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# Characterization of *quinonoid*-Dihydropteridine Reductase (*QDPR*) from the Lower Eukaryote *Leishmania major**

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**Lon-Fye Lye, Mark L. Cunningham, and Stephen M. Beverley‡**

*From the Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110*

**Biopterin is required for growth of the protozoan parasite *Leishmania* and is salvaged from the host through the activities of a novel biopterin transporter (BT1) and broad-spectrum pteridine reductase (PTR1). Here we characterize *Leishmania major quinonoid*-dihydropteridine reductase (LmQDPR), the key enzyme required for regeneration and maintenance of $H_4$biopterin pools. LmQDPR shows good homology to metazoan *quinonoid*-dihydropteridine reductase and conservation of domains implicated in catalysis and regulation. Unlike other organisms, LmQDPR is encoded by a tandemly repeated array of 8–9 copies containing *LmQDPR* plus two other genes. *QDPR* mRNA and enzymatic activity were expressed at similar levels throughout the infectious cycle. The pH optima, kinetic properties, and substrate specificity of purified LmQDPR were found to be similar to that of other *q*DPRs, although it lacked significant activity for non-*quinonoid* pteridines. These and other data suggest that LmQDPR is unlikely to encode the dihydrobiopterin reductase activity (PTR2) described previously. Similarly LmQDPR is not inhibited by a series of antifolates showing anti-leishmanial activity beyond that attributable to dihydrofolate reductase or PTR1 inhibition. *q*DPR activity was found in crude lysates of *Trypanosoma brucei* and *Trypanosoma cruzi*, further emphasizing the importance of $H_4$biopterin throughout this family of human parasites.**

*Leishmania* are trypanosomatid protozoan parasites that infect over 15 million people in tropical and subtropical regions of the world, with a further 350 million at risk (1). Leishmaniasis manifests as cutaneous lesions from minor to severe, or as a visceral form that, if untreated, has a high fatality rate. Existing chemotherapies are unsatisfactory, relying upon pentavalent antimonial compounds despite considerable host toxicity and some evidence for the emergence of parasite resistance (2). Presently no effective vaccine against leishmaniasis is available. *Leishmania* have a digenetic life cycle, first residing in the gut of phlebotomine sand flies where they replicate as a procyclic promastigote. As parasites enter stationary phase they differentiate into the infectious metacyclic promastigote, which is ultimately transmitted by the bite of a sand fly. Once parasites are introduced into the mammalian host, they are taken up by macrophages where they differentiate into amastigotes. Amastigotes reside and propagate within the phagolysosome, where they induce pathology and disease.

*Leishmania* and other trypanosomatid protozoan parasites are incapable of *de novo* synthesis of pteridines (folate and pterins) and must obtain them by salvage from their insect or mammalian hosts (3–6). To accomplish this, *Leishmania* express a versatile pteridine salvage network, consisting of transporters with specificity for folate and biopterin (FT1 and BT1, respectively; Refs. 7–10).[1] Following uptake, two pteridine reductases, one specific for folate (a bifunctional dihydrofolate reductase-thymidylate synthase; DHFR-TS)[2] and a second with broader specificity (pteridine reductase 1 or PTR1), reduce folate and biopterin, respectively, into the active forms, tetrahydrofolate ($H_4$folate) and tetrahydrobiopterin ($H_4$B; Refs. 11–13). The importance of folate in essential metabolic processes such as synthesis of thymidylate has been established firmly in *Leishmania* by pharmacological and genetic studies both *in vitro* and *in vivo* (14–16). Current data suggest that $H_4$B is essential for growth in *Leishmania* (12, 17, 18) and plays a role in parasite virulence and differentiation (19). $H_4$B has also been found to be a growth factor in *Crithidia fasciculata* and to effect proliferation and differentiation in various mammalian cell lines (20–22). While essential, the role(s) of $H_4$B in *Leishmania* metabolism is not well understood at present. *Leishmania* is auxotrophic for tyrosine and trypanosomatids have been reported to lack phenylalanine hydroxylase activity (23), however, the *Leishmania* genome encodes a protein with strong homology to amino acid hydroxylases.[3] NOS activity has been reported in *Leishmania* and trypanosomes (24, 25), but the *Leishmania* ether lipid cleavage activity utilizes NADPH rather than $H_4$B as a cofactor (26).

In other organisms, $H_4$B is metabolized to pterin-4$\alpha$-carbinolamine through the action of aromatic amino acid hydroxylases or NOS, or by spontaneous oxidation (27). Two enzymes are involved in its subsequent dehydration and reduction to $H_4$B: pterin-4$\alpha$-carbinolamine dehydratase (PCD) (28) and *quinonoid*-dihydropteridine reductase (*q*DPR; Ref. (29), respectively (Fig. 1). Regeneration of $H_4$B allows organisms to efficiently use biopterin cofactor in metabolism, and in humans *q*DPR deficiency is the second most common cause of hyper-

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF523363, AY141854, AF523371, AF523369, and AF523370.*

‡ To whom correspondence should be addressed: Dept. of Molecular Microbiology, Campus Box 8230, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-747-2630; Fax: 314-747-2634; E-mail: beverley@borcim.wustl.edu.

[1] J. Moore and S. M. Beverley, manuscript in preparation.

[2] The abbreviations used are: DHFR-TS, bifunctional dihydrofolate reductase-thymidylate synthase; *q*DPR, *quinonoid*-dihydropteridine reductase; *LmQDPR*/*LmQDPR*, *Leishmania major q*DPR gene/enzyme; $H_2$B, dihydrobiopterin; $H_4$B, tetrahydrobiopterin; ORF, open reading frame; PTR1, pteridine reductase 1; PTR2, postulated dihydrobiopterin reductase; PCD, pterin-4 $\alpha$-carbinolamine dehydratase; $DMPH_4$, dimethyl-5,6,7,8-tetrahydropterin; nt, nucleotide(s); NOS, nitric oxide synthase.

[3] L.-F. Lye, M. L. Cunningham, and S. M. Beverley, unpublished data.

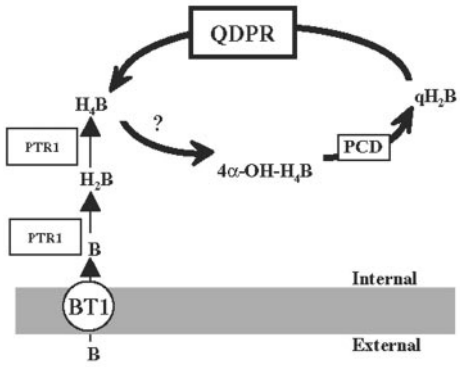


FIG. 1. **Proposed enzymatic pathways for the consumption and regeneration of $H_4B$ in *Leishmania*.** *B*, biopterin; *q*$H_2B$, *quinonoid*-dihydrobiopterin; 4α-OH-$H_4B$, pterin-4α-carbinolamine; and "?", predicted activity, either an unidentified enzyme or spontaneous oxidation.

phenylalanemia (30–32). *q*DPR has been extensively characterized in mammalian cells, with its three-dimensional structure placing it within the family of short-chain dehydrogenases (33, 34). The *q*DPRs from most species show a strong dependence for NADH as a cofactor and for *quinonoid*-dihydrobiopterin (*q*$H_2B$) as the pteridine substrate (35).

Outside of metazoans there are few studies of *q*DPRs. Some prokaryotes exhibit *q*DPR activity (36) and a *Pseudomonas* *q*DPR has been characterized (37). In trypanosomatids *q*DPR activity has been found in crude lysates of *Leishmania major* and its relative *Crithidia fasciculata* (12, 38). Because of the importance of $H_4B$ to *Leishmania* metabolism and virulence, we decided to characterize the *q*DPR gene and enzyme from *L. major* (*LmQDPR*).

## EXPERIMENTAL PROCEDURES

*Reagents*—Biopterin and $H_2B$ were purchased from Schircks Laboratories (Jona, Switzerland). 6,7-Dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$), NADPH, NADH, and horseradish peroxidase were purchased from Sigma. Folate-deficient medium was custom manufactured by Invitrogen and is identical to M199 except that it lacks folate and thymidine (18). All other reagents were of analytical grade. Several pteridine analogs were tested for inhibition of LmQDPR activity whose structures, provenance, and usage were described previously (39).

*Parasites and Transfection*—The following strains were used: *L. major* Friedlin V1 (MHOM/JL/80/Friedlin) and CC-1 (MHOM/IR/83/IR; Ref. 40), a null mutant CC-1 *Leishmania* lacking PTR1 (*ptr1*$^-$) described previously (12), *Leishmania donovani* Sudanese strain 1S2D (MHOM/S.D./00/1S-2D), and *Leishmania mexicana* (MNYC/BZ/62/M379). Wild-type promastigotes were maintained by serial passage in M199 medium supplemented with 10% fetal calf serum at 26 °C; *ptr1*$^-$ parasite media additionally contained 2 μg/ml $H_2B$ (7). For *L. major*, metacyclic promastigotes were isolated from stationary phase 48-h cultures by negative selection with peanut agglutinin as described (41) and amastigotes were harvested from BALB/c mouse footpad lesions 3 weeks postinfection. Axenic culture and differentiation of *L. mexicana* amastigote were previously described (7). The procyclic *Trypanosoma brucei* cell line YTAT 1.1 (a gift from E. Ullu, Yale University) was propagated in Cunningham's SM medium supplemented with 10% heat inactivated fetal calf serum. Methods for DNA transfection of *Leishmania* by transfection were previously described (40) and clonal populations were obtained by plating on 20 μg/ml G418.

*Sequencing LmQDPR*—The sequences of five random shotgun clones of *L. major* strain Friedlin V1 (42) spanning the *QDPR* repeating unit were completed (lm18b06, strain B4501; lm61b05, strain B4181; lm25d04, strain B4180; and lm78e09, strain B4254; and lm62d04, strain B4502; GenBank™ number AF523363 and AY141854). This missing ~200 nt of the 3.6-kb *LmQDPR* repeating unit was amplified by PCR using primers SMB1465 (5′-AACATTGAGCGGCAGAGGATGT) and SMB1466 (5′-TGATGGTGCGGCACTCGCGGTA), with DNA from cosmid 1c15-2 (strain B4259). The PCR product was A-tailed and cloned into pGEM™-T Easy (Promega, Madison, WI) and sequenced (strain B4506, GenBank™ accession number AF523363). Dideoxynucleotide sequencing reactions were performed using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA).

*Southern and Northern Analysis—Leishmania* genomic DNA was isolated from late logarithmic phase promastigotes by the LiCl method (43). *T. brucei* and *Trypanosoma cruzi* genomic DNAs were prepared by phenol extraction as described (44). Total RNA was isolated from early and late logarithmic phase promastigotes, metacyclic cells, and lesion amastigotes by using the phenol/guanidine isothyocyanate reagent TRIzol™ (Invitrogen) according to the manufacturer's instructions. Southern and Northern blots were performed following standard procedures (45), and a PCR-derived *QDPR* hybridization probe (described below) was labeled with [α-$^{32}$P]dCTP by the random-priming method (46). Quantitation was performed with a laser densitometer (Molecular Dynamics with ImageQuant™ version 3.0; Molecular Dynamics). A *L. major* Friedlin V1 cosmid library prepared in the shuttle vector cLHYG (47) was gridded onto nylon membranes, and hybridized with the *L. major* *QDPR* full-length coding region probe. Four different cosmids containing *QDPR* were obtained (c1e16-1, strain B4257; c1c15-2, strain B4259; c7c21-2, strain B4260; and c12d12-3, strain B4264). Chromosomes were prepared and separated by pulsed field electrophoresis with a Bio-Rad Chef Mapper as described (48, 49).

*Mapping the 5′ Terminus of the Mature QDPR Transcript*—The 5′ terminus of the mature *L. major* *QDPR* transcript was determined by reverse transcriptase-PCR (50), with primers specific for the *L. major* spliced leader sequence (SMB936: 5′-ACCGCTATATAAGTATCAGTTCTGTACTTTA) and the *QDPR* coding region (SMB 1036: 5′-TTCACCCTGCGTACTGAACACAT; the 1st base is located 332 nt downstream of the *LmQDPR* ATG). Complementary DNA was made from 5 μg of stationary promastigote RNA primed with oligo(dT) by Superscript II reverse transcriptase (Invitrogen) prior to PCR amplification, and the PCR product was purified and sequenced directly.

*Trypanosome QDPR Sequencing*—A partial *QDPR* product was obtained by PCR amplification using primers based upon EST sequences (GenBank™ accession number AL390114; SMB 1609, 5′-ATGGCCCAAAAGAGCGGATTGG; SMB1610, 5′-CTGCCGGCTTGCACCCTGGCCA); it was inserted into pGEM™-T Easy and sequenced (strain B4586; GenBank™ accession number AF523371). From comparisons with the *LmQDPR* repeat and flanking regions, we assembled a preliminary contig for the syntenic *T. brucei* region (see legend to Fig. 4); as this assembly exhibited several gaps, we designed PCR primers to obtain the sequence of these (GenBank™ accession numbers AF523369 and AF523370). The contig sequences are available from the authors on request. Sequence data for *L. major* was obtained from The Sanger Institute website at www.sanger.ac.uk/Projects/L_major and was accomplished as part of the *Leishmania* Genome Network with support by The Wellcome Trust.

*Overexpression of LmQDPR in L. major*—The 690-nt *LmQDPR* open reading frame was amplified by PCR with *Pfu* polymerase (Stratagene) using primers SMB1084 (5′-gcggat**cc**acc**ATGAAAAATGTACTCCTCATCG; the underlined sequence corresponds to a *Bam*HI site and the bold nucleotides correspond to a "Kozak" sequence), SMB1085 (5′-cgggatcCTACACAATAAAACGCGTCTT), and 50 ng of template DNA (clone lm61b05). This PCR product was also used as a probe for Southern and Northern blot hybridization. The amplified DNA fragment was digested and cloned into the *Bam*HI site of the *Leishmania* expression vector pXG1a (51) in both orientations; the *QDPR* sequences were confirmed by sequencing. The resulting constructs (sense and antisense constructs pXG-QDPR and pXG-RPDQ, respectively; strains B4102 and B4103), were transfected into *Leishmania*.

*Purification of the Recombinant L. major qDPR*—pET-15b DNA (Novagen, Madison, WI) was digested with *Nde*I, blunt-ended with T4 DNA polymerase, and ligated to the *Bam*HI fragment from pXG-QDPR (also blunt-ended), yielding pET-QDPR (strain B4184; confirmed by sequencing). This provided a *LmQDPR* fusion construct bearing an N-terminal His tag.

For protein expression, pET-QDPR was transformed into *Escherichia coli* strain BLR(DE3) pLys-S (strain B4244; Ref. 52). A 500-ml culture was grown in L-broth medium plus ampicillin (100 μg/ml) to $A_{600}$ of 0.6, at 37 °C; isopropyl-β-D-thiogalactoside was added to a 1 mM final concentration and the culture was further incubated at 37 °C for another 4 h. Cells were harvested and resuspended in 25 ml of phosphate-buffered saline (150 mM NaCl, 16 mM $Na_2HPO_4$, 4 mM $NaH_2PO_4$, pH 7.3), lysed by sonication, and the debris was precipitated by centrifugation at 4,000 × *g* for 10 min. Five ml of the supernatant was loaded onto 1 ml of the $Ni^{2+}$-nitriloacetic acid resin affinity column (Qiagen), which was previously equilibrated with 10 ml of wash buffer (50 mM $NaH_2PO_4$, pH 8.0, 300 mM NaCl, 20 mM imidazole). The recombinant

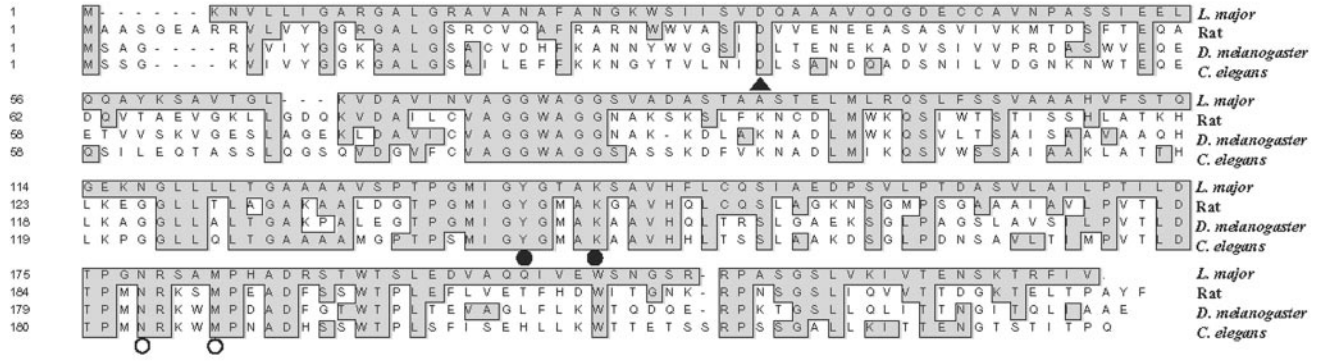


FIG. 2. **Alignment of eukaryotic *q*DPRs.** An amino acid sequence alignment of *q*DPRs from *L. major* (this work), rat (GenBank™ accession number P11348), *C. elegans* (T24395), and *D. melanogaster* (AAF50315) was performed using the Clustal algorithm implemented in the Lasergene software (DNASTAR, Inc.). Identical amino acids in all sequences are highlighted with a *gray background*. The residues interacting with NADH for hydride transfers are indicated by *filled circles*, the residues involved in substrate binding are indicated by *open circles*, and the residues involved in NADH binding preference are indicated by *filled triangles* (61–63).

protein was eluted with 2 ml of elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole). Proteins were analyzed by SDS-PAGE using a 15% acrylamide gel by standard protocols (53) and visualized by stained Coomassie Brilliant Blue.

*Preparation of Crude Leishmania, T. brucei, and T. cruzi Lysates*—*Leishmania* promastigotes and *T. brucei* procyclics were collected by centrifugation at 1,250 × *g* for 10 min at 4 °C, washed twice with phosphate-buffered saline, and resuspended at $2 \times 10^9$ cells/ml in 10 ml of Tris-Cl, pH 7.0, with 1 mM EDTA and a mixture of protease inhibitors as described (18). Frozen pellets of *T. cruzi* epimastigotes (Silvio strain) were generously provided by M. Pereira (Tufts University School of Medicine). Cells were lysed by three rounds of freeze thawing and sonication, and the extracts clarified by centrifugation at 15,000 × *g* for 30 min at 4 °C. Protein concentrations were determined by the Bradford method (54) with bovine serum albumin as a standard.

*Measurement of qDPR Activity and Inhibition Studies*—*q*DPR activity was measured at 25 °C as described (55) using the *quinonoid* form of 6,7-dimethyl-$H_2$-pterin (*q*$DMPH_2$). Because *quinonoid* pteridines are very unstable, they are continuously provided by the horseradish peroxidase-catalyzed oxidation of $DMPH_4$ in this assay. The standard reaction mixture contained 50 mM Tris-HCl, pH 7.2, 20 μg of horseradish peroxidase, 0.9 mM $H_2O_2$, 5–320 μM $DMPH_4$, 100 μM NADH, and 30 ng of purified *q*DPR, unless otherwise indicated. Experimentally, all components except $DMPH_4$ were incubated for 1 min prior to initiation of reaction by addition of $DMPH_4$. NADH consumption was measured by absorbance at 340 nm in a Beckman DU-640 spectrophotometer. The initial rates were obtained from the rate of decrease of absorbence at 340 nm ($\epsilon_{340}$ for NADH or NADPH is 6200 $M^{-1}$ $cm^{-1}$). The pH dependence was determined using two overlapping buffers: 50 mM sodium phosphate, pH 4.8–8.8, and 50 mM Tris-HCl, pH 7.0–11.1.

Inhibitor studies were performed with 10 μM inhibitor and 100 μM $DMPH_4$ and 100 μM NADH. All inhibitors except compound **66** were preincubated at 25 °C for 1 min in the presence of the complete reaction mixture containing 30 ng of purified LmQDPR, after which $DMPH_4$ was added to start the reaction. With compound **66**, addition of purified protein prior to $DMPH_4$ resulted in a decrease in absorbance; thus, purified LmQDPR was first added together with substrate, after which compound **66** was added. Water-insoluble compounds were dissolved in dimethyl sulfoxide ($Me_2SO$); in the final assay, the $Me_2SO$ concentration did not exceed 0.05%, a value that had no effect on *q*DPR activity (data not shown).

*Measurement of Intracellular Levels of Biopterin and $H_4B$*—Intracellular levels of biopterin and $H_4B$ were determined as described previously (7). Briefly, log phase promastigotes were isolated from the growth medium by centrifugation through a dibutylphthalate cushion. Cell pellets were subjected to either acid or alkaline oxidation and separated by high performance liquid chromatography. Under acidic conditions biopterin, $H_2B$, and $H_4B$ form biopterin, while under alkaline conditions biopterin and $H_2B$ form biopterin, whereas $H_4B$ forms pterin.

*Ferric Reductase Assay*—Ferric reductase activity was measured as described (56), with some modifications. Recombinant LmQDPR protein or sheep liver dihydropteridine reductase (Sigma) were added to a 250-μl reaction mixture containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM $NaH_2PO_4$, 5 mM Hepes, pH 7.4, 0.2 mM ferrozine, 50 μM ferric nitrilotriacetic acid (prepared as 1 mM ferric chloride, 4 mM nitrilotriacetic acid, 100 mM HCl, then the pH was adjusted to pH 6.8), with or without 100 μM NADH, in a microtiter plate. The rate of formation of NADH-dependent ferrozine complex was followed spectrophotometrically at 575 nm in a Molecular Devices Thermomax microtiter plate reader at 26 °C ($\epsilon$ = 27,900 $M^{-1}$ $cm^{-1}$). End point absorbance was measured after 60 min.

## RESULTS

### *Sequence Analysis of L. major QDPR*

In a shotgun survey of the *L. major* genome, we found several recombinants whose end sequences showed homology to *q*DPRs of other species (42). The sequences were completed, yielding a predicted LmQDPR protein encompassing 230 amino acids, with a predicted molecular mass of 25.6 kDa. Mapping of the 5′ end of the *LmQDPR* transcript confirmed the assignment of the start codon (described below). The predicted LmQDPR amino acid sequence was highly related to those of human, rat, *Caenorhabditis elegans,* and *Drosophila melanogaster*, showing 36–39% identity and 53–58% similarity (57–60) (Fig. 2). Significantly, key residues implicated by structural or mutational studies in substrate and cofactor binding in other *q*DPRs were conserved in the predicted LmQDPR. These included the Tyr-$(Xaa)_3$-Lys NADH binding motif (positions 138–42, marked by *solid circles* in Fig. 2), Asp-32, the residue implicated in preferential binding of NADH (marked by a *triangle* in Fig. 2), and two residues implicated in binding of *quinonoid* dihydropteridine (*open circles* in Fig. 1; Refs. 61–64). Additionally, distant relationships to the short-chain dehydrogenase protein family were detected, as expected for *q*DPRs (65, 66).

### *The LmQDPR Locus Contains a Tandemly Repeated Array of 8–9 Copies*

With an *LmQDPR* ORF probe and digestion with enzymes such as *Sma*I, *Not*I, or *Sac*I that do not cut within this probe, Southern blot analysis revealed strong hybridization to a band of 3.6 kb in each digest, as well as weaker hybridization to another band (Fig. 3*A*). This suggested the possibility that *LmQDPR* was organized as head-to-tail tandemly repeated genes. Consistent with this, Southern blot analysis using enzymes cutting once (*Ava*II, *Nae*I, and *Xho*I) or twice (*Pst*I) within the probe yielded patterns with two predominant hybridizing fragments, in each case adding to ~3.6 kb (Fig. 3*A*). Partial digestion with *Sac*I confirmed the presence of tandemly repeated *LmQDPR* genes (Fig. 3*B*), as Southern blot analysis yielded a ladder of fragments, ranging up to at least five 3.6-kb repeats (Fig. 3*B*). Preliminary mapping of four different cosmids each bearing ~40-kb inserts of *Leishmania* DNA span-

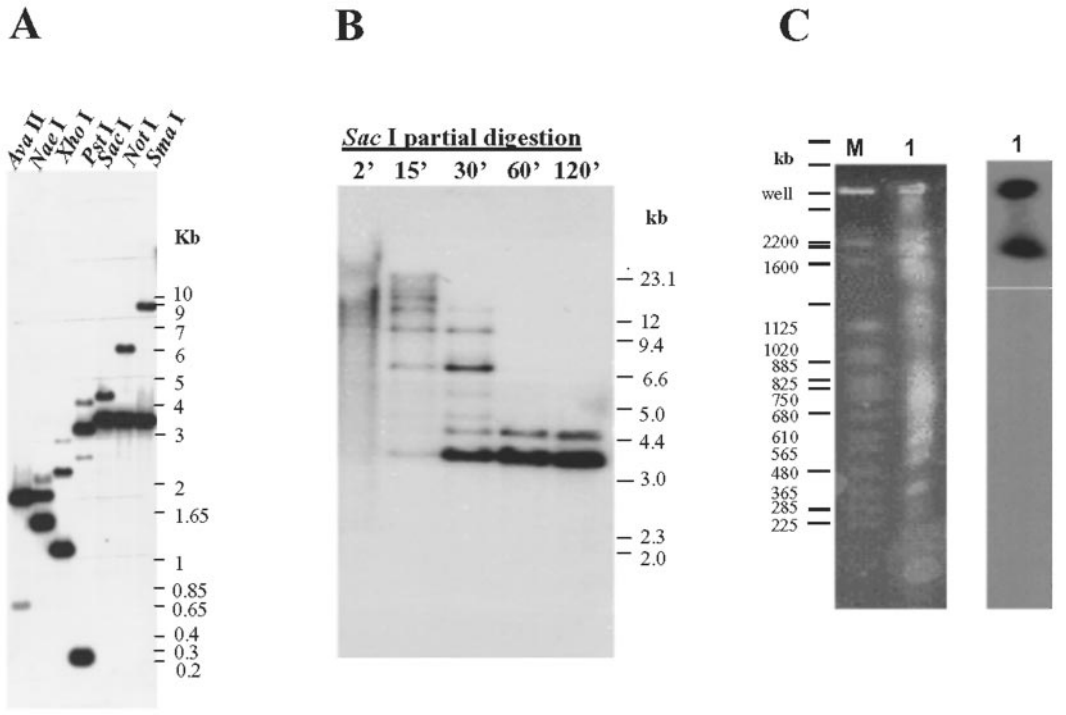


FIG. 3. **Southern blot analysis of the genomic organization of *L. major QDPR*.** In all panels, the hybridization probe was the *LmQDPR* ORF probe shown in Fig. 4*A*. *A*. Southern blot. *L. major* genomic DNA was completely digested with the indicated restriction enzymes, electrophoresed on a 0.8% agarose gel, blotted, and hybridized. The sizes of the molecular weight markers are shown. *B*, partial digestion with *Sac*I. A standard digestion of *L. major* genomic DNA was initiated (0.5 unit of *Sac*I, 7 $\mu$g of genomic DNA), and at the intervals indicated aliquots were withdrawn and the reactions stopped by adding EDTA (to 15 mM) and quick-freezing. Samples were electrophoresed on a 0.8% agarose gel, blotted, and hybridized. *C*, chromosomal mapping of *QDPR*. *L. major* chromosomes were resolved by pulsed field gel electrophoresis as described under "Experimental Procedures," blotted, and hybridized. The *left panel* shows the ethidium bromide-stained gel with *S. cerevisiae* chromosomal markers in kb (*M*), and the *right panel* shows the autoradiogram.

ning the *QDPR* locus also confirmed the presence of the 3.6-kb tandemly repeated array ("Experimental Procedures"; data not shown). Molecular karyotype analysis of separated *L. major* chromosomes showed hybridization to a single chromosome of about 2000 kb (Fig. 3*C*).

To estimate *LmQDPR* gene number, we compared the hybridization of the "unit" 3.6-kb fragment in the *Sac*I, *Not*I, or *Sma*I digests to that of the more weakly hybridizing band in each digest (Fig. 3*A*), which we reasoned corresponded to a single copy of the gene located at the end of the tandem array. By densitometry, the ratio of the "end" to the 3.6-kb unit fragments was 1 to 8.1, 7.5, or 7.2, respectively, suggesting that the *L. major QDPR* locus contains 8–9 copies.

### *The LmQDPR Repeating Unit Contains Two Other Unrelated Genes*

We identified molecular clones encompassing the entire *QDPR* repeating unit, determined their sequence ("Experimental Procedures"), and generated a consensus sequence for the 3541-bp *QDPR* repeating unit (Fig. 4*A*, GenBank™ accession number AF523363). In addition to *QDPR*, the repeating unit predicts the presence of two additional ORFs. One showed 70, 41, and 38% amino acid identity, respectively, to 20 S proteasome subunits from *T. brucei* ($\beta$7), humans, and *Arabidopsis* (GenBank™ accession numbers AF290945, D26600, and AF043538, respectively). The other ORF (ORF-q) comprised 112 amino acids, and did not show any relationship to other proteins in data base searches. ORF-q showed a high level of identity (86%) to a putative *T. brucei* ORF identified in the trypanosome genome project (data not shown), and was additionally identified by an EST from *L. major* amastigotes (GenBank™ accession number AA680881). While repeated gene families are common in *Leishmania*, ones whose repeating units include unrelated genes are relatively uncommon.

The sequences of the individual shotgun clones used to assemble the *QDPR* repeating unit were identical, except that there were 5 differences observed between the consensus and clone lm18b06. (GenBank™ accession no. AY141854.) One substitution was located between the *QDPR* and the 20 S proteasome $\beta$7 subunit intergenic region (marked by a *asterisk* in Fig. 4*A*). The other 4 differences were clustered and occurred in the 3′ half of the predicted 20 S proteasome $\beta$7 protein; as a consequence, a frameshift leading to the predicted formation of a subunit with a variant C′ terminus occurred (also marked by a *asterisk* in Fig. 4*A*). These data raise the possibility of microheterogeneity among the other genes encoded in the *QDPR* repeating unit.

### *Mapping the 5′ End of the LmQDPR mRNA*

In *Leishmania* and related protozoans, every mRNA contains a 39-nt "mini-exon" or "spliced leader" at its 5′ end that is added by *trans*-splicing (67). This enabled the mapping of the *LmQDPR* 5′ mRNA terminus by reverse transcriptase-PCR, using mini-exon and *QDPR*-specific primers. With an *LmQDPR* primer located 332 nt 3′ of the presumptive initiating ATG, a single ~500-nt product was obtained. Sequence analysis of this product mapped the 5′ splice acceptor to position −136 bp relative to the presumptive translation start codon (data not shown). No other ATG intervened between the *trans*-splice acceptor site and the initiating ATG for the *LmQDPR* ORF.

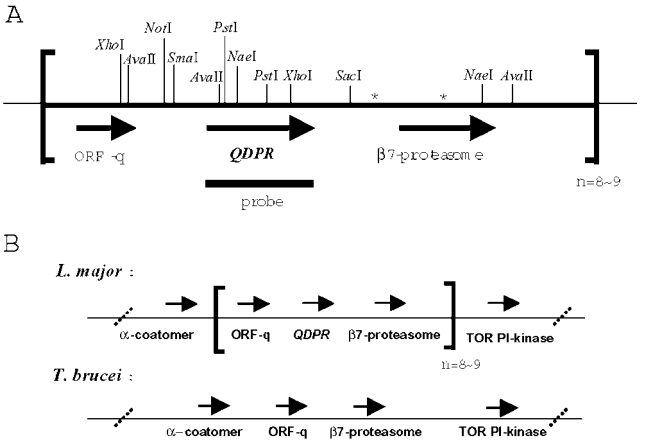


FIG. 4. **The *L. major QDPR* repeating unit contains two other genes while the syntenic region of *T. brucei* lacks *QDPR*.** *A*, map of the consensus *L. major QDPR* repeating unit. This was assembled from individual shotgun sequences as described in the text and is shown linearized arbitrarily at a *Bsa*BI site (GenBank™ accession numbers AF523363 and AY141854). *Arrows* show the coding regions for ORF-q, QDPR, and the 20 S proteasome β7 subunit. *A*, "*" indicates polymorphic sites as discussed in the text. *B*, comparison of the organization of the *QDPR* locus of *L. major* with a syntenic region of *T. brucei.* Preliminary contigs spanning the *LmQDPR* region or its equivalent in *T. brucei* were prepared using information deposited in GenBank™ or generously made available by the *Leishmania* and *T. brucei* genome projects (see "Acknowledgments"). The predicted gene order is shown; these regions are syntenic except for the absence of the *QDPR* in *T. brucei*. The contigs and specific sequence information used in their assembly are available from the authors by request.

### *Overexpression of LmQDPR in L. major*

The *LmQDPR* ORF was inserted in both orientations in the *Leishmania* expression vector pXG1a (51), which replicates as a multicopy episome. These constructs (as well as the empty vector) were transfected into wild-type CC-1 *L. major*. Assays of *q*DPR activity in crude lysates of these transfectants revealed 7–8-fold higher levels in the sense pXG-QDPR transfectants, when compared with the control or vector transfectants (Table I). Thus LmQDPR overexpression conferred elevated *q*DPR activity. Whereas the increase was less than typically seen by pXG-mediated overexpression of single copy genes (18), recall that there are 8–9 copies of *LmQDPR* already in the *Leishmania* genome (Fig. 3). No significant change in *q*DPR activity was seen with the antisense transfectants relative to controls (pXG-RPDQ; Table I), and the parasites grew normally.

### *qDPR Expression during the Leishmania Infectious Cycle*

Northern blots were used to determine *LmQDPR* mRNA levels throughout the *Leishmania* infectious cycle. A single 1.1-kb transcript was detected in all stages, with little variation during development (Fig. 5). After normalizing for total RNA loading with rRNA, the ratios of the intensities among early log, late log, and metacyclic promastigotes, and amastigotes were 1.3:1:1.8:1.4.

We measured *q*DPR activity in crude lysates from different growth phases or developmental stages of *L. major*, *L. mexicana,* and *L. donovani* (Table II). In stationary phase cultures, *Leishmania* promastigotes differentiate to the infective metacyclic stage that are most conveniently purified from *L. major* (68), although *L. mexicana* has the ability to differentiate to the amastigote stage *in vitro* ("Experimental Procedures"). In keeping with the mRNA levels (Fig. 5), there was little change in *q*DPR activity during the infectious cycle in *L. mexicana* or between log and stationary phase (Table II).

TABLE I
*QDPR activity in crude extracts of Leishmania LmQDPR transfectants*

Data presented are the mean ± S.D. from three experiments. Crude extracts of *Leishmania* were prepared as described under "Experimental Procedures" and the assay conditions were: 5–20 μg/ml soluble crude extract protein, $DMPH_4$ (100 μM) and NADH (100 μM).

| Parasite line | *q*DPR activity | Relative activity |
|---|---|---|
| | *nmol/min/mg* | |
| *L. major* CC1 (parent) | 163 ± 6 | 1 |
| pXG1a (empty vector) | 154 ± 7 | 1 |
| pXG-QDPR-clone 1 (sense) | 1100 ± 125 | 7 |
| pXG-QDPR-clone 2 (sense) | 1220 ± 100 | 8 |
| pXG-QDPR-clone 3 (sense) | 1190 ± 26 | 7 |
| pXG-RPDQ-clone 1 (antisense) | 156 ± 9 | 1 |

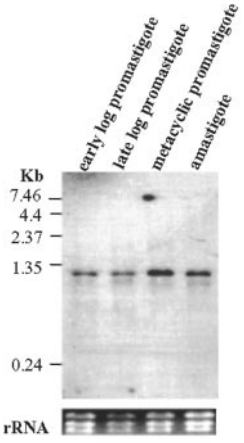


FIG. 5. **Expression of *QDPR* mRNA during the *Leishmania* infectious cycle.** Total RNAs (5 μg) were separated on a 1.5% formaldehyde-agarose gel, transferred to GeneScreen Plus, and hybridized with the *QDPR* probe (Fig. 4*A*). The autoradiogram is shown in the *upper panel* with the single ~1.1-kb *QDPR* transcript. Molecular weight standards (kb) are shown. The *lower panel* shows the ethidium bromide-stained gel region containing the three rRNAs as a loading control. Samples were RNAs from early logarithmic promastigotes (*lane 1*), late logarithmic promastigotes (*lane 2*), metacyclic promastigotes (*lane 3*), and amastigotes (*lane 4*).

### *Expression in E. coli and Enzymatic Characterization of Recombinant LmQDPR*

LmQDPR was expressed in *E. coli* as an N-terminal His-tagged fusion protein, under the control of an inducible T7 RNA polymerase system. After addition of isopropyl-1-thio-β-D-galactoside, a major protein band of about 28 kDa was observed following SDS-PAGE (Fig. 6*A*, *lane 3*), in agreement with the predicted size of the fusion protein. The recombinant His-tagged LmQDPR was purified by chromatography on nickel-agarose, yielding an apparently homogeneous protein (Fig. 6*A*, *lane 4*) with typical recoveries of ~0.2 mg of LmQDPR/500-ml bacterial culture. This preparation was active and its catalytic properties are summarized in Table III and below.

*Enzyme Activity*—The specific activity when assayed with the substrate *q*$DMPH_2$ was 2550 ± 145 μmol/min/mg (Fig. 6*C*). This was greater than that seen previously with rat and human *q*DPRs (300 and 450 μmol/min/mg, respectively; Ref. 34).

*pH Optimum*—Two overlapping buffers were used to assay *q*DPR activity over a pH range from 4.8 to 11.1, using saturating levels of NADH (100 μM) and *q*$DMPH_2$ (100 μM). Activity was maximal at pH 7.2 and dropped at more acid pH values, pH 4.8, and more gradually at more alkaline pH values (Fig. 6*B*). The pH optimum was similar to that of purified *q*DPRs from rat and *Pseudomomas* species (37, 62).

TABLE II
*qDPR activity in crude lysates of T. brucei, T. cruzi, and different Leishmania species in differing growth phases and developmental stages (nmol/min/mg)*

The mean ± S.D. of two independent experiences and six replicates are shown. Crude extracts were prepared as described under "Experimental Procedures." The assays included 100 μg of soluble crude extract protein per assay, NADH (100 μM) and $DMPH_4$ (10 μM). Whereas this concentration of substrate is below the $K_m$ for these species of *q*DPRs, it was necessary due to problems arising from interference we observed occurring in assays of crude extracts. Consistent with the results shown in Table I and below, preliminary studies using substrate concentrations of 320 μM $DMPH_4$ yielded specific activities of 177, 302, and 63 nmol/min/mg protein for log phase *L. major*, *T. brucei*, and *T. cruzi*, respectively.

| Parasite line | Log phase | Stationary phase | Axenic amastigotes |
|---|---|---|---|
| *L. major* Friedlin V1 | 42.1 ± 8.5 | 38.5 ± 9.0 | ND[a] |
| *L. major* CC1 | 22.6 ± 0.9 | 14.1 ± 1.8 | ND |
| *L. donovani* | 8.0 ± 2.0 | 8.9 ± 4.4 | ND |
| *L. mexicana* | 10.1 ± 3.6 | 5.7 ± 2.9 | 5.9 ± 1.3 |
| *T. brucei* (procyclics) | 151 ± 3.6 | 11.1 ± 6.0 | ND |
| *T. cruzi* (epimastigotes) | 14.2 ± 3.6 | 11.0 ± 1.9 | ND |

[a] ND, not determined.

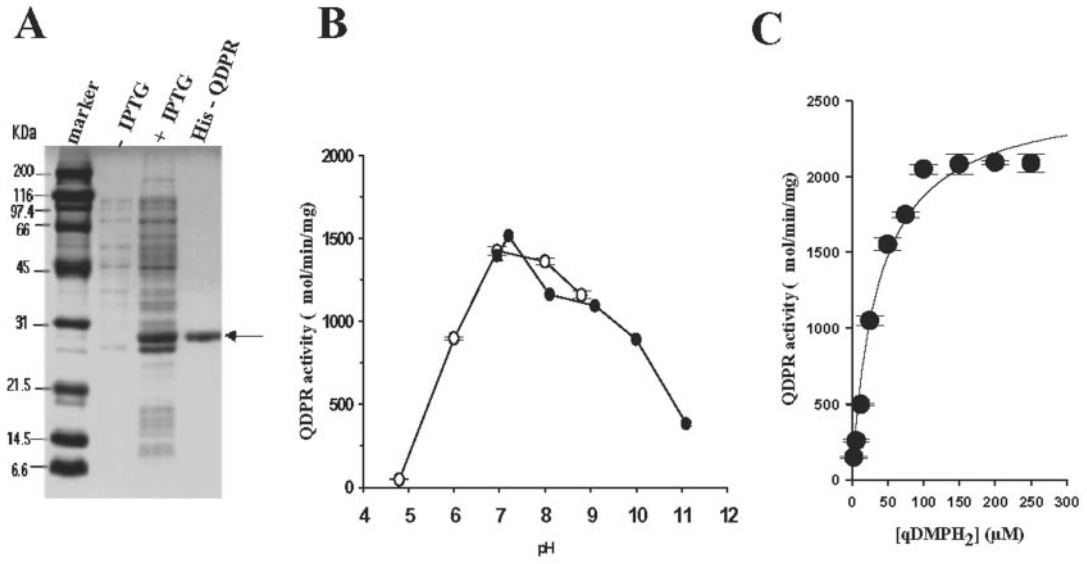


FIG. 6. **Expression and characterization of recombinant *L. major* QDPR.** *A*, purification of LmQDPR from *E. coli*. Samples were electrophoresed through a 15% SDS-polyacrylamide gel, and the gel was stained with Coomassie Brilliant Blue. *Lane 1*, molecular mass markers (in kDa); *lane 2*, crude extract of uninduced *E. coli*; *lane 3*, *E. coli* lysate after isopropyl-1-thio-β-D-galactoside induction; and *lane 4*, protein recovered after $Ni^{2+}$-nitrilotriacetic acid-agarose chromatography. The *arrow* indicates $His_6$-QDPR. *B*, pH profile. *q*DPR activity assays were performed with recombinant *L. major* QDPR (30 ng of purified recombinant protein/ml, 100 μM NADH, 100 μM $DMPH_4$) using two overlapping buffers at the indicated pH. ○, 50 mM sodium phosphate, and ●, 50 mM Tris-HCl. The values shown are the average of three independent experiments ± S.D. *C*, substrate dependence of *q*DPR activity. The activity of purified recombinant LmQDPR was assayed at the indicated concentrations of $qDMPH_2$.

TABLE III
*Enzymatic parameters of L. major and other qDPRs*

| Property | *L. major*[a] | Mammals[b] | *Pseudomonas*[c] |
|---|---|---|---|
| $K_m$ (NADH), μM | 23.1 ± 3.8[d] | 12 (11–13) | 90 |
| $K_m$ ($qDMPH_2$), μM | 36.5 ± 7.1[e] | 34 (27–41) | 340 |
| $V_{max}$ ($qDMPH_2$) (μmol/min/mg protein) | 2550 ± 145 | 330 | 5700 |
| Ferric reductase activity (nmol/min/mg protein) | 0.037 ± 0.005 | | |

[a] The mean ± S.D. from three experiments are shown.
[b] Data taken from Refs. 64 and 69.
[c] Data taken from Ref. 37; the substrate was 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine.
[d] 10 ng of purified recombinant protein, $DMPH_4$ fixed at 10 μM.
[e] 10 ng of purified recombinant protein, NADH fixed at 100 μM.

*Kinetic Parameters*—We determined a $K_m$ for $qDMPH_2$ of 36.5 ± 7.1 μM and for NADH of 23.1 ± 3.8 μM (Table III). These values are comparable with those reported previously for rat and human (11–13 μM for NADH and 27–41 μM for $qDMPH_2$) (64, 69).

*Substrate and Cofactor Specificity*—LmQDPR showed high specificity for the *quinonoid* substrates, as the activity for $H_2B$ was barely detectable (31.1 ± 0.8 nmol/min/mg) and about 66,000-fold less than obtained with *quinonoid* pteridine substrates. The specific activities when assayed with the substrate $qDMPH_2$ for NADH and NADPH were 1630 ± 12 and 10.2 ± 0.9 μmol/min/mg, respectively, yielding a substrate preference for NADH of ~160-fold.

*Ferric Reductase Activity*—Recently it was reported that mammalian *q*DPRs possess a pteridine-independent NADH-dependent ferric reductase activity (56). LmQDPR had detectable ferric reductase activity, although its specific activity was 308-fold less than that of the ferric reductase of *Mycobacterium paratuberculosis* (0.037 *versus* 11.4 nmol/min/mg, respectively; Table III) (70).

TABLE IV
*Biopterin levels in QDPR-overexpressing L. major*

Cells were grown in folate-deficient M199 (18) supplemented with 2 $\mu$g/ml $H_2B$ and collected in log phase. Total and $H_4$-biopterin were determined as described under "Experimental Procedures" from 6 replicates (mean ± S.D.).

| *Leishmania* cell line | Total biopterin | $H_4$-biopterin |
|---|---|---|
| | *pmol/10$^9$ cells* | |
| *L. major* CC1 | 2200 ± 290 | 1810 ± 200 |
| pXG1a | 2230 ± 240 | 1736 ± 90 |
| *ptr1*$^-$ | 1230 ± 160 | 756 ± 110 |
| *ptr1*$^-$ + pXG1a | 1110 ± 190 | 620 ± 120 |
| *ptr1*$^-$ + pXG-QDPR | 1000 ± 240 | 600 ± 80 |

### *LmQDPR Does Not Provide $H_2$biopterin Reductase Activity in Vivo*

Previous studies of *ptr1*$^-$ mutants suggested that *Leishmania* possesses a second activity capable of the reduction of $H_2B$ to $H_4B$, provisionally termed PTR2 (12, 18, 19). While LmQDPR had little $H_2B$ reductase activity, potentially even a low level of activity could lead to significant production of $H_4B$ over long periods of time *in vivo*. To test this idea, we introduced the pXG-QDPR construct into *ptr1*$^-$ null mutants, as the absence of PTR1-dependent $H_2B$ reductase activity would increase the ability to detect changes in $H_4B$ formation. Crude lysates from the *ptr1*$^-$/pXG-QDPR transfectants showed elevated *q*DPR activity, comparable with that of the wild-type pXG-QDPR transfectants (data not shown; Table I). Parasites were then grown in folate-deficient M199 medium with $H_2B$ as the sole exogenous pteridine source, and pteridine levels were determined during the logarithmic phase of the 4th passage by a high performance liquid chromatography-based method (7, 19). As seen previously, wild-type and vector control transfectant *Leishmania* contained ~2 nmol of biopterin/$10^9$ cells, of which 82% was present as $H_4B$, although the *ptr1*$^-$ mutant contains lower levels of biopterin, only 62% of which was $H_4B$ (Table IV). Despite expression of high levels of LmQDPR, and growth in $H_2B$ containing media, the *ptr1*$^-$/pXG-QDPR transfectants showed no elevation in total cellular biopterin or $H_4B$ levels, compared with the *ptr1*$^-$ mutant or control transfectants (Table IV). These findings suggest that LmQDPR is not the source of $H_2B$ reductase activity *in vivo*.

### *Tests of Candidate QDPR Inhibitors*

To date, only weak *q*DPR inhibitors have been identified (71, 72). Previously, we tested a collection of pteridine analogs for activity against *Leishmania* and purified pteridine reductases (39); several showed good activity against *Leishmania*, but in a PTR1 and DHFR-thymidylate synthase-independent manner. In contrast to their ability to inhibit *Leishmania* growth and the reductases significantly below 1 $\mu$M, these compounds showed minimal inhibition of LmQDPR, at best 60% inhibition with compounds **35**, **36**, **66,** and **70** when tested at 10 $\mu$M (Fig. 7). These data suggest that it is unlikely that LmQDPR is their target *in vivo*.

### *PCD and QDPR Homologs and QDPR Activity in Trypanosomes*

The presence of *q*DPR in *Leishmania* suggested that PCD, the first enzyme of the $H_4B$ regeneration cycle, should be present as well. A probable hit was found in the unannotated *L. major* sequence data base, and good candidates had been deposited previously for *T. brucei* (GenBank™ accession numbers T26730 and AL485250) and *T. cruzi* (GenBank™ accession number AI110297). In contrast, searches of the *T. brucei* genome with *q*DPRs of *Leishmania* or other species failed to identify a convincing hit, whereas a candidate *q*DPR EST was found in *T. cruzi* (GenBank™ accession numbers AA676052 and AF523371). In *T. brucei* we were able to identify homologs of the two other genes present in the *Leishmania QDPR* repeat (20 S proteasome $\beta$7 subunit and ORF-q), and from a combination of genomic project information as well as additional sequencing we were able to assemble a preliminary contig spanning this region. Unlike *Leishmania*, the 20 S proteasome $\beta$7 subunit and ORF-q genes appeared to be single copy, in agreement with previous studies of this locus (73). Comparisons with a preliminary contig of the *L. major QDPR* array and flanking regions with that of *T. brucei* showed considerable synteny, except for a gap occurring in *T. brucei* located where *QDPR* resided in *Leishmania* (Fig. 4*B* and data not shown). Southern blot data suggested that the *T. cruzi QDPR* may also be single copy (data not shown).

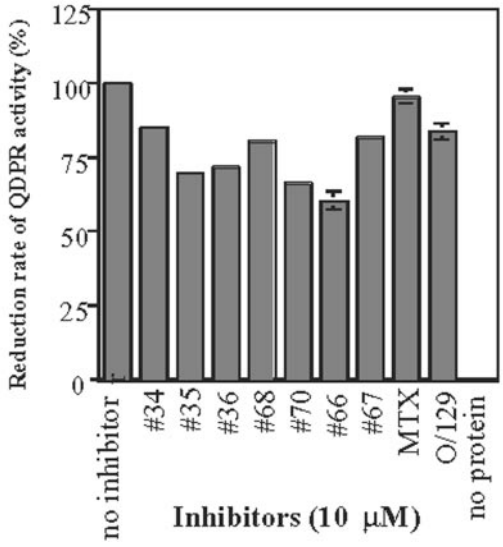


FIG. 7. **Tests of pteridine analogs for inhibition of purified recombinant LmQDPR.** *q*DPR activity was assayed in the presence of the indicated inhibitors whose structures can be found in Ref. 39. The results are expressed as percentage reduction activity with respect to a control without inhibition. The mean ± S.D. from three experiments are shown.

The above data raised the question of whether *T. brucei* possessed a *QDPR* gene. Because the genomes of neither *T. brucei* nor *T. cruzi* are completed as yet, we asked whether trypanosomes possessed *qDPR* activity. Lysates from log phase procyclic stage *T. brucei* and epimastigote stage *T. cruzi* showed good activity, with *T. brucei* being the highest (Table II). Preliminary studies suggested that the $K_m$ for *q*$DMPH_2$ of the trypanosomes was about 2–3-fold higher than that of recombinant LmQDPR (data not shown). Little developmental or growth phase regulation was observed in the three species of *Leishmania* or *T. cruzi*, in contrast to the 14-fold decreasing activity between log and stationary phases of *T. brucei* promastigotes (Table II).

## DISCUSSION

Nutritional and gene knockout studies have shown that $H_4B$ is essential for growth of *L. major in vitro* (12, 18) and that the levels of $H_4B$ are regulated and affect the ability of the parasite to differentiate into the infective metacyclic stage (7, 19). $H_4B$ levels are maintained primarily by regeneration of $H_4B$, which in mammals requires two enzymes, PCD and *q*DPR. In this report, we have shown that *Leishmania* possesses a *q*DPR whose sequence and enzymatic properties closely resemble those of mammalian *q*DPRs (Fig 2, Table III). *q*DPR activity was found at high levels throughout the parasite infectious cycle, confirming that *Leishmania* possesses an efficient $H_4B$ regeneration system akin to those of other organisms.

Northern blot and enzyme activity assays in three different *Leishmania* species show that *QDPR* is expressed constitutively throughout the infectious cycle (Fig. 5, Table II), as are the DHFR and PTR1 reductases required for activation of pteridines to the tetrahydro level (7). This is in keeping with the importance of $H_4B$ as a cofactor required for diverse aspects of parasite growth, differentiation, and virulence, raising the possibility that inhibition of parasite *q*DPR might be a potential target for chemotherapy. Therefore, we attempted to decrease *L. major q*DPR levels through expression of an antisense construct, however, this was unsuccessful (Table I) and the parasites grew normally. We also tested a panel of pteridine analogs that had previously been shown to inhibit *Leishmania* growth through PTR1- and DHFR-independent mechanisms (39), however, these were ineffective against LmQDPR (Fig. 7). As of yet, no strong inhibitors of *q*DPR in any species have been described (72). Thus, whereas we expect inhibition of *q*DPR to have important consequences to *Leishmania* growth, further studies involving gene inactivation or pharmacological inhibition will be required to formally establish this.

One unexpected finding was that *LmQDPR* is encoded as a tandemly repeated array bearing 8–9 copies of a unit encoding LmQDPR, an unidentified protein (ORF-q), and a 20 S proteasome $\beta$7 subunit (Figs. 3 and 4). While repeated gene families are common in *Leishmania* and trypanosomes, it is less common for the repeating units to contain unrelated genes. Because *q*DPR is encoded by a single copy gene in most species including *T. cruzi* (data not shown), it seems likely that *QDPR* underwent amplification in the lineage leading to *Leishmania*. Gene amplification in response to laboratory selective pressures or occurring spontaneously has been widely observed in *Leishmania*, especially for genes involved in pteridine metabolism (18, 74). While amplifications typically encompass contiguous regions bearing dozens of genes on circular or linear episomes, chromosomally integrated tandem repeats have been observed (74, 75).

These data invite speculations about the forces leading to amplification of *QDPR* in *Leishmania*. Potentially, it could be associated with a need for increased $H_4B$ levels accompanying adaptation of *Leishmania* to sand flies or inside of macrophages, as pteridine levels in these environments may be sufficiently limiting so as to force parasites to make the most efficient use of biopterin through regeneration. Another possibility comes from the finding that LmQDPR, like that of other species, exhibits ferric reductase activity (Table III). Iron levels frequently are limiting for the growth of pathogens, and ferric reductase has been shown to play an important role in iron acquisition in some species (76). However, the ferric reductase activity of recombinant LmQDPR is low (Table III). From its specific activity in crude extracts we calculate that the total ferric reductase activity conferred by *Leishmania* QDPR would be less than 0.002% of that found in *M. paratuberculosis*, where this activity has been found to contribute to virulence (70), thus casting doubt on this scenario in *Leishmania*. It is also possible that the driving force for amplification may not be directed at LmQDPR at all, but instead could arise from pressures involving the 20 S proteasome $\beta$7 subunit or ORF-q, both of which are highly conserved in trypanosomatids.

While not the focus of this work, more limited data with trypanosomes showed that these parasites also possess an efficient $H_4B$ regeneration system. Cell-free lysates derived from the insect stages of *T. brucei* and *T. cruzi* revealed that these parasites had substantial *q*DPR activity, with *T. brucei* being the highest and *T. cruzi* being lowest. While a *T. brucei QDPR* gene has not been found, a partial sequence for the *T. cruzi QDPR* was identified. Additionally, we were able to identify candidate *PCD* genes in the emerging genome project data bases for all three trypanosomatid species.

In summary, our studies of *LmQDPR* gene structure and enzymatic activity in *Leishmania* (as well as more limited data in trypanosomes) show that these parasites possess a potent system for regenerating $H_4B$, in common with other eukaryotes. This further serves to emphasize the importance of $H_4B$ metabolism in these organisms, although as yet the precise role of $H_4B$ remains to be established. Because $H_4B$ has been shown to be required for growth of several species of *Leishmania in vitro* and *in vivo* (12, 17, 18), our studies suggest that parasite *q*DPRs may prove to be useful targets for chemotherapy in the future.

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