. Turanov, A. A., Xu, X. M., Carlson, B. A., Yoo, M. H., Gladyshev, V. N., and Hatfield, D. L. (2011) Biosynthesis of selenocysteine, the 21st amino acid in the genetic code, and a novel pathway for cysteine biosynthesis. Adv. Nutr. 2, 122–128
2. Lobanov, A. V., Hatfield, D. L., and Gladyshev, V. N. (2009) Eukaryotic selenoproteins and selenoproteomes. Biochim. Biophys. Acta 1790, 1424–1428
3. Lu, J., and Holmgren, A. (2009) Selenoproteins. J. Biol. Chem. 284, 723–727
4. Ganiychkin, O. M., Xu, X. M., Carlson, B. A., Mix, H., Hatfield, D. L., Gladyshev, V. N., and Wahl, M. C. (2008) Structure and catalytic mechanism of eukaryotic selenocysteine synthase. J. Biol. Chem. 283, 5849–5865
5. Small-Howard, A., Morozova, N., Stoytcheva, Z., Forry, E. P., Mansell, J. B., Harney, J. W., Carlson, B. A., Xu, X. M., Hatfield, D. L., and Berry, M. J. (2006) Supramolecular complexes mediate selenocysteine incorporation in vivo. Mol. Cell. Biol. 26, 2337–2346
6. Xu, X. M., Carlson, B. A., Mix, H., Zhang, Y., Saira, K., Glass, R. S., Berry, M. J., Gladyshev, V. N., and Hatfield, D. L. (2007) Biosynthesis of selenocysteine on its tRNA in eukaryotes. PLoS Biol. 5, e4
7. Yuan, J., Palourea, S., Salazar, J. C., Su, D., O’Donoghue, P., Hohn, M. J., Cardoso, A. M., Whitman, W. B., and Söll, D. (2006) RNA-dependent
conversion of phosphoserine forms selenocysteine in eukaryotes and archaea. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 18923–18927

8. Araiso, Y., Paloura, S., Ishitani, R., Sherrer, R. L., O’Donoghue, P., Yuan, J., Oshikane, H., Domae, N., Defranco, I., Söll, D., and Nureki, O. (2008) Structural insights into RNA-dependent eukaryal and archaeal selenocysteine formation. *Nucleic Acids Res.* **36**, 1187–1199

9. Paloura, S., Sherrer, R. L., Steitz, T. A., Söll, D., and Simonovic, M. (2009) The human SepSecS-tRNAsec complex reveals the mechanism of selenocysteine formation. *Science* **325**, 321–325

10. Aebi, E., Paloura, S., Pusnik, M., Marazzi, J., Lieberman, A., Ullu, E., Söll, D., and Schneider, A. (2009) The canonical pathway for selenocysteine insertion is dispensable in Trypanosomes. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 5088–5092

11. Cassago, A., Rodrigues, E. M., Prieto, E. L., Gaston, K. W., Alfonso, J. D., Iribar, M. P., Berry, M. J., Cruz, A. K., and Thiemann, O. H. (2006) Identification of Leishmania selenoproteins and SECIS element. *Mol. Biochem. Parasitol.* **149**, 128–134

12. Lobanov, A. V., Gromer, S., Salinas, G., and Gladyshev, V. N. (2006) Selenium metabolism in *Trypanosoma*: characterization of selenoproteomes and identification of a Kinetoplastida-specific selenoprotein. *Nucleic Acids Res.* **34**, 4012–4024

13. Sculaccio, S. A., Rodrigues, E. M., Cordeiro, A. T., Magalhães, A., Braga, A. L., Alberto, E. E., and Thiemann, O. H. (2008) Selenocysteine incorporation in Kinetoplastid: selenophosphate synthetase (SELD) from *Leishmania major* and *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **162**, 165–171

14. Shchedrina, V. A., Everley, R. A., Zhang, Y., Gygi, S. P., Hatfield, D. L., and Gladyshev, V. N. (2011) Selenoprotein K binds multiprotein complexes and is involved in the regulation of endoplasmic reticulum homeostasis. *J. Biol. Chem.* **286**, 42937–42948

15. Grumolato, L., Ghzili, H., Montero-Hadjadje, M., Gasman, S., Lesage, J., Tanguy, Y., Galas, L., Alt-Ali, D., Leprince, J., Guérineau, N. C., Elkahlon, A. G., Fournier, A., Vieau, D., Vaudry, H., and Anouar, Y. (2008) Selenoprotein T is a PACAP-regulated gene involved in intracellular Ca2+ mobilization and neuroendocrine secretion. *FASEB J.* **22**, 1756–1768

16. Sengupta, A., Carlson, B. A., Labunskyy, V. M., Gladyshev, V. N., and Hatfield, D. L. (2009) Selenoprotein T deficiency alters cell adhesion and elevates selenoprotein W expression in murine fibroblast cells. *Biochem. Cell Biol.* **87**, 953–961

17. Gowri, V. S., Ghosh, L., Sharma, A., and Madhubala, R. (2012) Unusual domain architecture of aminoacyl-tRNA synthetases and their paralogs from *Leishmania major*. BMC Genomics **13**, 621

18. Aslett, M., Aurrecoechea, C., Berriman, M., Brestelli, J., Brunk, B. P., Carrington, M., Depledge, D. P., Fischer, S., Gajria, B., Gao, X., Gardner, M. J., Gingle, A., Grant, G., Harb, O. S., Heiges, M., Hui, S., Iodice, J., Kissinger, J. C., Kraemer, E. T., Li, W., Pinney, D. F., Pitts, B., Roos, D. S., Srinivasamoorthy, G., Stoeckert, C. J., Jr., Wang, H., and Warrenfeltz, S. (2013) EuPathDB: the eukaryotic pathogen database. *Nucleic Acids Res.* **41**, D684–691

19. Aurrecoechea, C., Barreto, A., Brestelli, J., Brunk, B. P., Cade, S., Doherty, R., Fischer, S., Gajria, B., Gao, X., Gingle, A., Grant, G., Harb, O. S., Heiges, M., Hui, S., Iodice, J., Kissinger, J. C., Kraemer, E. T., Li, W., Pinney, D. F., Pitts, B., Roos, D. S., Srinivasamoorthy, G., Stoeckert, C. J., Jr., Wang, H., and Warrenfeltz, S. (2013) EuPathDB: a portal to eukaryotic pathogen databases. *Nucleic Acids Res.* **38**, D415–419

20. Holm, L., and Rosenström, P. (2010) Dali server: conservation mapping in 3D. *Nucleic Acids Res.* **38**, W545–549

21. Schneidman-Duhovny, D., Inbar, Y., Nussinov, R., and Wolfson, H. J. (2005) PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucleic Acids Res.* **33**, W363–367

22. Jackson-Rosario, S., Cowart, D., Myers, A., Tarrien, R., Levine, R. L., Scott, R. A., and Self, W. T. (2009) Auranofin disrupts selenium metabolism in *Clostridium difficile* by forming a stable Au-Se adduct. *J. Biol. Inorg. Chem.* **14**, 507–519

23. Talbot, S., Nelson, R., and Self, W. T. (2008) Arsenic trioxide and auranofin inhibit selenoprotein synthesis: implications for chemotherapy for acute promyelocytic leukaemia. *Br. J. Pharmacol.* **154**, 940–948

24. Di Sarra, F., Fresch, B., Bini, R., Saielli, G., and Bagno, A. (2013) Reactivity of auranofin with selenols and thiols: implications for the anticancer activity of gold(I) compounds. *Eur. J. Inorg. Chem.* **2013**, 2718–2727

25. Aebi, E., Seidel, V., and Schneider, A. (2009) The selenoproteome is dispensable in bloodstream forms of *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **168**, 191–193

26. Bates, P. A. (1994) Complete developmental cycle of *Leishmania mexicana* in axenic culture. *Parasitology**108**, 1–9

27. Debnath, A., Parsonage, D., Andrade, R. M., He, C., Cobo, E. R., Hirata, K., Chen, S., García-Rivera, G., Orozco, E., Martínez, M. B., Gunatilleke, S. S., Barrios, A. M., Arkin, M. R., Poole, I. B., McKerrow, J. H., and Reed, S. L. (2012) A high-throughput drug screen for *Entamoeba histolytica* identifies a new lead and target. *Nat. Med.* **18**, 956–960
Selenocysteinyl-tRNA Synthase of L. donovani

45. Harbut, M. B., Vilchèze, C., Luo, X., Hensler, M. E., Guo, H., Yang, B., Chatterjee, A. K., Nizet, V., Jacobs, W. R., Jr., Schultz, P. G., and Wang, F. (2015) Auranofin exerts broad-spectrum bactericidal activities by targeting thiol-redox homeostasis. Proc. Natl. Acad. Sci. U.S.A. 112, 4453–4458

46. Ilari, A., Baiocco, P., Messori, L., Fiorillo, A., Boffi, A., Gramiccia, M., Di Muccio, T., and Colotti, G. (2012) A gold-containing drug against parasitic polyamine metabolism: the X-ray structure of trypanothione reductase from Leishmania infantum in complex with auranofin reveals a dual mechanism of enzyme inhibition. Amino Acids 42, 803–811

47. Herkel, J., Heidrich, B., Nieraad, N., Wies, I., Rother, M., and Lohse, A. W. (2002) Fine specificity of autoantibodies to soluble liver antigen and liver/pancreas. Hepatology 35, 403–408