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

Bioterpin is required for growth of the protozoan parasite Leishmania and is salvaged from the host through the activities of a novel biotin protein 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$_4$biotin 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 QRPs, although it lacked significant activity for non-quinonoid pteridines. These and other data suggest that LmQDPR is unlikely to encode the dihydrobiotin 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. QDPR activity was found in crude lysates of Trypanosoma brucei and Trypanosoma cruzi, further emphasizing the importance of H$_4$biotin 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 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 biotin (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 biotin, respectively, into the active forms, tetrahydrofolate (H$_4$folate) and tetrahydrobiotin (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-4α-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$_2$B: pterin-4α-carbinolamine dehydratase (PCD) (28) and quinonoid-dihydropteridine reductase (qDPR; Ref. 29, respectively (Fig. 1). Regeneration of H$_4$B allows organisms to efficiently use biotin cofactor in metabolism, and in humans qDPR 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

‡ To whom correspondence should be addressed: Dept. of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110.

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

2 The abbreviations used are: DHFR-TS; bifunctional dihydrofolate reductase-thymidylate synthase; qDPR, quinonoid-dihydropteridine reductase; LmQDPR/LmQDPR. Leishmania major qDPR gene/sequence; H$_4$B, dihydrobiotin; H$_4$B, tetrahydrobiotin; ORF, open reading frame; PTR1, pteridine reductase 1; PTR2, postulated dihydrobiotin reductase; PCD, pterin-4 α-carbinolamine dehydratase; DMPH$_4$ di-methyl-5,6,7,8-tetrahydropterin; nt, nucleotide(s); NOS, nitric oxide synthase.

phenylalanemia (30–32). qDPR has been extensively characterized in mammalian cells, with its three-dimensional structure placing it within the family of short-chain dehydrogenases (33, 34). The qDPRs from most species show a strong dependence for NADH as a cofactor and for quinoid-dihydrobipterin (qH₂B) as the pteridine substrate (35).

Outside of metazoa there are few studies of qDPRs. Some prokaryotes exhibit qDPR activity (36) and a Pseudomonas qDPR has been characterized (37). In trypanosomatids qDPR activity has been found in crude lysates of Leishmania major and its relative Crithidia fasciculata (12, 38). Because of the importance of H₂B to Leishmania metabolism and virulence, we decided to characterize the qDPR gene and enzyme from L. major (LmQDPR).

EXPERIMENTAL PROCEDURES

Reagents—Biotin and H₂B were purchased from Schircks Laboratories (Jona, Switzerland). 6,7-Dimethyl-5,6,7,8-tetrahydropterin (dH₂B) and horseradish peroxidase were purchased from Sigma. Folate-deficient medium was custom manufactured by Invitrogen and is identical to M99 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 predicted activity, either an unidentified enzyme or spontaneous dimerization at 4°C (39). For protein expression, pET-QDPR was transformed into E. coli (Novagen, Madison, WI) was digested with BamHI; the underlined sequence corresponds to a BamHI site and the restrictionenzyme “Kozak” sequence (5’-CAGGAGTACACATG), 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 BamHI site of the bacteriophage λ expression vector pXG1a (51) in both orientations; the resulting constructs (sense and antisense constructs pXG-QDPR and pXG-RPDQ, respectively; strains B4102 and B4103), were transfected into Leishmania.

Oxidized 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 B4259).

Sequence LmQDPR—The sequences of five random shotgun clones of L. major strain Friedlin V1 (42) spanning the qDPR repeating unit were completed (ln1806, strain B4501; ln1605, strain B4181; ln25604, strain B4180; and ln7609, strain B4254; and ln62604, strain B4502. GenBank™ accession number AF523363 and AY141854).

This missing ~200 nt of the 3.6-kb LmQDPR repeating unit was amplified by PCR using primers SMB1465 (5’-AACATTGAGCGCCAGGAGTGT) and SMB1466 (5’-TGATGTTGCGGACACTGGGTA), with DNA from cosmids 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).

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protein was eluted with 2 ml of elution buffer (50 mM Na2HPO4, 300 mM NaCl, 20 mM Tris-Cl, pH 7.0), 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 procyclids were collected by centrifugation at 1,250 × g for 10 min at 4 °C, washed twice with phosphate-buffered saline, and resuspended at 2 × 10⁷ cells/ml in 1 ml of Tris-Cl, pH 7.0, with 1 mM EDTA and a mixture of protease inhibitors as described (18). Frozen pellets of T. brucei epimastigotes (Silvio strain) were generously provided by M. Pereira (Tufts University School of Medicine). Cells were lysed by three rounds of freezing 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.

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 QDPRs 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 QDPRs were conserved in the predicted LmQDPR. These included the Tyr(Xaa)3-Lys NADH binding motif (positions 138–142, marked by solid circles in Fig. 2), Asp-32, the residue implicated in preference binding of NADH (marked by open circles 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 QDPRs (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 SmaI, NotI, or SacI 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. 3A). 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 (AvaII, NaeI, and XhoI) or twice (PstI) within the probe yielded patterns with two predominant hybridizing fragments, in each case adding to ~3.6 kb (Fig. 3A). Partial digestion with SacI confirmed the presence of tandemly repeated LmQDPR genes (Fig. 3B), as Southern blot analysis yielded a ladder of fragments, ranging up to at least five 3.6-kb repeats (Fig. 3B). Preliminary mapping of four different cosmids each bearing ~40-kb inserts of Leishmania DNA span-
A standard digestion of *L. major* bridization of the “...”mental Procedures...” the 3541-bp QDPR accession number AF523363). In addition to QDPR electrophoresed on a 0.8% agarose gel, blotted, and hybridized. The sizes of the molecular weight markers are shown. ORF probe shown in Fig. 4A 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.

To estimate *LmQDPR* gene number, we compared the hybridization of the “unit” 3.6-kb fragment in the *SacI*, *NotI*, or *SmaI* digests to that of the more weakly hybridizing band in each digest (Fig. 3A), 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. 4A, 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 β7 subunit from *T. brucei* (β7), 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 database 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 Im18b06. (GenBank™ accession no. AY141854.) One substitution was located between the QDPR and the 20 S proteasome β7 subunit intergenic region (marked by a asterisk in Fig. 4A). The other 4 differences were clustered and occurred in the 3′ half of the predicted 20 S proteasome β7 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. 4A). 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 BsoBI site (GenBank™ accession numbers AF523363 and AY141854). Arrows show the coding regions for ORF-q, QDPR, and the 20 S proteasome β subunit. A, n=1 indicates polymorphic sites as described 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 an episome. These constructs (as well as the empty vector) were transfected into wild-type CC-1 L. major. Assays of qDPR 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 qDPR activity. Whereas the level 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 qDPR 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 the different stages were 1.3:1:1.8:1.4.

We measured qDPR 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 qDPR activity during the infectious cycle in L. mexicana or between log and stationary phase (Table II).

Table I

<table>
<thead>
<tr>
<th>Parasite line</th>
<th>qDPR activity</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. major CC1 (parent)</td>
<td>163 ± 6</td>
<td>1</td>
</tr>
<tr>
<td>pXG1a (empty vector)</td>
<td>154 ± 7</td>
<td>1</td>
</tr>
<tr>
<td>pXG-QDPR-clone 1 (sense)</td>
<td>1100 ± 125</td>
<td>7</td>
</tr>
<tr>
<td>pXG-QDPR-clone 2 (sense)</td>
<td>1220 ± 100</td>
<td>8</td>
</tr>
<tr>
<td>pXG-QDPR-clone 3 (sense)</td>
<td>1190 ± 26</td>
<td>7</td>
</tr>
<tr>
<td>pXG-RPDQ-clone 1 (antisense)</td>
<td>156 ± 9</td>
<td>1</td>
</tr>
</tbody>
</table>

Expression in E. coli and Enzymatic Characterization of Recombinant LmQDPR

LmQDPR was expressed in E. coli as an N-terminal Histagged fusion protein, under the control of an inducible T7 RNA polymerase system. After addition of isoprpyl-1-thio-β-D-galactoside, a major protein band of about 28 kDa was observed following SDS-PAGE (Fig. 6A, 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. 6A, lane 4) with typical recoveries of approximately 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 qDMPH₂ was 2550 ± 145 μmol/min/mg (Fig. 6C). This was greater than that seen previously with rat and human qDPRs (300 and 450 μmol/min/mg, respectively; Ref. 34).

pH Optimum—Two overlapping buffers were used to assay qDPR activity over a pH range from 4.8 to 11.1, using saturating levels of NADH (100 μM) and qDMPH₂ (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. 6B). The pH optimum was similar to that of purified qDPRs from rat and Pseudomonas 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 (10 µM). Whereas this concentration of substrate is below the Km for these species of qDPRs, 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 yielded specific activities of 177, 302, and 63 nmol/min/mg protein for log phase L. major, T. brucei, and T. cruzi, respectively.

<table>
<thead>
<tr>
<th>Parasite line</th>
<th>Log phase</th>
<th>Stationary phase</th>
<th>Axenic amastigotes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. major</em> Friedlin V1</td>
<td>42.1 ± 8.5</td>
<td>38.5 ± 9.0</td>
<td>ND*</td>
</tr>
<tr>
<td><em>L. major</em> CC1</td>
<td>22.6 ± 0.9</td>
<td>14.1 ± 1.8</td>
<td>ND</td>
</tr>
<tr>
<td><em>L. donovani</em></td>
<td>8.0 ± 2.0</td>
<td>8.9 ± 4.4</td>
<td>ND</td>
</tr>
<tr>
<td><em>L. mexicana</em></td>
<td>10.1 ± 3.6</td>
<td>5.7 ± 2.9</td>
<td>5.9 ± 1.3</td>
</tr>
<tr>
<td><em>T. brucei</em> (procyclics)</td>
<td>151 ± 3.6</td>
<td>11.1 ± 6.0</td>
<td>ND</td>
</tr>
<tr>
<td><em>T. cruzi</em> (epimastigotes)</td>
<td>14.2 ± 3.6</td>
<td>11.0 ± 1.9</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined.

![Image of electrophoresis gel](image_url)

**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 Ni²⁺-nitrilotriacetic acid-agarose chromatography. The arrow indicates His₆-qDPR. B, pH profile. qDPR activity assays were performed with recombinant *L. major* QDPR (30 ng of purified recombinant protein/ml, 100 µM NADH, 100 µM DMPH) 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 qDPR activity. The activity of purified recombinant LmQDPR was assayed at the indicated concentrations of DMPH₂.

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

<table>
<thead>
<tr>
<th>Property</th>
<th><em>L. major</em>⁺</th>
<th>Mammals⁺⁺</th>
<th><em>Pseudomonas</em>⁺⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kₑa (NADH), µM</td>
<td>23.1 ± 3.8⁺</td>
<td>12 (11–13)</td>
<td>90</td>
</tr>
<tr>
<td>Kₑa (qDMPH₂), µM</td>
<td>36.5 ± 7.1⁺</td>
<td>34 (27–41)</td>
<td>340</td>
</tr>
<tr>
<td>Vₘ₉₉ (qDMPH₂) (µmol/min/mg protein)</td>
<td>2550 ± 145</td>
<td>330</td>
<td>5700</td>
</tr>
<tr>
<td>Ferric reductase activity (nmol/min/mg protein)</td>
<td>0.037 ± 0.005</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The mean ± S.D. from three experiments are shown.
⁺ Data taken from Refs. 64 and 69.
⁺⁺ Data taken from Ref. 37; the substrate was 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine.
⁺⁺⁺ 10 ng of purified recombinant protein, DMPH fixed at 10 µM.
⁺⁺⁺⁺ 10 ng of purified recombinant protein, NADH fixed at 100 µM.

**Kinetic Parameters**—We determined a Kₑa for qDMPH₂ 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 qDMPH₂) (64, 69).

**Substrate and Cofactor Specificity**—LmQDPR showed high specificity for the quinonoid substrates, as the activity for H₂B 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 qDMPH₂ 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 qDPRs 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

<table>
<thead>
<tr>
<th>Leishmania cell line</th>
<th>Total biopterin pmol/10^6 cells</th>
<th>H4-biopterin pmol/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. major CC1</td>
<td>2200 ± 290</td>
<td>1810 ± 200</td>
</tr>
<tr>
<td>pXG1a</td>
<td>2230 ± 240</td>
<td>1736 ± 90</td>
</tr>
<tr>
<td>ptr1</td>
<td>1230 ± 160</td>
<td>756 ± 110</td>
</tr>
<tr>
<td>ptr1&lt;sup&gt;+&lt;/sup&gt; + pXG1a</td>
<td>1110 ± 190</td>
<td>620 ± 120</td>
</tr>
<tr>
<td>ptr1&lt;sup&gt;+&lt;/sup&gt; + pXG-QDPR</td>
<td>1000 ± 240</td>
<td>600 ± 80</td>
</tr>
</tbody>
</table>

LmQDPR Does Not Provide H₄-biopterin Reductase Activity in Vivo

Previous studies of ptr<sup>1</sup> mutants suggested that Leishmania possesses a second activity capable of the reduction of H₂B to H₄B, provisionally termed PTR2 (12, 18, 19). While LmQDPR had little H₂B reductase activity, potentially even a low level of activity could lead to significant production of H₂B over long periods of time in vivo. To test this idea, we introduced the pXG-QDPR construct into ptr<sup>1</sup>-null mutants, as the absence of PTR1-dependent H₂B reductase activity would increase the ability to detect changes in H₂B formation. Crude lysates from the ptr<sup>1</sup>-/pXG-QDPR transfectants showed elevated qDPR 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 H₂B 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 transfec -

tants (Table IV). These findings suggest that LmQDPR is not the source of H₂B reductase activity in vivo.

Tests of Candidate QDPR Inhibitors

To date, only weak qDPR 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 μM, these compounds showed minimal inhibition or LmQDPR at best 60% inhibition with compounds 35, 36, 66, and 70 when tested at 10 μM (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 qDPR in Leishmania suggested that PCD, the first enzyme of the H₂B 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 qDPRs of Leishmania or other species failed to identify a convincing hit, whereas a candidate qDPR 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 β7 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 β7 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. 4B and data not shown). Southern blot data suggested that the T. cruzi QDPR may also be single copy (data not 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<sub>m</sub> for qDMPH<sub>2</sub> 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 H₂B is essential for growth of L. major in vitro (12, 18) and that the levels of H₂B are regulated and affect the ability of the parasite to differentiate into the infective metacyclic stage (7, 19). H₂B levels are maintained primarily by regeneration of H₂B, which in mammals requires two enzymes, PCD and qDPR. In this report, we have shown that Leishmania possesses a QDPR whose sequence and enzymatic properties closely resemble those of mammalian qDPRs (Fig 2, Table III). qDPR activity was found at high levels throughout the parasite infectious cycle, confirming that Leishmania possesses an efficient H₂B 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$_4$B as a cofactor for diverse aspects of parasite growth, differentiation, and virulence, raising the possibility that inhibition of parasite *qDPR* might be a potential target for chemotherapy. Therefore, we attempted to decrease *L. major* *qDPR* 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 *qDPR* in any species have been described (72). Thus, whereas we expect inhibition of *qDPR* 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 proteosome $\beta\beta$ 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 *qDPR* 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 speculation about the forces leading to amplification of *QDPR* in *Leishmania*. Potentially, it could be associated with a need for increased H$_4$B 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. tuberculosis*, 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 proteosome $\beta\beta$ subunit or ORF-q, both of which are highly conserved in trypanosomatids.

While not the focus of this work, more limited data with trypanosomals showed that these parasites also possess an efficient H$_4$B regeneration system. Cell-free lysates derived from the insect stages of *T. brucei* and *T. cruzi* revealed that these parasites had substantial *qDPR* 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$_4$B, in common with other eukaryotes. This further serves to emphasize the importance of H$_4$B metabolism in these organisms, although as yet the precise role of H$_4$B remains to be established. Because H$_4$B has been shown to be required for regulation of several species of *Leishmania in vitro* and *in vivo* (12, 17, 18), our studies suggest that parasite *qDPRs* may prove to be useful targets for chemotherapy in the future.

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11. T. brucei


