Leishmania donovani Possess a NADPH-Dependent Alkylglycerol Cleavage Enzyme

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Leishmania parasites possess an abundance of ether-linked hydrocarbons as components of phospholipids and glycosylphosphatidylinositol anchors of glycoproteins and polysaccharides, including important surface molecules such as lipophosphoglycan (LPG) and glycosylinositolphospholipids (GIPLs). Cleavage of the ether bond is an important feature in the turnover pathway of alkylglycerols. In mammals, ether lipid cleavage activity requires a pteridine cofactor (H_4-biopterin), suggesting the potential for linkage between the unusual *Leishmania* pteridine metabolic pathways and lipid metabolism. In this study, we partially purified and characterized an activity in *L. donovani* capable of cleaving the ether lipid 1-*O*-alkyl[^{3}H]glycol. Unlike the mammalian enzyme but like that of *Tetrahymena,* the *Leishmania* enzyme required NADPH rather than H_4-biopterin. The use of divergent cofactors by the parasite and mammalian enzymes may provide a basis for the design of anti-parasitic drugs targeting ether-linked lipid metabolism.   © 1996 Academic Press, Inc.

Key determinants of *Leishmania* infectivity and survival in hostile environments of the host are its surface glycoconjugates lipophosphoglycan (LPG) and glycosylinositolphospholipids (GIPLs). LPG is a polymorphic glycosylphosphatidylinositol (GPI)-anchored polysaccharide. The lipid portion of LPG characteristically contains a 1-*O*-alkyl-2-*lyso*-phosphatidylinositol in which the aliphatic chain consists of either a C-24 or C-26 saturated, unbranched hydrocarbon (1,2). While all of the GIPLs have the identical 1-*O*-alkylglycerol moiety, some possess an esterified fatty acid on C-2 of the glycerol backbone.

In mammalian cells, the turnover of 1-*O*-alkylglycerol requires a glyceryl ether monooxygenase (1-*O*-alkyl-*sn*-glycerol, tetrahydropteridine:oxygen oxidoreductase, EC 1.14.16.5) that catalyzes the oxidative cleavage of the lipid to fatty aldehyde and glycerol (3). The monooxygenase can likewise degrade alkylglycol. This reaction requires molecular oxygen and tetrahydrobiopterin to hydroxylate the aliphatic carbon adjacent to the ether bond. The product of the initial reaction is a hemiacetal which spontaneously hydrolyzes to yield the fatty aldehyde and free glycerol. Relatively low concentration of alkylglycerol can be efficiently catabolized by *Leishmania* whereas high concentrations ($\geq 25\ \mu$M) of alkylglycerol are toxic (4). Due to the abundance of the alkylglycerol-anchored LPG and GIPLs on the surface of *Leishmania* promastigotes and the toxicity of alkylglycerol to the parasites, we investigated the nature of the parasitic enzyme that degrades alkylglycerol. In this paper, we report the discovery of an enzyme in *Leishmania* capable of catalyzing the cleavage of alkylglycol. The enzyme was
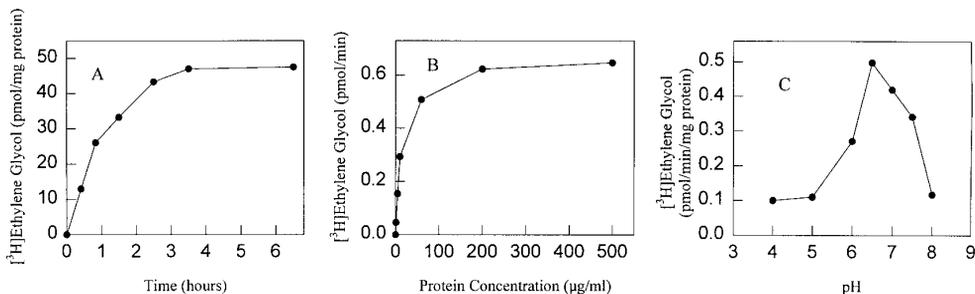
FIG. 1. Analysis of alkylglycerol cleavage activity in a cell-free system of *L. donovani*. Membranes (2 mg/ml) were incubated with 0.01 μCi of 1-*O*-octadecyl[^{3}H]glycol at 35°C for 3.5 h unless otherwise noted. At the appropriate time, aliquots of the reaction mixture were removed and extracted as described in Materials and Methods. A, time course. B, protein concentration. C, pH curve.

partially purified and characterized and found to require NADPH as a coenzyme unlike its mammalian counterpart.

MATERIALS AND METHODS

Materials. All materials were obtained as follows: Dulbecco's modified Eagle's medium (DME 430-2100) and fetal calf serum from Gibco; Bacto-Brain Heart Infusion (0037-05-2) from Difco; 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydroxychloride from Aldrich Chem. Co., 1-*O*-octyldecylglycerol, QAE-cellulose, NADPH, catalase, and glutathione from Sigma, (6R) 5,6,7,8,-tetrahydrobiopterin from RBI.

Parasites. Promastigotes of *L. donovani* were maintained as described elsewhere (5). For isolation and partial purification of enzyme, one-liter cultures of parasites were grown in Brain-Heart Infusion (6).

Preparation of 1-O-octadecyl[^{3}H]glycol. 1-*O*-octadecyl[^{3}H]glycol was prepared by a modification of the procedure of Kaufman et al. (7). Batyl alcohol (25 mg) was dissolved at 5 mg/ml in 90% acetic acid containing 50 mM sodium periodate. After a 1 hour incubation at room temperature in darkness, 5 ml of water was added and the reaction mixture was extracted twice with 1 vol. hexane/diethyl ether (1:1, v/v). The upper phase was dried under nitrogen, redissolved in absolute ethanol, and transferred to a tube containing 5 mCi of NaB[^{3}H]$_4$. After a 12 hour incubation at room temperature, 25 mg NaBH$_4$ was added to the solution and then incubated for another 2 hours. One volume of 0.5 M HCl was then added and the mixture was extracted twice with hexane/diethyl ether (1:1, v/v). The radioactive product in the upper phase was concentrated to 1-2 ml by rotary evaporation, and further purified by preparative TLC on silica developed in diethyl ether/methanol (90:10). The band corresponding to 1-*O*-octadecyl[^{3}H]glycol was scraped and extracted twice with chloroform. The combined supernatants were evaporated to dryness, resuspended in absolute ethanol, and stored at −20 °C.

Standard enzyme assay and lipid extraction. Membranes were obtained by disruption of stationary phase *L. donovani* promastigotes (harvested at a density of 3-5 × 10^7 cells/ml) by nitrogen cavitation at 1500 psi for 25 minutes at 4°C (8). Large organelles and debris were removed by centrifugation at 10,000 × g and the microsomal membranes were pelleted by centrifugation at 100,000 × g. Cleavage activity was assayed by measuring the formation of [^{3}H]ethylene glycol from 1-*O*-octadecyl[^{3}H]glycol cleavage. Standard incubation mixtures (200 μl) contained: membranes (1.5-2.0 mg protein), 50 mM MES buffer, pH 7.0 (0.1 M Tris/HCl, 0.05 M MES, 0.05 M acetic acid, 0.25 M sucrose, 0.1 M KCl, 1 mM EDTA, 1 mM EGTA, 1μg/ml leupeptin, 0.1 mM TLCK), 5 mM (NH$_4$)$_2$SO$_4$ containing 20 U/μl catalase, 1.0 mM NADPH, 0.1 mM DTT, and 0.01-0.02 μCi 1-*O*-octadecyl[^{3}H]glycol. The incubation mixture was incubated at 35°C for 3.5 hours and was terminated by addition of 0.75 ml of chloroform/ methanol (1:2). Radioactivity in the aqueous phase was measured by scintillation counting.

Analytical methods. Protein concentration was determined using the method of bicinchoninic acid microassay (9), bovine serum albumin was used for a standard.

RESULTS

In vitro analysis of alkylglycerol cleavage activity. In a previous report by Kaufman et al. (7), an assay was developed for assaying alkylglycerol cleavage in a rat liver system using alkylglycol as a substrate. Based on this assay, a cell lysate from *L. donovani* was examined for the capability of hydrolyzing 1-*O*-octadecyl[^{3}H]glycol. The *in vitro* cleavage of the lipid substrate to form [^{3}H]ethylene glycol was linear with time up to 1 h (Fig. 1A) and was protein

TABLE I

Effects of Cofactors on Alkylglycol Cleavage Activity

NADPH (1 mM)	BPTH$_4$ (1 mM)	PTR1 (0.25 mg/ml)	[^{3}H]Ethylene glycol (pmol/min/mg protein)
−	−	−	1.8 ± 0.4
+	−	−	12.5 ± 0.4
+	+	−	12.7 ± 1.2
+	+	+	13.4 ± 0.4
−	+	+	1.2 ± 0.1

Enzyme activity was assayed as described in Materials and Methods. Background cpm (boiled enzyme control) was subtracted from the values shown.

concentration-dependent (Fig. 1B). Several possible reasons for why product formation was not linearly proportional to the amount of protein are inhibitory effects of increasing amounts of lipid (from the membranes) in the assay, consumption of substrate or cofactors, or increased amounts of degradative enzymes. The pH optimum of the cleavage activity was 6.5-7 (Fig. 1C). In contrast to the mammalian system, pterin cofactors had no stimulatory effect on cleavage of the radioactive substrate whereas NADPH did stimulate *Leishmania* cleavage activity (described below).

Solubilization and partial purification of the alkylglycerol cleavage enzyme. The cleavage activity was pelleted by centrifugation at 100,000 × g, indicative of a membrane-bound enzyme. To solubilize the enzyme, this pellet was incubated with CHAPS at a final concentration of 2.0% for 30 min at 4°C, and then was diluted to a CHAPS concentration of 0.3% for an additional 2 h. After centrifugation at 100,000 × g for 1 hour, approximately 80% of the enzymatic activity was recovered in the supernatant. The solubilized enzyme was subjected to ion exchange chromatography on a column of QAE-cellulose pre-equilibrated with 0.1% CHAPS in 20 mM Tris-HCl, pH 7.5. The bulk of the enzyme bound to the anionic support and was displaced by isocratic elution with 0.1 M Tris/HCl, pH 7.5. The fractions containing the main peak of enzymatic activity were pooled, and the enrichment in specific activity was judged to be 10-fold.

Cofactor requirements for alkylglycerol cleavage activity. The cofactor requirements of the partially purified alkylglycerol cleavage enzyme were investigated. NADPH stimulated monooxygenase activity (Table I) with an apparent K_m around 10 μM. NADH (1 mM) was approximately 50% as effective as NADPH in stimulating cleavage activity. Addition of tetrahydrobiopterin and/or pteridine reductase (PTR1) (10) had no effect on cleavage activity in the absence of NADPH. Moreover, the addition of related pteridine derivatives tetrahydropterin with or without PTR1, dimethyltetrahydropteridine and 5,6,7,8-tetrahydropteridine or the pteridine reductase inhibitor methotrexate (1 mM) failed to alter ether bond cleavage (data not shown). Glutathione also had no effect on the activity. Similar cofactor results were obtained using a solubilized preparation of the enzyme which had not been fractionated on QAE cellulose. Collectively, these results were consistent with the presence of a NADPH-dependent alkylglycerol cleavage enzyme in *L. donovani*.

Substrates of the alkylglycerol cleavage enzyme. Since the radioactive substrate for the alkylglycerol cleavage assay was not a physiological substance, it was important to confirm that the enzyme hydrolyzes a biologically-relevant substrate. 1-*O*-octadecylglycerol is present in the parasite and is a shorter chain version of the alkylglycerol portion of LPG and GIPLs. When 1-*O*-octadecylglycerol was used as the substrate in the *in vitro* assay, the cleavage of
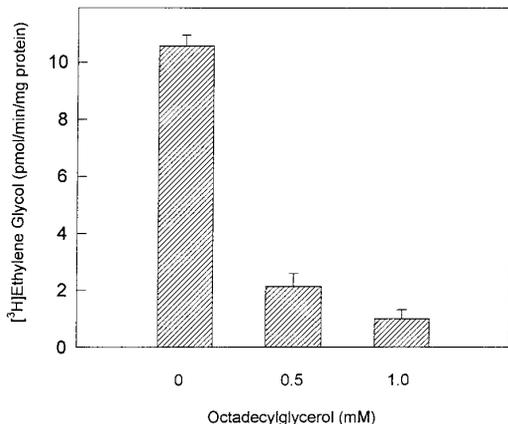
FIG. 2. Inhibition of 1-*O*-octadecyl[³H]glycol cleavage by 1-*O*-octadecylglycerol. Unlabeled 1-*O*-octadecylglycerol using the designated concentrations was added to the standard enzyme assay and the amount of [³H]ethylene glycol was measured.

the ether bond was also observed and the aqueous-soluble product was identified as glycerol by paper chromatography. 1-*O*-octadecylglycerol was also examined for inhibition of cleavage of the 1-*O*-[³H]octadecylglycol analog in the alkylglycerol cleavage assay using QAE-cellulose-purified enzyme. Increasing the concentration of unlabeled 1-*O*-octadecylglycerol resulted in a decrease in cleavage of radiolabeled substrate (Fig. 2). At 1 mM of 1-*O*-octadecylglycerol, more than 95% of the radiolabeled glycol substrate remained uncleaved. These results indicated that the same alkylglycerol cleavage enzyme that catalyzed the cleavage of the alkylglycol was also responsible for the degradation of alkylglycerol in *L. donovani.*

DISCUSSION

Tietz et al. (3) first reported the finding of a rat liver microsomal enzyme that could cleave 1-*O*-alkylglycerol. Similar to phenylalanine, tyrosine and tryptophan hydroxylases, this mammalian enzyme had a strict requirement for tetrahydrobiopterin and molecular oxygen. Addition of pteridine reductase, which functions to reduce the ''oxidized pteridine'' intermediate to the tetrahydro level, could stimulate enzyme activity. The cofactor-dependence of the liver glyceryl ether monooxygenase was later shown to require one of several different tetrahydropterins. The most effective pterins were the unconjugated tetrahydropterins rather than conjugated derivatives, such as tetrahydrofolate. Unlike the rat liver enzyme system, our results indicated a major difference in *Leishmania* with respect to the requirement of reducing cofactors. NADPH was found to be the necessary cofactor for ether-cleaving activity. Neither tetrahydrobiopterin or related pteridines was involved in the reaction. Glyceryl ether monooxygenase activity has also been investigated in *Tetrahymena pyriformis*. Similar to the *Leishmania* enzyme, cell-free preparations of *T. pyriformis* required NADPH to cleave the ether bond (11).

We observed other differences between the rat liver and *Leishmania* alkylglycerol cleaving enzymes. Glutathione was necessary for expression of full enzyme activity in rat liver microsomes (12). Since N-ethylmaleimide inhibits the rat liver enzyme activity, it was believed that the role of glutathione was probably to maintain the sulfhydryl groups of the enzyme. In contrast, omission of glutathione in the *Leishmania* system had no effect on the enzyme activity. The pH and temperature optimum of the *L. donovani* enzyme were also different from those of the rat liver monooxygenase. All these results indicated that a distinct enzyme in *L. donovani* was responsible for the cleavage of the ether bond.

The role of the monooxygenase in the parasite is unknown. Interestingly, the *Leishmania* alkylglycerol cleavage enzyme was unable to hydrolyze the alkylglycerol anchor of purified, intact LPG or GIPLs. This observation suggests that alkylglycerol cleavage does not initiate LPG and GIPL turnover, but may be a later step in GPI lipid anchor metabolism. Alternatively, LPG and GIPLs may first need to be preprocessed or associated with another molecule prior to cleavage of the lipid anchor by the monooxygenase. It is also possible that the enzyme described in this study might not be involved in GPI lipid anchor turnover, but rather in the metabolism of alkylphospholipids, which are abundantly present in *Leishmania* (13). The difference in cofactor requirements for the mammalian and parasitic alkylglycerol monooxy-genase may represent a potential target for effective therapeutic protocols for the treatment of this disease if the enzyme is shown to be essential for parasitic metabolism. Full characterization and purification of the *Leishmania* alkylglycerol monooxygenase should facilitate studies in the generation of specific inhibitors of the enzyme.

ACKNOWLEDGMENTS

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