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Biochemistry

PTR1: A reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major*

(DNA transfection/gene targeting/null mutant/primaquine)

ALEXANDRE R. BELLO*†, BAKELA NARE*, DANIEL FREEDMAN*‡, LARRY HARDY§, AND STEPHEN M. BEVERLEY*

*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115; and §Department of Pharmacology and Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605

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ABSTRACT **Trypanosomatid protozoans are pterin auxotrophs, a finding noted decades ago which heralded the discovery of key metabolic roles played by pteridines in eukaryotes. We have now identified the enzyme mediating unconjugated pteridine salvage in the human parasite *Leishmania major*, PTR1 (*p*teridine *r*eductase *1*, formerly hmtx^r or ltdh). *PTR1* is the gene in the amplified H region responsible for methotrexate (MTX) resistance, and belongs to a large family of oxidoreductases with diverse substrates and roles. We generated *Leishmania* lacking *PTR1* by homologous gene targeting, and these *ptr1*$^-$ mutants required reduced biopterin (dihydro- or tetrahydrobiopterin) for growth. PTR1 purified from engineered *Escherichia coli* exhibited a MTX-sensitive, NADPH-dependent biopterin reductase activity. PTR1 showed good activity with folate and significant activity with dihydrofolate and dihydrobiopterin, but not with quinonoid dihydrobiopterin. PTR1 thus differs considerably from previously reported pteridine reductases of trypanosomatids and vertebrates. Pteridine reductase activity was diminished in *ptr1*$^-$ *Leishmania* and was elevated in transfected parasites bearing multiple copies of *PTR1*; correspondingly, *ptr1*$^-$ was MTX-hypersensitive whereas the multicopy transfectant was MTX-resistant. The concordance of the biochemical and genetic properties of PTR1 suggests that this is the primary enzyme mediating pteridine salvage. These findings suggest several possible mechanisms for PTR1-mediated MTX resistance and should aid in the design of rational chemotherapy.**

Leishmania is a genus of trypanosomatid protozoan parasites causing a spectrum of tropical diseases collectively termed leishmaniasis, varying in severity from mild cutaneous to fatal visceral disease. Currently the primary treatment consists of pentavalent antimonials, whose efficacy is often compromised by toxicity and whose mode of action remains unknown. There is an urgent need for an increased understanding of *Leishmania* biochemistry in order to permit the development of new drugs.

Trypanosomatids including *Leishmania* show many unusual features in pteridine metabolism. Historically, the eukaryotic metabolic requirement for unconjugated pterins was first shown in the related protozoan *Crithidia* (1, 2). This led to the discovery of the key roles of pteridines in hydroxylations, cleavages, and more recently as cofactors for NO synthase (3–5). Novel aspects of *Leishmania* pteridine metabolism include the presence of a bifunctional dihydrofolate reductase–thymidylate synthase (DHFR-TS), a growth requirement for exogenous pterins, *de novo* synthesis of folate from exogenous pterin, the modulation of methotrexate (MTX) potency over a $>10^5$-fold range by exogenous folate, and the ability of sublethal MTX concentrations to serve as a growth factor (6–13). Other than DHFR-TS, little is known about the enzymes involved.

PTR1 (*p*teridine *r*eductase *1*; formerly *hmtx*^{r} or *ltdh*) was identified as the gene within the *Leishmania* H region responsible for mediating MTX resistance following overexpression by gene amplification or DNA transfection (14, 15). The predicted PTR1 protein showed homology to a large family of aldo/keto reductases and short-chain dehydrogenases, including several enzymes involved in pteridine metabolism such as sepiapterin reductase (14) and dihydropteridine reductase (DHPR; ref. 16). Some DHPRs show dihydrofolate (H_2-folate) reductase activity and are inhibited by MTX (17), suggesting that PTR1 could provide an alternative source of H_2-folate. However, *Crithidia* DHPR does not reduce H_2-folate and is insensitive to MTX (18), and PTR1 is no more related to DHPR than to non-pteridine-metabolizing enzymes, especially in the C-terminal half implicated in substrate recognition (19, 20). We have used complementary biochemical and genetic approaches to dissect the role of PTR1 and have found that PTR1 possesses an oxidized-pteridine reductase activity responsible for pteridine salvage.

METHODS

Cell Culture and Transfection. All lines were derived from clone CC-1 of LT252 *Leishmania major* (21). Cells were grown in M199 medium containing 10% heat-inactivated fetal bovine serum (21). Transfections were performed by electroporation and plating on M199 semisolid medium containing selective drugs (21–23). Transfections intended to yield homologous replacements used linear DNA fragments whose cohesive ends had been filled in with T4 DNA polymerase (24). Additional medium supplements were tetrahydrobiopterin (H_4-biopterin) (Research Biochemicals), dihydrobiopterin (H_2-biopterin) (Schircks Laboratories, Jona, Switzerland), and thymidine, biopterin, folate, H_2-folate, tetrahydrofolate (H_4-folate), and leucovorin (5-formyl-H_4-folate) (all from Sigma). Pteridine stocks were made freshly in 40 mM Hepes, pH 7.4/5 mM dithiothreitol.

Molecular Techniques. *Leishmania* chromosomes were prepared in agarose plugs, separated by pulsed-field gel electrophoresis using a Bio-Rad CHEF Mapper, stained with ethidium bromide, and transferred to nylon membranes for Southern blot hybridization (25, 26).

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Abbreviations: H_2-biopterin, dihydrobiopterin; H_4-biopterin, tetrahydrobiopterin; MTX, methotrexate; DHFR-TS, dihydrofolate reductase–thymidylate synthase; DHPR, dihydropteridine reductase; H_2-folate, dihydrofolate; H_4-folate, tetrahydrofolate.

†Present address: Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 20560 Brazil.
‡Present address: Immucell Inc., Portland, ME 04103.

Expression of *PTR1* in *Escherichia coli*. The *PTR1* coding region was amplified by PCR using the primers 5′-cgcggatcccatATGACTGCTCCGACC-3′ and 5′-ggcggatccT-CAGGCCCGGGTAAGGCTGTA-3′ (lowercase letters represent bases not present in *PTR1*) with 100 ng of plasmid pSNBR-SstA (14) and 10 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C. The expected 0.9-kb product was purified by binding to glass beads, digested with *Bam*HI, and ligated into the *Bam*HI site of *Leishmania* expression vector pX63NEO (27) which had been digested with *Bam*HI and dephosphorylated. This construct (pX63NEO-PTR1) was transfected into *L. major* CC-1, and colonies obtained were further selected for resistance to G418 (512 μg/ml). These lines (such as CC-1/pX63NEO-PTR1 clone 2.3) were 180-fold resistant to MTX. The modified *PTR1* was excised from pX63NEO-PTR1 with *Nde* I and *Bam*HI and inserted into *E. coli* expression vector pET3a (28), yielding pET3a-PTR1. This was transformed into *E. coli* BL21(DE3)/pLysS (28).

Transformed *E. coli* were grown at 37°C and induced with isopropyl β-D-thiogalactopyranoside (28). Cells were harvested, resuspended in 50 mM Tris·HCl, pH 7.0/20 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride, and frozen at −80°C. Subsequent procedures were carried out at 4°C. Lysis was initiated by thawing and sonication, and debris was removed by centrifugation (8000 × *g*, 15 min). Proteins precipitated by ammonium sulfate (0.17 g/ml of lysate) were recovered by centrifugation and suspended in and dialyzed against 20 mM Tris·HCl (pH 7.0). Proteins were separated on a DEAE-cellulose column equilibrated with 20 mM Tris·HCl (pH 7.0) with a 0–0.3 M NaCl gradient. PTR1 was eluted at 0.12 M NaCl and the fractions with highest activity were used.

***Leishmania* Extracts.** Late-logarithmic-phase promastigotes were collected by centrifugation, washed twice, and frozen at −80°C. Cells were suspended and sonicated in 50 mM Tris·HCl, pH 7.0/1 mM EDTA/5 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/10% glycerol. Lysates were centrifuged at 15,000 × *g* for 20 min and the supernatants were taken for assay.

Pteridine Reductase Assays. PTR1 activity was measured at 30°C in 20 mM Tris·HCl (pH 7.0) with 0.1 mM NADPH and 0.1 mM pteridine substrate; NADPH oxidation was followed at 340 nm. As pteridines exhibit absorbance changes when reduced, the following extinction coefficients (compiled from the literature or determined empirically) were used for the coupled oxidation/reduction of NADPH/pteridine (biopterin, 7230; H_2-biopterin, 10420; folate, 6460; H_2-folate, 12260 $M^{-1}\cdot cm^{-1}$). Protein concentrations were determined by dye binding assay (29).

RESULTS

Homologous Replacement of *PTR1*. We described (23) the constructs pHM-NEO, pHM-HYG, and pHM-PAC), where the *PTR1* coding region was replaced with the selectable markers *NEO* (neomycin resistance), *HYG* (hygromycin resistance), and *PAC* (puromycin resistance), respectively. These contain 7.4- to 7.8-kb *Sst* I fragments bearing 2 kb of 5′ and 4.6 kb of 3′ genomic DNA flanking the *PTR1* coding region. These targeting fragments were transfected into *L. major* by protocols which favor homologous replacement (24). Since sexual crosses are not yet feasible in this diploid parasite, two rounds were required (22). Parasites were first transfected with the *HYG* targeting fragment. Southern blot hybridization of separated *Leishmania* chromosomes showed that all eight colonies examined contained the *HYG* marker integrated into a 900-kb chromosome indistinguishable in size from the wild-type *PTR1* chromosome (data not shown). These lines grew normally and retained a copy of the wild-type *PTR1*-bearing chromosome (Fig. 1, lane 5 vs. lanes 4 and 6), and PCR analysis confirmed the presence of both *HYG* and *PTR1* (data not shown). These transfectants were the planned *PTR1/HYG* heterozygote.

Since the second targeting round was expected to eliminate *PTR1*, there was concern about cell viability. We hypothesized that products arising from pterin and/or folate metabolic pathways would rescue *ptr1⁻* "knockout" mutants and used a supplemented medium (M199/BLT) containing H_4-biopterin, leucovorin, and thymidine (all at 10 μg/ml). Transfection of a *PTR1/HYG* heterozygote (clone HTH-2) with the *NEO* and *PAC* targeting fragments in the presence of hygromycin B and a second drug yielded colonies with good efficiencies, about 70% that of the circular DNA controls (10 vs. 15 colonies/μg of DNA). The DNA content of all lines was the same as that of CC-1 (data not shown; ref. 25).

Transfectants were analyzed by Southern blot hybridization, and PCR-based assays with primer pairs situated within the coding regions of *PTR1* and the three markers or flanking the *PTR1* coding region. All G418-resistant transfectants contained *NEO* and *HYG* while retaining *PTR1* (Group B; data not shown), and Southern blot analysis with a *PTR1* probe confirmed that the G418-resistant transfectants contained a normal 900-kb chromosome bearing *PTR1* (Fig. 1). Since episomal DNA was not detected, we presume that these lines have integrated the *NEO* fragment without replacement of *PTR1*, as observed previously with *DHFR-TS* (24, 25).

PCR analysis showed that the puromycin-resistant colonies contained *HYG* and *PAC* but lacked *PTR1* (data not shown). Southern blot analysis confirmed that the puromycin-resistant transfectants completely lacked *PTR1* (Fig. 1, lanes 1–3 vs. lanes 4–9). These double transfectants now contained *PAC* on a 900-kb chromosome, identical in size to the normal *PTR1* chromosome (data not shown). The *HYG/PAC* transfectants thus represented the planned double replacement mutant (hereafter referred to as *ptr1⁻*).

Phenotype of *ptr1⁻* Cells. When tested in unsupplemented M199 medium, the *ptr1⁻* mutant grew slowly through >20 serial passages (>120 cell doublings). The decrease in growth was variable (2- to 5-fold) and most noticeable in "aged"

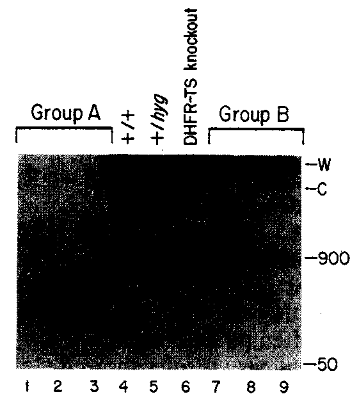

FIG. 1. Molecular karyotype analysis of *PTR1* "knockout" mutants. *Leishmania* chromosomes were separated by pulsed-field electrophoresis (Bio-Rad CHEF Mapper). Ethidium bromide staining revealed that the samples possessed similar amounts of DNA (data not shown). The autoradiogram of a Southern blot hybridization with a *PTR1* coding region probe (the *PTR1* PCR product) is shown. Group A (lanes 1–3, clones HTH-NT-1, -2, and -3, respectively) contains transfectants of the *PTR1/HYG* heterozygote (lane 5) which contain the *PAC* targeting fragment. Group B (lanes 7–9; clones HTH-NT-11, -16, and -17, respectively) contains transfectants of the *PTR1/HYG* heterozygote with the *NEO* targeting fragment. Other lines analyzed are wild-type *L. major* clone CC-1 (lane 4) the *PTR1/HYG* heterozygote clone HTH-2 (lane 5), and the *dhfr-ts⁻* clone E10-5A3 (22) (lane 6). W, sample well; C, compression zone; 900 and 50, size in kilobases.

M199 medium, suggesting that a labile component was involved. Addition of H_2- or H_4-biopterin at 1 μg/ml restored growth to the level of wild-type cells, whereas biopterin, folate, thymidine, or leucovorin at 9 μg/ml was ineffective (note that H_4-biopterin is very unstable and rapidly decays into a mixture of H_2-biopterin and other pteridines; ref. 30). In M199 medium prepared with 10% dialyzed serum (dM199, containing folate at 10 ng/ml), the *ptr1*$^-$ cells grew well in the first passage, poorly in the second, and failed to grow thereafter; wild-type cells grew normally (Fig. 2*A*). Wild-type growth was restored by H_2- or H_4-biopterin at 100–300 ng/ml, but not by thymidine, folate, H_2-folate, H_4-folate, or leucovorin at 9 μg/ml (Fig. 2*A*). This finding implicated *PTR1* in the salvage of oxidized biopterin.

We transfected *ptr1*$^-$ cells with pX63NEO-PTR1 and selected for G418 resistance. These transfectants now grew normally in dM199 medium, confirming that the lack of PTR1 was solely responsible for the reduced pterin requirement or the *ptr1*$^-$ mutant.

MTX Hypersensitivity of *ptr1*$^-$ Cells. Since PTR1 overproduction mediated by transfection or H-region amplification confers MTX resistance (14, 15, 31, 32), the MTX sensitivity of *ptr1*$^-$ cells was tested in medium where reduced pteridines were present but limiting for growth. The *ptr1*$^-$ mutant was hypersensitive to MTX, with an EC_{50} of 10–15 nM, compared with 150–300 nM for wild-type *L. major* (Fig. 2*B*). Inclusion of H_4-biopterin at 1 μg/ml reversed the sensitivity of *ptr1*$^-$ cells to 300 nM, whereas the wild-type EC_{50} remained unchanged (Fig. 2*B*). *ptr1*$^-$ cells in which high levels of *PTR1* expression had been restored by transfection of pX63NEO-PTR1 showed elevated MTX resistance (EC_{50} > 10 μM; Fig. 2*B*).

Amplification of *PTR1* and the *Leishmania* H region has been induced in response to other drugs such as terbinafine and primaquine (33). No differences in terbinafine sensitivity were observed in any of the *PTR1*-modified lines described here, and the *PTR1* overexpressor showed little resistance to primaquine (Fig. 2*C*). However, the *ptr1*$^-$ mutant showed hypersensitivity to primaquine (EC_{50}, 3 vs. 13 μg/ml; Fig. 2*C*).

Heterologous Expression of *PTR1* in *E. coli.* *PTR1* was expressed in *E. coli* using the T7 RNA polymerase system (28). Induced extracts showed the appearance of an abundant 30-kDa protein, as expected (14). This protein constituted up to 30% of the induced-cell soluble protein (Fig. 3, lane 3). PTR1 was soluble and stable and was purified (>95%) by a combination of ammonium sulfate precipitation and DEAE column chromatography (Fig. 3, lane 4; more recent preparations show only a single 30-kDa band whose properties are identical to those described below).

Assays of purified PTR1 revealed an NADPH-dependent biopterin reductase activity that was maximal at pH 4.7 and declined to 20% at pH 7.0 (Fig. 4*A*). Biopterin reductase activity with NADH was <5% the NADPH-dependent activity. PTR1 exhibited no detectable activity with quinonoid H_2-biopterin (preliminary results showed clear DHPR activity in crude *Leishmania* extracts). The biopterin reductase activity of PTR1 was sensitive to MTX (assayed with 100 μM biopterin, 30 nM PTR1, and 20–100 nM MTX). The K_i was estimated as 8 nM from a graphical plot of fractional activity vs. MTX concentration, as recommended for tight-binding inhibitors (35). No inhibition of biopterin reductase activity was observed with primaquine (10 μM).

PTR1 also showed significant activity with H_2-biopterin, folate, and H_2-folate at all pH values tested (Fig. 4*A*). Relative to biopterin, H_2-biopterin reductase activity was about 10–20% and was also maximal at pH 4.7. Folate reductase activity was about 30–100% that observed with biopterin, whereas H_2-folate reductase activity was about 50% that with H_2-biopterin. However, with both folates PTR1 activity was maximal at pH 6. PTR1 H_2-folate reductase activity was inhibited only 50% by 1.25 μM MTX (enzyme concentration, 0.13 μM), indicating that it does not arise from a contaminating DHFR.

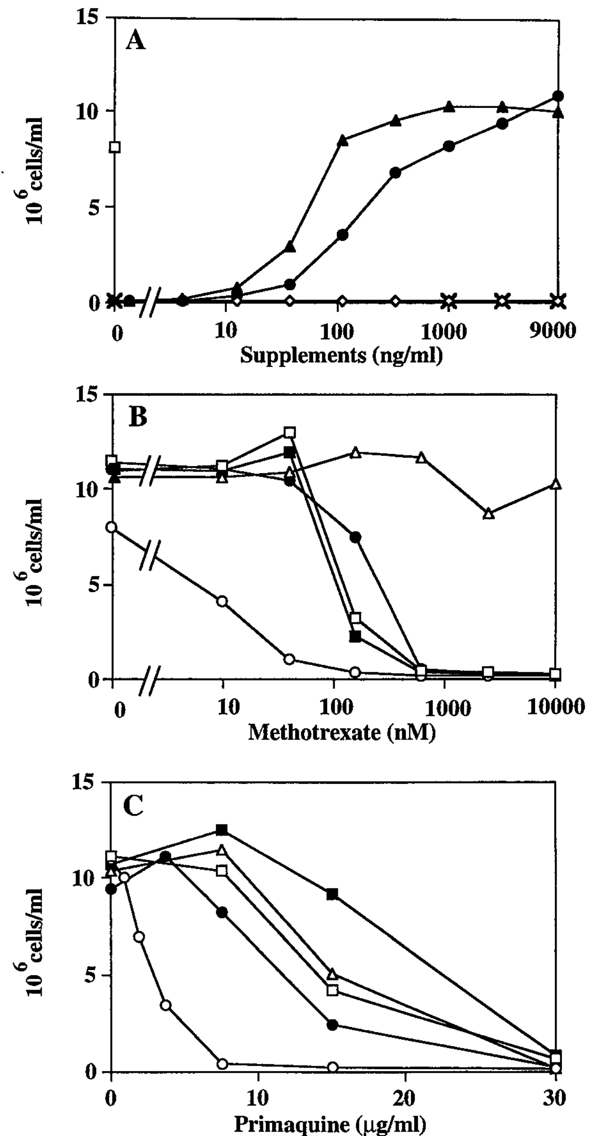

FIG. 2. Growth requirements and MTX inhibition of *PTR1* knockout mutants. The standard deviation for the number of cells is typically ±10%, and each experiment was performed at least three times with similar results; a representative experiment is shown. (*A*) Effect of metabolic supplements. *ptr1*$^-$ clone 3.1 was grown one passage (1:100 dilution) in dM199 to deplete reduced pterin pools and then inoculated at 10^5 cells per ml into dM199 containing the indicated supplements and grown for 96 hr, and counted with a Coulter Counter. Medium supplements were none or thymidine, folate, H_2-folate, H_4-folate or leucovorin (X, these values are identical and combined for clarity); biopterin (◇), H_2-biopterin (▲), or H_4-biopterin (●). The growth of wild-type CC-1 in unsupplemented medium is also shown (□). Similar results were obtained with cells grown in "aged" M199 medium. (*B*) Wild-type *L. major* (clone CC-1) and the *ptr1*$^-$ clone 3.1 were passaged twice (1:100 dilutions) in dM199 medium and then inoculated at 10^5 cells per ml in M199 containing MTX as indicated without (open symbols) or with H_4-biopterin (filled symbols). Lines are CC-1 (squares), *ptr1*$^-$ (circles), and *ptr1*$^-$ transfected with pX63NEO-PTR1 (triangles). A representative experiment is shown. Similar results were obtained with cells grown in "aged" M199 medium. (*C*) These experiments were performed as described in *B*, with primaquine instead of MTX.

PTR1 Activity in *Leishmania.* Biopterin reductase activity in crude *Leishmania* extracts correlated well with the pre-

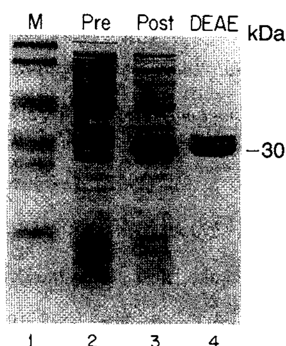

FIG. 3. Purification of *L. major* PTR1 synthesized in *E. coli*. Coomassie blue-stained proteins separated by SDS/10% polyacrylamide electrophoresis (34) are shown. Lane 1, molecular size markers; lane 2, extract of pET3a-PTR1-transformed *E. coli* prior to induction; lane 3, extract of pET3a-PTR1-transformed *E. coli* after induction; lane 4, purified PTR1 (from DEAE column).

dicted expression from *PTR1*. The activity in wild-type *L. major* was 1.3 nmol/min per mg of protein, whereas it was decreased to about 22% in the $ptr1^-$ mutant (Fig. 4*B*). The residual activity in $ptr1^-$ cells may reflect difficulties in assaying crude extracts, or the presence of other enzymes. In $ptr1^-$ cells transfected with pX63NEO-PTR1, biopterin reductase activity was increased to 320% (Fig. 4*B*). These data

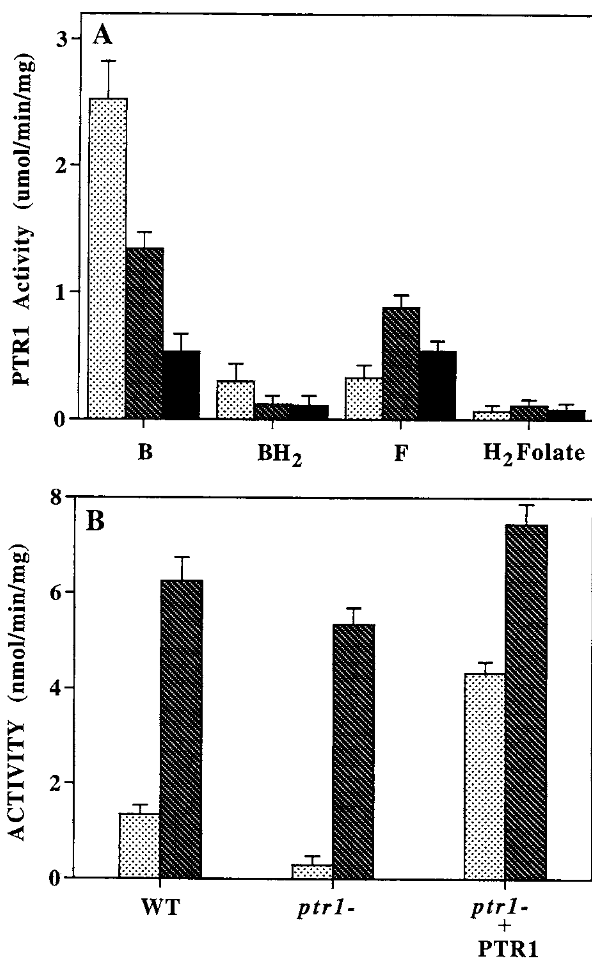

FIG. 4. Enzymatic assays with pteridine substrates. (*A*) Purified PTR1 was assayed with the biopterin (B), H_2-biopterin (BH_2), folate (F), or H_2-folate as substrate at pH 4.7 (20 mM Na acetate, stippled bars), pH 6.0 (20 mM $NaPO_4$, hatched bars), and pH 7.0 (20 mM Tris·HCl, filled bars). The concentration of NADPH and all pteridines was 100 μM, and 2–5 μg of purified PTR1 was used. Assays were performed in triplicate on several different enzyme preparations; the standard errors are shown. (*B*) Assay of crude *Leishmania* extracts. Total cellular extracts were made from *L. major* lines CC-1, $ptr1^-$, and $ptr1^-$ transfected with pX63NEO-PTR1 and were assayed at pH 7.0 for biopterin (stippled bars) and H_2-folate (hatched bars) reductase activity. Assays at other pH values were impossible due to interference from substrate-independent absorbance changes.

confirm that PTR1 is responsible for pteridine reduction within *Leishmania* extracts. Similar results were obtained with H_2-folate reductase activity, although the PTR1-dependent activity is superimposed upon a higher level of activity probably arising from DHFR-TS (Fig. 4*B*).

DISCUSSION

We have shown by biochemical and genetic criteria that PTR1 (formerly hmtx^r) mediates salvage of oxidized pteridines in *Leishmania*. Purified PTR1 exhibits a broad pteridine reductase activity, capable of reducing both unconjugated (biopterin) and conjugated (folate) pteridines from either the oxidized or the dihydro state. PTR1 activity was NADPH-dependent and highly sensitive to MTX (K_i = 8 nM). Quinonoid H_2-biopterin was not a substrate, indicating that PTR1 is not a DHPR despite its homology to this enzyme (16).

Complementing the biochemical analysis, a *PTR1*-null mutant ($ptr1^-$) exhibited an absolute requirement for reduced biopterin, a product of PTR1. Low levels of H_2- or H_4-biopterin (100 ng/ml) were sufficient to sustain growth. An excellent correlation was obtained with PTR1 activity and *PTR1* gene dosage. Correspondingly, the $ptr1^-$ mutant was hypersensitive to MTX whereas *PTR1* overexpressors were MTX-resistant. Given the enzymatic activities of PTR1, one would not predict that H_2-biopterin would rescue the $ptr1^-$ mutant. However, DHFR from many species has H_2-biopterin reductase activity (reviewed in ref. 36), and it is possible that *Leishmania* DHFR-TS or another pteridine reductase may compensate for the absence of PTR1-derived H_2-biopterin reductase activity.

Similar MTX sensitivity results were obtained with the *L. tarentolae PTR1* (*ltdh*) null mutants and overexpressors (32). Since reduced biopterins were not tested, no specific rescue of the null mutant was observed. Although it was suggested that the *L. tarentolae* enzyme mediated interconversions of folates and pterins, we have recently found that the purified *L. tarentolae* enzyme possesses the same activities shown here for *L. major* PTR1 (unpublished work).

The properties of PTR1 suggest several ways that its overproduction could mediate MTX resistance. As PTR1 binds MTX tightly, it could sequester MTX from DHFR-TS. However, since PTR1 is less sensitive to MTX (K_i = 8 vs. 0.13 nM; ref. 37), we believe that its diverse enzymatic activities are better candidates. PTR1 H_2-folate reductase activity could metabolically bypass DHFR(-TS). While the specific activity of PTR1 with H_2-folate is low, ≈10% of the total H_2-folate reductase activity in wild-type *Leishmania* extracts is attributable to PTR1 (Fig. 4). Overexpression by transfection or amplification could provide sufficient H_2-folate reductase activity to overcome MTX inhibition. Another possibility arises from the folate reductase activity of PTR1, which, by increasing H_2-folate levels, could relieve MTX inhibition and increase the metabolic flux through both PTR1 and DHFR-TS (38). Both of these mechanisms postulate a protective role for PTR1 in MTX inhibition of DHFR-TS, consistent with the MTX hypersensitivity of the $ptr1^-$ mutant. Finally, PTR1 overexpression should also increase H_4-biopterin pools and alleviate inhibition from lack of reduced unconjugated pteridines. While the role of H_4-biopterin in *Leishmania* and related trypanosomatids is unknown, a role in cleavage of surface glycolipids has been postulated (25). These models are not mutually exclusive and several could contribute to MTX resistance.

The enzymatic characterization of PTR1 provides some perspective on other aspects of pteridine metabolism and inhibition in *Leishmania*. Unlike mammalian cells, the potency of MTX can be modulated by a factor of 10^5 by increasing external folate to 4 μg/ml, a level found in many

common culture media (refs. 8, 9, 11, and 31; unpublished results). *Leishmania* can efficiently transport folate, and although this transport is sensitive to MTX the K_1 is very high (2 μM; refs. 39 and 40). Once transported, folates are retained by polyglutamylation whereas MTX is not, instead being destroyed by a potent MTX hydrolase (8, 41, 42). Under conditions of low or limiting folate, the ability of PTR1 to synthesize both H_2-folate and H_4-folate could readily modulate the potency of MTX as outlined above.

Amplification of the H region was observed following primaquine selection, although the resistance was weak (33). While the multicopy *PTR1* transfectants show little resistance, the *ptr1*$^-$ mutant shows clear hypersensitivity to primaquine (Fig. 2*C*). These data support the role of the H region and *PTR1* in primaquine resistance. Primaquine did not inhibit PTR1 activity, and it is unknown how it inhibits *Leishmania* growth or interacts with pteridine metabolism. One speculation is that primaquine-induced oxidant stress could be countered by reduced pteridines arising from PTR1 activity. The magnitude of the effect seen in the null mutant relative to the overexpressor suggests that tests on null mutants may be more sensitive indicators of drug interactions.

Although PTR1 shows significant homology with mammalian DHPRs, it lacks DHPR activity and displays properties that are unique relative to other known pteridine reductases. DHFR-TS, DHPR, and another pteridine reductase have been purified from the related trypanosomatid *Crithidia fasciculata*. DHFR-TS possesses activity with H_2-folate but not with folate, biopterin, or H_2-biopterin (43), whereas the pteridine reductase shows activity with H_2-folate and H_2-biopterin but not biopterin or folate (44–46). Both of these enzymes (and PTR1) are MTX-sensitive, whereas DHPR is insensitive (18). Relative to H_2-folate, mammalian DHFRs display moderate activity with H_2-biopterin, weak activity with folate, and little if any activity with biopterin (47). In contrast, PTR1 has a potent biopterin reductase activity. In conjunction with its distant evolutionary position and distinctive properties evident in this work, PTR1 will be attractive for detailed studies of structure and catalytic mechanism in comparison to previously studied pteridine reductases.

Historically, the requirement of *Crithidia* for exogenous pteridines was the first clue leading to the realization of the importance of unconjugated pterins in intermediary metabolism (see Introduction). The properties of PTR1 provide a missing link in this story, as our data suggest that PTR1 is the previously undetected enzyme responsible for efficient salvage of oxidized pteridines in trypanosomatids. This is a significant metabolic duty, as *Leishmania* and *Crithidia* are incapable of the *de novo* synthesis of pteridines and must obtain them exclusively by salvage. This suggests that inhibition of PTR1 could be a logical target for chemotherapy, especially in combination with DHFR-TS inhibitors.

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