Leishmania major Elongation Factor 1B Complex Has Trypanothione S-Transferase and Peroxidase Activity*

Received for publication, July 14, 2004, and in revised form, August 12, 2004

Published, JBC Papers in Press, August 18, 2004, DOI 10.1074/jbc.M407958200

Tim J. Vickers, Susan Wyllie, and Alan H. Fairlamb‡

From the Division of Biological Chemistry and Molecular Microbiology, Wellcome Trust Biocentre, School of Life Sciences, University of Dundee, Dundee, DD1 5EH, Scotland, United Kingdom

In the Trypanosomatidae, trypanothione has subsumed many of the roles of glutathione in defense against chemical and oxidant stress. Crithidia fasciculata lacks glutathione S-transferase, but contains an unusual trypanothione S-transferase activity that is associated with eukaryotic translation elongation factor 1B (eEF1B). Here we describe the cloning, expression, and reconstitution of the purified α, β, and γ subunits of eEF1B from Leishmania major. Individual subunits lacked trypanothione S-transferase activity. Only eEF1B, formed by reconstitution or co-expression of the three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathionyl-three subunits, was able to conjugate a variety of electrophilic substrates to trypanothione or glutathione. In contrast to the C. fasciculata eEF1B, the L. major enzyme also displayed peroxidase activity against a variety of organic hydroperoxides. The enzyme showed no activity with hydrogen peroxide and greatest activity with linoleic acid hydroperoxide (1 unit mg⁻¹). Kinetic studies suggest a ternary complex mechanism, with Kₘ values of 140 μM for trypanothione and 7.4 mM for cumene hydroperoxide and k_cat = 25 s⁻¹. Immunofluorescence studies indicate that the enzyme may be localized to the surface of the endoplasmic reticulum. These results suggest that, in addition to its role in protein synthesis, the Leishmania eEF1B may help protect the parasite from lipid peroxidation.

Parasitic protozoa of the family trypanosomatidae cause disease and death throughout the tropical and subtropical world. Trypanosoma brucei infections are estimated to cause ~400,000 cases of sleeping sickness per year; Trypanosoma cruzi, the cause of Chagas’ disease, chronically infects ~17 million people and Leishmania spp. are thought to cause 2 million cases of leishmaniasis per year.¹ Current chemotherapies for these diseases are, on the whole, ineffective and toxic (2, 3), whereas effective vaccines may never be developed. The development of effective treatments for these infections is therefore an urgent necessity.

New anti-parasitic drugs can be developed from inhibitors of biochemical pathways that are essential for parasite survival but absent from the host. One such target is the thiol metabolism of trypanosomatids. Uniquely, this is dependent upon trypanothione (N³,N³-bis(glutathionyl)spermidine or T[SH]₂)² (4), whereas their human hosts use glutathione ((γ-L-glutamyl-L-cysteinylglycine or GSH). Trypanothione is involved in protective processes, defending against oxidative stress through peroxidase systems (5), against reactive aldehydes through the glyoxalase system (6, 7), and against toxic xenobiotics through the trypanothione S-transferase (TST) (8). All these processes depend upon trypanothione being maintained in its dithiol form by trypanothione reductase. This NADPH-dependent flavoenzyme is essential to the trypanosomatids (9, 10) as, in these organisms, it is the only known route for the transfer of reducing equivalents from NADPH to low molecular mass thiols. In addition, because these parasites lack an equivalent to thioredoxin reductase, T[SH]₂ also reduces their orthologues of thioredoxins, the tryparedoxins (TryX) (5).

The role of T[SH]₂ in the defense against oxidative stress is particularly important, as reactive oxygen species are critical to the host-parasite interaction (11). Furthermore, several of the clinically used drugs, such as the antimonials used to treat leishmaniasis, and the nitroimidazoles and nitrofurans used to treat Chagas’ disease, may all act through the induction of oxidative stress (12). Trypanosomatids lack catalase and selenium-dependent peroxidases and are unusually dependent upon a set of thiol-dependent peroxidases (5). These enzymes fall into two classes, both of which are thought to contain a redox-active cysteine residue in their active sites that is reduced by their first substrate and then oxidized by the hydroperoxide secondary substrate (13, 14). The best characterized of these are the tryparedoxin peroxidases (13, 15–17). These are a group of peroxiredoxins that accept electrons from TryX and effectively reduce hydrogen peroxide. However, they vary in their abilities to metabolize aryl and alkyl hydroperoxides, such as cumene hydroperoxide (CuOOH). Recently, a second class of peroxidases has been discovered in T. cruzi (18, 19) and T. brucei (14) with higher activity toward hydrophobic hydroperoxides. These are homologues of the selenoprotein glutathione peroxidase (GPX), with the active site selenocysteine being replaced by a cysteine residue. However, with the exception of the T. cruzi GPX II (20), these enzymes use TryX, rather than GSH as their preferred reductant (14, 19).

In mammalian cells, another class of enzymes that are im-

* This work was supported by the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AY677167 (eEF1Ba gene), AY677168 (eEF1Bβ gene), and AY677169 (eEF1Bγ gene).

† To whom correspondence should be addressed. Tel.: 44-1382-345155; Fax: 44-1382-345542; E-mail: a.h.fairlamb@dundee.ac.uk.

World Health Organization (www.who.int/en/).

² The abbreviations used are: T[SH]₂ and T[S]₂, trypanothione and trypanothione disulfide, respectively; TCEP, tris(2-carboxyethyl)phosphine; GST, glutathione S-transferase; TST, trypanothione S-transferase; TryX, tryparedoxin; GPX, glutathione peroxidase; eEF1B, eukaryotic elongation factor 1B complex (formerly eEF-1β); eEF1Ba formerly eEF-1β; eEF1Bβ formerly eEF-1β; eEF1Bγ formerly eEF-1γ; CuOHH, cumene hydroperoxide; ER, endoplasmic reticulum; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PBS, phosphate-buffered saline; BiP, immunoglobulin heavy chain-binding protein; CDNB, 1-chloro-2,4-dinitrobenzene.
portant in the metabolism of hydrophobic hydroperoxides are the glutathione S-transferases or GSTs. The GSTs are detoxification enzymes that catalyze the nucleophilic attack of GSH on a wide variety of hydrophobic substrates (23). These enzymes therefore not only reduce the phospholipid hydroperoxides generated during lipid peroxidation (24, 25), but they can also detoxify the reactive aldehydes such as 4-hydroxynonenol-2-enal produced by the breakdown of these oxidized lipids (26).

Recently, a trypanothione S-transferase (TST) activity was detected in several members of the trypanosomatidae and purified from Crithidia fasciculata. The TST complex was identified as the eukaryotic translation elongation factor 1B (eEF1B), which is involved in ribosomal protein synthesis, and the TST active site localized to the γ subunit (eEF1Bγ) (8). Previous studies in other organisms had shown that the other two subunits (eEF1Ba and β) of eEF1B act to recycle translation elongation factor 1A complexed with GDP back to the active GTP-complexed form (27, 28). The eEF1B holocomplex, but not the isolated subunits, was able to catalyze the conjugation of TSH₂ to a variety of electrophiles, being most active with hydrophobic hydroperoxides.

**EXPERIMENTAL PROCEDURES**

**Materials**—Thyminine hydroperoxide was synthesized from 5-hydroxymethyluracil and purified as described (29). Linoleic acid hydroperoxide (13S-hydroxy-9(Z,11E)-octadecadienoic acid) was synthesized using soybean lipoxidase and purified as described (30). Recombinant T. cruzi trypanothione reductase was purified as previously described (31). L. major Friedlin A1 clone promastigotes and C. fasciculata chlamastigotes were cultured as described (32).

**Cloning of L. major eEF1B Subunits**—The genes encoding the L. major eEF1B subunits were identified in the L. major genome data base (www.genedb.org). The eEF1Ba gene was amplified by PCR from genomic DNA, using the sense primer 5′-CATATGGCCACCTCAGAGTGCAAC-3′ and the antisense primer 5′-GGATCCTTAAATCTT-3′. The PCR product was then cloned into the pCR-Blunt II-TOPO plasmid using the Zero Blunt TOPO PCR cloning kit (Invitrogen) and fully sequenced. Similarly, an eEF1Ba gene was acquired from T. brucei (15) by using the sense primer 5′-GGACGTTGACGAAGAAG-3′ and the antisense primer 5′-GGATCGTCGAGTATGTTCGAGCCGCA-3′ and the eEF1Ba gene using the sense primer 5′-CATATGACCTCAAGCTCCTCCGCCC-3′ and the antisense primer 5′-GGATCGTCGAGTATGTTCGAGCCGCA-3′. These primers added 5′ NdeI sites and 3′ BamHI sites (underlined), allowing subcloning of these genes into the expression plasmid pET3aTr (33) and, in addition, the eEF1Ba gene into pET15b (Novagen).

The polyclonal expression construct pST39.eEF1B was assembled by the insertion of these three genes into translation cassette 1, 2, and 4 of the polyclonal expression vector pST39 (33). First, the eEF1Bβ gene was excised from pET15b.eEF1Bβ by digestion with XbaI and BamHI, this removed a fragment containing the plasmid 5′-untranslated region and the eEF1Bβ gene as an in-frame fusion with the vector-derived N-terminal hexahistidine tag. This fragment was then ligated into the corresponding sites of pST39, to create the plasmid pST39.eEF1Bβ. Similarly, the eEF1Ba gene was excised by EcoRI and HindIII digestion from pET3aTr.eEF1Ba and ligated into these sites in the pST39.eEF1Bβ plasmid, to create pST39.eEF1Baβ. The eEF1Bγ gene was then re-amplified, using the pET3aTr.eEF1Bγ plasmid as template, and the antisense primer 5′-CCGGTGTTCGATGACCATTTA-3′ and the sense primer 5′-CCGGTGTTCGATGACCATTTA-3′. These primers amplified a fragment containing the pET3aTr 5′-untranslated region and added a 5′ XhoI site and a 3′ XhoI site (underlined), allowing subcloning into the BspEI and Xhol sites of the pST39.eEF1Bβ plasmid, to create pST39.eEF1B.

**Expression and Purification of eEF1B Subunits**—The pET3aTr.eEF1Ba, β, and γ plasmids were transformed into BL21(DE3)pLysS E. coli. These were grown at 37 °C in LB media containing 100 μg ml⁻¹ carbenicillin and 12.5 μg ml⁻¹ chloramphenicol to an OD of 0.6. After cooling to 25 °C, expression was induced for 4 h with 1 mm isopropyl β-D-galactopyranoside and the cells then harvested and frozen.

All three eEF1B subunits were expressed as untagged proteins from pET3aTr and purified by anion-exchange and size-exclusion chromatography. Unless otherwise specified, all procedures were carried out at 4 °C. Cells were resuspended in a final volume of 30 ml of lysis buffer (75 mM (Na⁺) phosphate, pH 7.5, 1 mM benzamidine, 5 μM β-mercaptoethanol, 250 μM 1,2-aminoethoxybenzenesulfonfluoride, 1 μM pepstatin A) to which dithiothreitol and EDTA were added to a final concentration of 1 mM. The cells were then lysed by sonication (10 × 30 s bursts), with cooling to <4 °C between pulses. After centrifugation (45,000 × g, 1 h), the supernatant was applied at 2 ml min⁻¹ to a 25-ml (1.6 × 8 cm) Q-Sepharose HP anion-exchange column equilibrated in either buffer A (20 mM (Na⁺) histidine, pH 6) for pET3aTr and β, or buffer B (25 mM (Na⁺) bis-Tris, pH 6.5) for pET39.eEF1Bγ. The column was washed for 30 min and bound proteins were then eluted with a linear gradient of 0–1 M NaCl, in the same buffer. Fractions containing the recombinant protein were pooled and concentrated to 5 ml. This sample was then applied to a 319-ml (2.6 × 60 cm) Superdex 200 26/60 size-exclusion column equilibrated with buffer C (50 mM (Na⁺) HEPES, pH 7.5, 300 mM NaCl, 0.01% (w/v) NaN₃) at a flow rate of 1 ml min⁻¹ to a 1-ml (0.64 × 3 cm) Resource Q anion-exchange column (Amersham Biosciences) equilibrated in buffer B. Bound proteins were then eluted with a linear gradient of 0–0.5 M NaCl in buffer B.

**Polyclonal Expression and Complex Purification—**BL21(DE3) pLysS E. coli, transformed with pST39.eEF1B, were grown and protein expression was induced, as before. The cells were lysed, as described above, in lysis buffer modified by the addition of 500 mM NaCl and 1 mM 2-mercaptoethanol. After centrifugation (45,000 × g, 1 h), the supernatant was applied at 1 ml min⁻¹ to a 25-ml (1.6 × 8 cm) Q-Sepharose column (Amersham Biosciences) in buffer D (50 mM (Na⁺) phosphate, pH 7.5, 200 mM NaCl) and bound proteins were eluted with a linear gradient of 0–500 mM imidazole in buffer D. Fractions containing pET39.eEF1B were pooled and dialyzed overnight against 2 liters of buffer B containing 1 mM dithiothreitol and further purified by anion-exchange chromatography using a 25-ml Q-Sepharose HP column, in buffer B, as before.

**Physical Characterization of Proteins—**Subunit masses were measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry on a Voyager-DE STR (PerSeptive Biosystems). The relative molecular masses (M) of complexes were measured by size-exclusion chromatography using a 24-ml (1 × 30 cm) Superdex 200 HR 10/30 column equilibrated with buffer C containing eEF1B were pooled and dialyzed overnight against 2 liters of buffer B containing 1 mM dithiothreitol, and then further purified by anion-exchange chromatography using a 25-ml Q-Sepharose HP column, in buffer B, as before.

**Production of Antiserum Against C. fasciculata eEF1B and Immunoblot Analysis—**A polyclonal antiserum was raised in BALB/c mice against the purified C. fasciculata eEF1B complex (34). Cells (5 × 10⁶) from C. fasciculata and L. major cultures were harvested and resuspended in SDS-PAGE sample buffer. The proteins were then separated by SDS-PAGE and transferred onto Sequi-Blot polyvinylidene difluoride membranes (Bio-Rad). Immunoblotting was then performed, essentially as described (35), using a 1:10,000 dilution of the primary polyclonal antiserum and a 1:5,000 dilution of secondary antibody (Molecular Probe, GiG Sigma). Bound antibodies were detected using the ECL detection kit (Amersham Biosciences), according to the manufacturer's instructions.

**Immunolocalization of L. major eEF1B—**L. major promastigotes were washed twice in PBS before being dried onto polylysine-coated microscope slides. The cells were then fixed in 4% (w/v) paraformaldehyde in PBS for 10 min, followed by methanol at −20 °C for 2 min. Following dehydration in a series of 50%, 70%, 90%, and 100% (v/v) ethanol solutions, followed by 1 mg ml⁻¹ heat-treated RNase, the fixed cells were incubated in PBS containing 5% (w/v) fetal calf serum for 5 min. The cells were then co-labeled with anti-C. fasciculata eEF1B antiserum (1:500 in PBS) and anti-T. brucei BiP antisem (1:200 in PBS); raised in rabbits against the T. brucei homologue of immunoglobulin heavy chain-binding protein (BiP); a gift from James D. Bangs, University of Wisconsin-Madison Medical School, Madison, WI). Following incubation for 1 h in a...
dark humid chamber, the slides were washed with PBS and incubated for 1 h in fluorescein isothiocyanate-conjugated goat anti-mouse IgG antiserum (1:500 in PBS, Sigma) and tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG antiserum (1:500 in PBS, Sigma). The slides were washed with PBS before being mounted using the SlowFade Light Antifade kit with 4, 6-diamidino-2-phenylindole (Molecular Probes), according to the manufacturer’s instructions. Images were collected using a Zeiss Axiovert 200 M fluorescence microscope.

**Enzyme Assay—** TST was assayed at 25 °C in 100 mM (Na⁺) phosphate, pH 6.5, using either a Shimadzu UV-2401 PC or a Beckman DU640 spectrophotometer. In the standard assay T[S]₂ was produced immediately before use by mixing T[S]₂ (Bachem) with a 2-fold excess of purified products of these genes (23.2, 25.5, and 46.1 kDa, respectively). In addition, the masses of the predicted kDa, respectively) (8). All three subunits of size-exclusion chromatography to more than 95% purity (Fig. 2). The simple purification of these proteins by anion-exchange and P34715, respectively. In addition, the masses of the predicted subunits were co-incubated at 1:1 ratios in three pairwise combinations and one 1:1:1 eEF1Bα, -β, and -γ combination. Initially, these mixtures were analyzed by size-exclusion chromatography (Fig. 2). Only the ternary mixture formed a novel complex of Mₜ, 540,000 (Fig. 2, closed squares), whereas no additional high molecular mass peaks were observed in any of the binary combinations. However, as separation of the individual subunits was not possible because of the similar Mₛ values of their homocomplexes, these data do not exclude the formation of other heterocomplexes. The four protein mixtures were therefore also separated by anion-exchange chromatography and this showed that an aβ heterocomplex was formed (data not shown). The subunit stoichiometries of the purified aβ and aβγ complexes were determined by SDS-PAGE and densitometry. This indicated that the aβ species contained in the subunits in a 1:1 ratio and that the subunits in the aβγ complex were at a 1:1:2 ratio (Fig. 1). In combination with the Mₛ values of these complexes, this suggested that the species formed were an (aβ)₃ octamer and an [aβγ]₁₄ hexadecamer (Table I). The latter agrees with our previous analysis of eEF1B from C. fasciculata (8). Only the [aβγ]₁₄ holocomplex showed TST activity with CDNB or CuOOH substrates (Table I).

**Polycistronic Expression of Recombinant L. major eEF1B Complex—** As these reconstitution experiments indicated that all three subunits were required to form a complex with S-transferase activity, the three subunits were cloned into the pPST39 polycistronic expression vector. The fusion of an N-terminal hexahistidine tag to eEF1B allowed the simple purification of the eEF1B holocomplex by metal-affinity chromatography followed by anion-exchange chromatography (Fig. 3A). The purified complex is more than 95% pure but appears to show some proteolytic degradation of the eEF1Bα subunit. The Mₛ of the recombinant complex was 570,000 and is identical, within the precision of the method, with the Mₛ of the holocomplex formed by reconstitution from the isolated subunits.

**Subcellular Localization of eEF1B—** An antiserum was produced that detected all three subunits of the C. fasciculata eEF1B (Fig. 3B, lane 1). Immunoblotting of L. major extracts (Fig. 3B, lane 2) and recombinant L. major eEF1B (data not shown) showed that this antiserum cross-reacted with the eEF1Bβ and -γ subunits, but not the eEF1Bα subunit. This antiserum was therefore used to determine the subcellular localization of the L. major eEF1B by immunofluorescence microscopy. Initial studies showed the complex in the anterior of the parasite, particularly around the nucleus and kinetoplast. This pattern is characteristic of proteins located to the endoplasmic reticulum (ER) (20, 39). Double labeling of parasites with anti-C fasciculata eEF1B antiserum and an antiserum raised against the T. brucei homologue of the ER-localized chaperone BiP (39) was therefore performed. The resulting images showed labeling of these proteins that was again con-
centrated at the anterior end of the parasite, around the nucleus and kinetoplast (Fig. 4, panels A–D). No labeling of the nucleus, kinetoplast, or flagellum was observed. When the images were superimposed, the labeling of the two proteins was shown to be largely identical (Fig. 4, panels E and F, yellow staining). These data indicate that the L. major eEF1B is localized on or in the ER, but do not exclude the possibility that the complex may be present in other structures in this region, such as the Golgi apparatus or flagellar pocket.

Table I

| Subunit(s) | Molecular mass (kDa) | Relative molecular mass | Predicted species | TST activity (milliunits mg⁻¹) |
|------------|----------------------|------------------------|-------------------|-------------------------------|
| α          | 23,169               | 160,000                | Heptamer          | <1⁰                           |
| β          | 25,514               | 159,000                | Hexamer           | <1⁰                           |
| γ          | 46,104               | 171,000                | Tetramer          | <1⁰                           |
| αβ complex | 194,000              | 540,000                | αβγ               | 285 ± 1                       |
| αβγ complex|                     |                        | (αβγ)             | 96 ± 8                        |

a Mass of protein after removal of N-terminal methionine.
b Less than the limits of detection.
transfase activity was found with GSSG, insulin, or 2-hydroxyethyl disulfide as substrates. The eEF1B complex did, however, catalyze the aromatic nucleophilic substitution reactions between T[SH]2, CDNB, and 2-chloro-5-nitrobenzonitrile (RCI in Reaction 1), the thiolysis of p-nitrophenyl acetate by T[SH]2 (Reaction 2), Michael addition of T[SH]2 to 4-hydroxy-2-nonenal (Reaction 3), and the reduction of thymine hydroperoxide, trans-5-phenyl-4-pentenyl-(E)-hydroperoxide, CuOOH, and linoleic acid hydroperoxide by T[SH]2 (ROOH in Reaction 4).

\[
\text{RCI} + \text{T[SH]}_2 \rightarrow \text{T[SH]}_2 \text{SH} + \text{Cl}^- + \text{H}^+ \\
\text{REACTION 1}
\]

\[
p\text{-NO}_2\text{C}_6\text{H}_4\text{OCOCH}_3 + \text{T[SH]}_2 \rightarrow p\text{-NO}_2\text{C}_6\text{H}_4\text{OH} + \text{T[SCOCH}_3\text{]}_2\text{SH} \\
\text{REACTION 2}
\]

\[
\text{C}_6\text{H}_5\text{CH(OH)CH=CHCHO} + \text{T[SH]}_2 \rightarrow \text{C}_6\text{H}_5\text{CH(OH)CH(STSH)}_2\text{CHCHO} \\
\text{REACTION 3}
\]

\[
\text{ROOH} + \text{T[SH]}_2 \rightarrow \text{ROH} + \text{T[Sl]}_2 + \text{H}_2\text{O} \\
\text{REACTION 4}
\]

Under these assay conditions, the most effective substrates were the hydrophobic hydroperoxides, with the linoleic acid hydroperoxide being the most active (Table II). No activity was detected with any of the above substrates when T[SH]2 was replaced with GSH.

Using CuOOH as a model substrate, the mechanism of recombinant L. major eEF1B peroxidase reaction was studied in more detail. Substrate-mediated inactivation of the enzyme does not appear to occur, as linear reaction rates were observed at high concentrations of CuOOH, with assays containing 2 mM peroxide giving linear rates until NADPH was completely consumed (data not shown). Initial rates were measured over four fixed concentrations of CuOOH with varying concentrations of T[SH]2, or its metabolic precursor, N^1-glutathionylspermidine. Lineweaver-Burk plots of individual fits of the resulting data to the Michaelis-Menten equation were clearly not parallel. Rather, they intersected at or near the x axis, indicating that a ternary complex is formed (Fig. 5). Both random and ordered ternary complex mechanisms were fitted to the data, and a rapid equilibrium random order mechanism was selected for this preliminary kinetic analysis, as it gave the best fit. Further studies on this enzyme will be required to distinguish between the range of possible ternary complex mechanisms.

**FIG. 5. Kinetic pattern of L. major eEF1B peroxidase activity.** Trypanothione-dependent peroxidase activity of recombinant L. major eEF1B with T[SH]2 and cumene hydroperoxide substrates. Trypanothione concentrations were varied at fixed concentrations of 0.5 mM (open circles), 0.75 mM (closed circles), 1 mM (open squares), and 1.5 mM (closed squares) cumene hydroperoxide. The lines show the fit to the rapid-equilibrium random order mechanism.

The kinetic constants obtained with these two thiol substrates were identical, within experimental error (Table III).

**DISCUSSION**

The L. major eEF1B complex possesses trypanothione S-transferase activity against a range of xenobiotic compounds and is most active with hydrophobic hydroperoxides. The inability of the isolated L. major eEF1B subunit to catalyze these reactions is consistent with the complete loss of TST activity after separation of the subunits of the C. fasciculata eEF1B (8). These results indicate that the enzymatically active conformation of eEF1Bγ is only achieved in the eEF1B complex. Interactions between the eEF1B subunits can affect their enzymatic activities, with the nucleotide-exchange activity of Artemia salina eEF1Bα being increased 2-fold by the addition of equimolar amounts of eEF1Bγ (28). However, the absence of TST activity in C. fasciculata and L. major eEF1Bγ contrasts with the GST activity of the recombinant rice eEF1Bγ (40). This eEF1Bγ protein has a GST activity with 1-chloro-2,4-dinitrobenzene that is comparable with that of the native eEF1Bγβγγ complex. Unfortunately, although the rice eEF1Bγ was shown not to be active with t-butyl hydroperoxide as a substrate (in common with the L. major eEF1B), no assays were performed with other hydrophobic hydroperoxides that are substrates for the L. major eEF1B. Further studies to test the specificity of the rice eEF1Bγ and determine whether the mammalian eEF1Bγ proteins have GST activity might therefore be desirable.

**Table II. Specific activities of the trypanothione S-transferase activity of recombinant L. major eEF1B with various electrophilic substrates.**

| Electrophilic substrate | Assay conditions | Specific activity |
|-------------------------|-----------------|------------------|
|                         | [Electrophile]  | [Thiol]          | µM | milliunits mg⁻¹ |
| CDNB                    | 400             | 400              | <2.5 | 93 ± 2 |
| 2-Chloro-5-nitrobenzonitrile | 1000           | 400              | <1 | 22.5 ± 0.1 |
| p-Nitrophenyl acetate   | 400             | 400              | <1 | 9.15 ± 0.8 |
| 4-Hydroxy-2-nonenal     | 25              | 200              | <5 | 26 ± 1 |
| Thymine hydroperoxide   | 400             | 400              | <1 | 44 ± 6 |
| Trans-5-phenyl-4-pentenyl-(E)-hydroperoxide | 400    | 400              | <1 | 116 ± 1 |
| Cumene hydroperoxide    | 1000            | 400              | <0.75 | 291 ± 3 |
| Linoleic acid hydroperoxide | 50           | 400              | <1 | 950 ± 40 |

*The absorbance coefficient for the T[SDNB]₂ conjugate of 9.2 mM cm⁻¹ per pm sulfhydryl group at 340 nm was used for T[SH]₂ (8), whereas the published value for GS-DNB of 9.6 mM cm⁻¹ at 340 nm was used for GSH (62). The published absorbance coefficient of 9.6 mM cm⁻¹ at 340 nm for the GSH adduct was used for both thiol substrates (1). An absorbance coefficient of 5.12 mM cm⁻¹ at 400 nm for p-nitrophenol at pH 6.5 was calculated from the published pKₐ and absorbance coefficient of the p-nitrophenolate anion (22). The published absorbance coefficient of 13.75 mM cm⁻¹ at 224 nm for the GSH adduct was used for both thiol substrates (21).
Interestingly, the assembly process of the \emph{L. major} eEF1B complex appears to be significantly different from that of the mammalian complex. The \(\alpha\) and \(\beta\) subunits of the rabbit eEF1B do not interact in the absence of the eEF1\(\beta\gamma\) subunit (41). However, rabbit eEF1\(\beta\gamma\) can bind to either of these subunits to form eEF1\(\beta\alpha\gamma\) and eEF1\(\beta\beta\gamma\) complexes that may be intermediates in the assembly process. This contrasts with the \emph{L. major} proteins, where the eEF1\(\alpha\) and -\(\beta\) subunits appear to be capable of binding to eEF1\(\beta\gamma\) only after forming an octameric eEF1\(\alpha\beta\gamma\) complex.

The ability of the \emph{L. major} eEF1B to utilize hydrophobic hydroperoxides is highly significant, as they are not substrates of the previously characterized \emph{C. fasciculata} TST (8). Moreover, these compounds may not be effectively detoxified by the TryX/tryparedoxin peroxidases pathway, which, in \emph{L. major}, prefers hydrogen peroxide as substrate and does not use cumene hydroperoxide at all (42). This may be because hydrophobic hydroperoxides can rapidly inactivate tryparedoxin peroxidases by causing the overoxidation of a redox-active cysteine in the active site (43, 44). This inactivation reaction is common to most eukaryotic peroxidoredoxins and has been proposed to function in oxidative stress signaling (45). The observed insensitivity of the TST to inactivation by hydroperoxides could result from the enzyme catalyzing the direct attack of T[SH]₂ on hydroperoxides, with no formation of a sulfenic acid intermediate on an active-site cysteine. The TST activity of the eEF1B complex may therefore be particularly important as a backup system under conditions of severe oxidative stress. However, other peroxidases may also complement the tryparedoxin peroxidases system in trypanosomatids, with non-selenium GPX homologues recently being characterized in \emph{T. cruzi} (18) and \emph{T. brucei} (14). Interestingly, the \emph{T. cruzi} GPX II was reported to be reduced directly by GSH and not by TryX or T[SH]₂ (20). Moreover, GPX II appeared to be specific for fatty acid and lipid hydroperoxides and to be localized to the ER. However, the activity of this enzyme was 70-fold less with linoleic acid hydroperoxide than that of the \emph{L. major} eEF1B. The relative roles of GPX II and any \emph{T. cruzi} eEF1B S-transferase activity in antioxidant metabolism are therefore unclear. Recently, the \emph{T. cruzi} GPX I (19) and the \emph{T. brucei} GPX (14) were shown to be most active with TryX as a electron donor, but the activity of these enzymes with fatty acid and lipid peroxides was not reported. The physiological role and substrate specificity of the \emph{L. major} GPX homologue LmjF36.3010 cannot therefore be predicted. Significantly, the activity of all TryX-dependent peroxidases may be limited under even moderate levels of oxidative stress by the slow rate of reduction of TryX by T[SH]₂ (13, 19). As the TST activity of eEF1B does not require TryX, its peroxidase activity will not be limited by this reaction. Moreover, eEF1B may contribute a significant proportion of the \emph{L. major} cellular lipid peroxidase activity even under non-stress conditions, as its relatively low activity could be compensated for by its abundance, with this complex forming 0.4% of total soluble protein in \emph{C. fasciculata} (8). The activity of the \emph{L. major} eEF1B of 0.95 unit mg⁻¹ with linoleic acid hydroperoxide is, however, comparable with that of the mammalian GSTs, with the specific activities of the rat isoenzymes ranging from 0.06 to 5.3 units mg⁻¹ with this substrate (46).

A \emph{Leishmania} TST has been previously proposed to be the uncharacterized T[SH]₂-dependent activity required for high-level antimony resistance (47). The characterization of a \emph{Leishmania} TST will therefore allow studies to directly address any function of this enzyme in the resistance phenotype. Interestingly, overexpression in \emph{T. cruzi} of their eEF1\(\beta\gamma\) gene confers resistance to the trypanothione reductase inhibitor clopimidine (48), a compound that might therefore be expected to induce oxidative stress in the parasite. However, the sensitivity of \emph{T. cruzi} to other chemotherapeutic compounds that induce oxidative stress was not affected.

The localization of the eEF1B complex to the outer surface of the ER has previously been observed in human fibroblasts (49) and \emph{Xenopus laevis} oocytes (50). This localization may be the result of an interaction between the ER-resident integral membrane protein kinectin and eEF1\(\beta\) (51). Interestingly, the association of eEF1B to the ER membrane has also been proposed to involve a hydrophobic domain of eEF1\(\beta\gamma\) (28). This affinity for membranes would be consistent with a role for eEF1\(\beta\gamma\) in the metabolism of the lipid hydroperoxides and cysteic acid aldehydes produced during lipid peroxidation. The targeting of peroxidases to the ER might also relate to a reduced ability of antioxidant systems in the ER lumen to protect these membranes from oxidative damage, resulting from the oxidizing environment that is maintained within this cellular compartment (52, 53). Moreover, the ER membranes may be exposed to oxidative species produced in the ER lumen during the oxidation of cysteine residues during the folding of secreted proteins. This is catalyzed by the flavoprotein Ero1p (54) and has been proposed to be a major source of reactive oxygen species (55).

The eEF1B complex may also mediate translational control in response to cellular oxidative stress. Protein synthesis is potently and reversibly inhibited by glutathione disulfide (56) and an \textit{in vivo} study has indicated that translation elongation is the step in this process that is most sensitive to oxidative stress (57). Interestingly, the eEF1\(\beta\) subunit is glutathionylated in response to oxidative stress (58) and the eEF1\(\beta\gamma\) subunit contains a redox-active pair of cysteine residues (59). These post-translational modifications might affect protein synthesis either by direct alterations in the nucleotide-exchange activity of eEF1B, or through the allosteric effects that interactions with this complex have on tRNA synthetases (60, 61).

The identification of a TST activity in the \emph{L. major} eEF1B complex has implications for translational control and the mechanisms of resistance to chemical and oxidative stress in these pathogens. Future studies on the activities of host and parasite eEF1\(\beta\gamma\) proteins may therefore reveal further important roles of these elongation factors in cellular physiology.

\textbf{Acknowledgments—} We thank Dr. Song Tan, Pennsylvania State University, for the kind gift of the pET3aTr and pST39 plasmids and Douglas Lamont for performing mass spectrometry. We also thank Ahilan

\begin{table}[h]
\centering
\caption{Kinetic parameters of recombinant \emph{L. major} eEF1B trypanothione-dependent peroxidase activity.} \label{table3}
\begin{tabular}{llllll}
\hline
Thiol substrate & \(K_{m, \text{Thiol}}\) & \(K_{m, \text{Peroxide}}\) & \(k_{cat}\) & \(h_{cat}/K_{m, \text{Thiol}}\) \\
& \(\mu\text{M}\) & \(\text{M} - \text{min}^{-1} \text{M}^{-1}\) & & \\
Trypanothione & 140 \(\pm\) 7 & 7400 \(\pm\) 1700 & 25 \(\pm\) 5 & 1.8 \(\times\) 10³ \\
Glutathionylspermidine & 150 \(\pm\) 7 & 6400 \(\pm\) 1000 & 25 \(\pm\) 3 & 1.7 \(\times\) 10³ \\
\hline
\end{tabular}
\footnotesize{\textsuperscript{a} Calculated using the concentration of the eEF1B complex.}
\end{table}
Saravanamuthu for the gift of trypanothione reductase and the production of the anti-C. fasciculata eEF1B antiseraum. The use of the genome sequence data from the Wellcome Trust Sanger Institute Pathogen Sequencing Unit (www.genedb.org) is gratefully acknowledged.

REFERENCES

1. van der Aar, E. M., Buikema, D., Commandeur, J. N., te Koppele, J. M., van Oostveen, C., van Bladeren, P. J., and Vermeulen, N. P. (1996) Xenobiota 26, 143–155
2. Fairall, A. H. (2003) Trends Parasitol. 19, 488–494
3. Croft, S. L., and Coombs, G. H. (2003) Trends Parasitol. 19, 592–598
4. Fairall, A. H. Blackburn, P., Urich, P., Chat, B. T., and Cerami, A. (1985) Science 227, 1485–1487
5. Flohe, L., Hecht, H. J., and Steiner, P. (1999) Free Radic. Biol. Med. 27, 986–984
6. Irsch, T., and Krauth-Siegel, R. L. (2004) J. Biol. Chem. 279, 371–377
7. Vickers, T. J., Greig, N., and Fairall, A. H. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 13386–13391
8. Vickers, T. J., and Fairall, A. H. (2004) J. Biol. Chem. 279, 27246–27256
9. Tovar, J., Wilkinson, S., Mottram, J. C., and Fairall, A. H. (1998) Mol. Microbiol. 29, 653–660
10. Dumas, C., Ouellette, M., Tovar, J., Cunningham, M. L., Fairall, A. H., Tamar, S., Olivier, M., and Papadopoulou, B. (1997) EMBO J. 16, 2590–2598
11. Nathan, C., and Shinlo, M. U. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 8841–8848
12. Fries, D. S., and Fairall, A. H. (2003) in Burger’s Medicinal Chemistry and Drug Discovery: Chemotherapeutic Agents (Abraham, D. J., ed) pp. 1033–1087, John Wiley & Sons, New York
13. Nogoecke, E., Gommel, D. U., Kiess, M., Kalisz, H. M., and Flohe, L. (1997) Biochim. Biophys. Acta 1376, 827–836
14. Hillebrand, H., Schmid, A., and Krauth-Siegel, R. L. (2003) J. Biol. Chem. 278, 6809–6815
15. Wilkinson, S. R., Temperton, N. J., Mondragon, A., and Kelly, J. M. (2000) J. Biol. Chem. 275, 8220–8225
16. Tetaud, E., Giroud, C., Prescott, A. R., Parkin, D. W., Baltz, D., Betteau, N., Baltz, T., and Fairall, A. H. (2001) Mol. Biochem. Parasitol. 116, 171–183
17. Castro, H., Sousa, C., Santos, M., Cordeiro-da-Silva, A., Flohe, L., and Tomas, A. M. (2000) Free Radic. Biol. Med. 28, 324–335
18. Wilkinson, S. R., Meyer, D. J., and Kelly, J. M. (2002) J. Biol. Chem. 277, 310–311
19. Fairall, A. H. (2003) Trends Parasitol. 19, 224–234
20. Cooper, H. M., and Paterson, Y. (1995) in Current Protocols in Immunology, Volume 3 (Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober, W., eds) Vol. 1, pp. 2.4.1–2.4.9, John Wiley & Sons, Inc., New York
21. Alin, P., Danielson, U. H., and Mannervik, B. (1985) Biochem. J. 231, 224–234
22. Han, S. B., and Wang, S. Y. (1976) J. Biol. Chem. 251, 1719–1724
23. van Damme, H. T., Amon, R., Kassies, R., Timmers, C. J. J., Janssen, G. M., and Moller, W. (1990) Biochim. Biophys. Acta 1056, 241–247
24. Janssen, G. M., and Moller, W. (1988) Eur. J. Biochem. 171, 119–129
25. Maiorino, M., Gregolin, C., and Ursini, F. (1990) Methods Enzymol. 186, 448–457
26. Borges, A., Cunningham, M. L., Tovar, J., and Fairall, A. H. (1995) Eur. J. Biochem. 228, 745–752
27. Ariyanayagam, M. R., and Fairall, A. H. (2001) Mol. Biochem. Parasitol. 115, 189–198
28. Han, J., Yen, S., Han, G., and Han, P. (1996) Anal. Biochem. 234, 107–109
29. Collier, B. (1973) Anal. Biochem. 56, 310–311
30. Berhane, K., Widersten, M., Engstrom, A., Khakh, M., and Mannervik, B. (2002) Biochem. Biophys. Res. Commun. 298, 509–514
31. Sheu, G. T., and Traugh, J. A. (1997) J. Biol. Chem. 272, 33290–33297
32. Levi, M. P., Tetaud, E., Fairall, A. H., and Blackwell, J. M. (1998) Mol. Biochem. Parasitol. 96, 125–137
33. Flushe, L. Budde, H., Bruns, K., Castro, H., Clos, J., Hofmann, B., Kansal-Kalavar, S., Krumme, D., Menge, U., Plank-Schumacher, K., Sztajer, H., Wissing, J., Wylegalla, C., and Hecht, H. J. (2002) Arch. Biochem. Biophys. 397, 324–335
34. Fairall, A. H. (2003) Trends Parasitol. 19, 488–494
35. Saravanamuthu for the gift of trypanothione reductase and the production of the anti-C. fasciculata eEF1B antiseraum. The use of the genome sequence data from the Wellcome Trust Sanger Institute Pathogen Sequencing Unit (www.genedb.org) is gratefully acknowledged.
