Phenylalanine hydroxylase (PAH) from the lower eukaryote Leishmania major

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ABSTRACT
Aromatic amino acid hydroxylases (AAAH) typically use tetrahydrobiopterin (H4B) as the cofactor. The protozoan parasite Leishmania major requires biopterin for growth and expresses strong salvage and regeneration systems to maintain H4B levels. Here we explored the consequences of genetic manipulation of the sole L. major phenylalanine hydroxylase (PAH) to explore whether it could account for the Leishmania H4B requirement. L. major PAH resembles AAAHs of other organisms, bearing eukaryotic-type domain organization, and conservation of key catalytic residues including those implicated in pteridine binding. A pah− null mutant and an episomal complemented overexpressing derivative (pah−/+) were readily obtained, and metabolic labeling studies established that PAH was required to hydroxylate Phe to Tyr. Neither WT nor overexpressing lines were able to hydroxylate radiolabeled tyrosine or tryptophan, nor to synthesize catecholamines. WT but not pah− parasites showed reactivity with an antibody to melanin when grown with l-3,4-dihydroxyphenylalanine (l-DOPA), although the reactive product is unlikely to be melanin sensu strictu. WT was auxotrophic for Phe, Trp and Tyr, suggesting that PAH activity was insufficient to meet normal Tyr requirements. However, pah− showed an increased sensitivity to Tyr deprivation, while the pah−/+PAH overexpressor showed increased survival and could be adapted to grow well without added Tyr. pah− showed no alterations in H4B-dependent differentiation, as established by in vitro metacyclogenesis, or survival in mouse or macrophage infections. Thus Leishmania PAH may mitigate but not alleviate Tyr auxotrophy, but plays no essential role in the steps of the parasite infectious cycle. These findings suggest PAH is unlikely to explain the Leishmania requirement for biopterin.

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1. Introduction
Leishmania is a genus of trypanosomatid protozoan parasites comprising more than 20 species responsible for a number of severe diseases of humans worldwide, infecting over 12 million people [1]. Leishmania are transmitted by the bite of Phlebotomine sand flies, and in the mammalian host reside primarily in an acidified phagolysosome of macrophages, where they must deflect host defenses and immune responses in order to survive [2]. As a deep-branching eukaryotic lineage, Leishmania exhibits considerable divergence in its metabolic enzyme repertoire [3], providing opportunities for basic studies as well as more practical efforts towards improved chemotherapy.

Here we focus on genetic studies of null and overexpression mutants of the sole Leishmania gene related to aromatic amino acid hydroxylases (AAAH). Our interest in AAAHs arose because their cofactor biopterin is an essential growth factor, distinct from folate, for several trypanosomatid species. In mammals tetrahydrobiopterin (H4B) is required by several enzymes of critical metabolic importance, including amino acid hydroxylases (AAAHs), nitric oxide synthase and the ether lipid cleavage monoxygenase (reviewed in [4,5]). Leishmania and other trypanosomatids are general auxotrophs for pteridines [6,7], and acquire biopterin from the host by salvage, first by uptake by the transporter BT1 [8,9] and then reduction to H4B by the broad spectrum pteridine reductase PTR1 [10], reviewed by [11,12]). The direct pteridine product of AAAH action is 4-oxo-H4B (carbinolamine) which is returned to H4B through the successive action of pteridine-4-carbinolamine dehydratase (PCD) and quinonoid dihydropteridine reductase [QDPR; 4]. Trypanosomatids encode functional forms of both genes [13,14].

Despite knowledge of H4B metabolism and requirements in Leishmania, the essential metabolic role of H4B is not established [15]. In other species, AAAH activity requires the

Abbreviations: PAH, phenylalanine hydroxylase; AAAH, aromatic amino acid hydroxylase; LmjPAH/LmPAH, Leishmania major phenylalanine hydroxylase gene/enzyme; H4B, dihydrobiopterin; H2B, tetrahydrobiopterin; bp, base pair(s); ORF, open reading frame; nt, nucleotide(s); PCR, polymerase chain reaction; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; WT, wild-type; l-DOPA, L-dihydroxyphenylalanine.

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amino acid substrate, molecular oxygen, and the reduced cofactor H4B (6-keto-erythro-5,6,7,8-tetrahydropterin; [16]). AAAHs catalyze the conversion of phenylalanine, tyrosine and/or tryptophan to tyrosine, 3,4-dihydroxyphenylalanine (L-DOPA), or 5-hydroxytryptophan, respectively. Typically these activities are carried out by different enzymes, phenylalanine hydroxylase (PAH; EC 1.14.16.1), tyrosine hydroxylase (TyRh; EC 1.14.16.2) and tryptophan hydroxylase (TrpH; EC 1.14.16.4), although in some organisms these hydroxylases can show broader specificities (reviewed in [16,17]). AAAHs are widespread in evolution, being found in prokaryotes, protozoans, plants, fungi, and animals. In metazoans AAAHs play critical roles in metabolism and neurotransmitter synthesis, and PAH participates in Phe catabolism in bacteria [18].

The identification of a putative H2B-dependent AAAH in the *Leishmania* genome raised the possibility that this activity might account for its H2B biopterin requirement. We report here studies that confirm the activity of Phe hydroxylase (PAH) in *L. major*, and characterize its role in parasite metabolism and infectivity.

2. Materials and methods

2.1. Chemicals and reagents

Dihydrobiopterin (H2B) was from Schircks Laboratories (Jona, Switzerland). Biopterin, epinephrine, norepinephrine, propanolol and RPMI 1640 vitamin solutions were from Sigma–Aldrich (St. Louis, USA). L-5-[3-3H]tryptophan, L-3-[3,5-3H]tyrosine, and L-2-[6,3-3H]phenylalanine) were obtained from Amersham Biosciences Corp. Phenol red solution and penicillin/streptomycin was from Invitrogen. Fetal calf serum was purchased from Bio-Whittaker and bovine serum albumin (Fraction V) from U.S. Biochemical Corp. M199 with Hank's salts was from US Biologicals. Folate-deficient medium (fdM199) was custom manufactured by Invitrogen and is identical to M199 except lacking folate and thymidine [19].

2.2. Parasite and growth media

*L. major* Friedlin V1 (MHOM/JL/80/Friedlin) recovered from infected animals were used within 10 serial passages in vitro. Promastigotes were grown in M199 medium supplemented with 10% heat-inactivated fetal bovine serum at 26 °C [19]; for routine culture 2 µg/ml -1 biopterin was added. Metacyclic promastigotes were purified from a stationary phase cells using the negative selection procedure [22]. Genomic DNA was digested with the appropriate restriction enzymes and transferred to nylon membranes. Total RNA of *L. major* promastigotes and amastigotes was isolated using the phenol/guanidine isothiocyanate reagent TRIzol™ (Invitrogen) according to the manufacturer’s instructions. Southern and Northern were performed following standard procedures and the hybridization probes were labeled with a [α-32P] dCTP by random-priming [23].

2.3. Southern and Northern blot analysis

*L. major* FV1 genomic DNA was isolated from the late logarithmic phase promastigotes by the LiCl method [22]. Genomic DNA was digested with the appropriate restriction enzymes and electrophoresed on 0.8% agarose gels and transferred to nylon membranes. Total RNA of *L. major* promastigotes and amastigotes was isolated using the phenol/guanidine isothiocyanate reagent TRIzol™ (Invitrogen) according to the manufacturer’s instructions. Southern and Northern were performed following standard procedures and the hybridization probes were labeled with a [α-32P] dCTP by random-priming [23].

2.4. PAH gene cloning and sequencing

A fragment spanning 870 nt to 953 nt of the *PAH* ORF was obtained by PCR amplification (primers SMB1076 5′-GGCGGAC ATGTTCTACGACAATC and SMB1077 5′-AGGCCCATGCTGTCGTG AGC) and GSS clone Inv48b04 [24] DNA template. This DNA was radiolabeled and used to screen an *L. major* Friedlin V1 cosmider library [25]. Three different cosmid clones containing *PAH* gene were obtained (c11p6, strain B4089; c10m17, strain B4086; c909, strain B4087). Following subcloning and mapping, the sequence of the *PAH* was determined using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA).

2.5. Mapping the *S*`- terminus of the mature PAH transcript

To amplify the 5′-end of *PAH*, *L. major* total RNA was used as a template for RT-PCR. Reverse transcription was performed with Superscript II reverse transcriptase (Invitrogen) following the manufacturer’s protocol. PCR was performed using Taq polymerase (Roche) with 30 cycles of denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min. Primers specific for the *L. major* spliced leader sequence (SMB936: 5′-ACCGCTATATAAG TATCAGTTCGTGACTT); and two specific primers, SMB1122 and 1117 located within the *PAH* coding region (SMB1122: 5′-TGCAAG GACCTGGCCACCGCG and SMB1117: 5′-GTGGGGTACGCCGG GTCAAGT). The PCR products were purified and sequenced directly.

2.6. Overexpression of PAH

The 1362-nt *PAH* open reading frame was amplified by PCR with Pfu polymerase (Stratagene) using primers SMB 1155 (5′-GCGGATCCACATCGCTGTCGCCAGTCCGTGTT; the under-line sequence corresponds to a *BamHI* site and the bold nucleotides correspond to a "Kozak" sequence), SMB 1156 (5′-GGCGGATCCCTTGAAGGTGAAGTTTGTTCG), and 50 ng of template genomic DNA. The amplified DNA fragment was digested and cloned into the *BamHI* site of the *Leishmania* expression vector pXG1a [26], yielding pXG-PAH (strain B4193), whose sequence was confirmed.

2.7. Molecular constructs for replacement of *PAH* alleles and transfection

The 5′-flanking region of the *PAH* gene was amplified by PCR using the primers SMB1589 (5′-CCGGGTACGACGACGACGACGGCC and SMB1590 (5′-CATCGATCATTATCTCGAGGAAGGTGAAGTTTGTTCG) and added KpnI/Clal sites, while the 3′ flanking region was PCR amplified using primers SMB1608 and 1592 adding BamHI/NotI sites. These fragments were digested with the appropriate enzymes and inserted successively into pBSKl (Promega, Madison, WI), yielding (pBS5′3′PAH). Then, BSD and HYG resistance
ORFs were amplified individually with primers SMB1665 (5′-CTGATGCGCCAAGGGTTTGTCCGAG) and SMB1667 (5′-CGGACATCCCTAGGCCCCACCTTTGAAC) or SMB1665 (5′-CCCAAAGTGTAGAAAGGCTTGAACCTCGG) and SMB1594 (5′-CGGGATCCATTTACCTTGTCCCCTGGAGA), respectively, which added HindIII and BamHI sites, and templates pXG-HYG [27] and pXG-BSD [28]. These fragments were digested appropriately and inserted into HindIII/BamHI digested pBS3′PAH, yielding the final replacement constructs pBS3′BSD PAH and pBS3′HYG PAH (strain B4696, strain B4692). DNAs were cut with NotI and KpnI, and the targeting fragment purified. WT parasites were elec-
troporated with the PAH::SOH targeting fragment derived from B4692 as described [21]. Transfectant colonies were obtained after plating on semisolid M199 medium containing hygromycin B (50 μg ml−1), with typical frequencies. Three bearing heterozygous replacements (formally PAH::PAH::ΔHYG) were passaged through animals where they showed typical infectivity. One (clone 2) was used for transfection experiments, and one (clone KO4-AB2) was selected where they showed typical infectivity, and one (clone KO4-AB2) was selected and electroporated with the targeting fragment, and the targeting fragment purified. WT parasites were elec-
troporated with the PAH::BSD targeting fragment, and transfectant colonies were obtained after plating on semisolid M199 medium containing blasticidin (10 μg ml−1) and hygromycin B (50 μg ml−1) again at normal frequencies. Following molecular confirmation several lines (formally PAH::PAH::ΔHYG, referred to as pah− hereafter) were used to infect animals where they showed typical infectivity, as described elsewhere in the text. One of these (KO4) was selected and electroporated with pXG-PAH, and transfectant colonies obtained following plating on semisolid media containing G418 (10 μg ml−1), blasticidin (10 μg ml−1) and hygromycin B (50 μg ml−1). Several lines (formally PAH::ΔHYG::ΔBSD [pXG-PAH], referred to as pah−[pah−] hereafter) were passaged through animals where they showed typical infectivity, and one (KO4-AB2) was selected.

2.8. Metabolic labeling

Leishmania major were grown in M199 medium until early log phase (2 × 106/ml), harvested by centrifugation, and washed twice and incubated further in modified RPMI 1640 medium whose aromatic amino acid contents was adjusted as follows. For Phe labeling, 235M Phe, 285M Trp were used. Cells were labeled 235M Phe, 480Ci/mmol, Tyr 55 Ci/mmol or Trp, 32 Ci/mmol. Cells were washed twice with 1 ml of medium at final concentration of 106/ml, and 105 parasites per mice[32]. Lesions were monitored by measuring the thickness of the footpad with a Vernier caliper. Lesion parasites were enumerated in the infected tissue by a limiting dilution assay [33].

2.12. Mouse infectivity tests

BALB/c mice were from Charles River Laboratories Inc. (Wilm-
ington, MA) and CBA/J mice from the Jackson Laboratory (Bar Harbor, Me). All studies were performed using protocols approved by the institutional animal studies committee. Parasites were grown to stationary phase in M199 medium, and groups of BALB/c mice were infected subcutaneously (s.c.) into the footpads with 106, 105, 104 parasites per mouse [32]. Lesions were monitored by measuring the thickness of the footpad with a Vernier caliper. Lesion parasites were enumerated in the infected tissue by a limiting dilution assay [33].

2.13. Macrophage Infections

Infection of peritoneal macrophages with C3-opsonized para-
sites with performed as described [34]. The medium was changed daily, and at days 1, 2, 3 postinfection, intracellular parasites were visualized in formaldehyde-fixed macrophages by nuclear staining with Hoechst 33342 (0.5 μg ml−1).
major cosmid library, and the relevant regions of several cosmids were sequenced. The gene encoded a predicted protein of 453 aa/50.4 kDa (Fig. 1) and was given the gene symbol PAH, as it was subsequently shown below to be required for PAH activity. Southern blot analysis showed it to be present in a single copy (Fig. S1), in good agreement with the current L. major genome assembly, where PAH bears the systematic gene identifier LmjF28.1280 [35].

L. major PAH displayed an overall 42–51% identity to AAAH from mammals, Drosophila or C. elegans. Mammalian AAAHs contain a variable N terminal domain comprising about 1/3 of the protein that mediates regulatory functions, a central conserved catalytic domain, and a short C-terminal domain mediating oligomerization (reviewed in [16,17]). Similarly, LmjPAH showed a divergent N terminal domain comprising amino acids 1–123, a more conserved catalytic domain comprising amino acids 124–436, followed by a divergent C-terminal domain (Fig. 1). Recently two PAHs were described in Toxoplasma gondii, whose predicted proteins bearing signal sequences potentially indicative of secretion [36]. However, L. major PAH lacks an N-terminal extension (Fig. 1) and several computational methods did not predict a signal sequence for the Leishmania PAH.

Structural motif characteristic of aromatic amino acid hydroxylases were found in the central catalytic region of LmjPAH, including the iron binding site (His295, His301, and Glu331 in LmjPAH) [37], and conserved substrate and cofactor binding sites (residues 274–301) [38]. LmjPAH contains Asp at the position corresponding to Glu286 of rat PheH, which has been implicated in H4B binding [39]. While amino acid hydroxylases often show cross-activity to tryptophan hydroxylase were not found in Leishmania PAH. These include the position corresponding to Y235 and F313 in tryptophan hydroxylase [40] and D455 of tyrosine hydroxylase [41].

3.2. Structure and expression of PAH mRNA during the infectious cycle

In Leishmania and related trypanosomatid protozoans, every mRNA contains a 39-nt ‘spliced-leader’ at its 5’ end added by trans-splicing [42]. We used RT-PCR with a spliced-leader and two primers specific for PAH to map the 5’ trans-splice acceptor site of the PAH mRNA to an AG dinucleotide 141 nt 5’ of the predicted AUG initiation codon, which was also the first AUG (data not shown). Northern blots revealed a single 4 kb transcript in all stages of the infectious cycle (early log promastigote, late log promastigote, metacyclic promastigote and amastigote) with little variation during development (Fig. 2). A similar result was obtained with the mRNA encoding the H4B regenerating enzyme QDPR [13].

3.3. Generation of PAH null mutants by targeted gene deletion

We were unable to express PAH in an enzymatically active form in E. coli, or in an active form in vitro, despite many attempts. These included varying expression conditions or host cells, tests of alternative pteridines including folates, or tests of truncations removing the poorly conserved N terminal ‘regulatory’ domain, or this as well as the C-terminal domain; several such variations had proven successful in studies of other species’ AAAH. Thus we turned to studies of PAH null mutants, or parasites over-expressing PAH, to explore its potential roles and activities in vivo.

As Leishmania species are predominantly diploid, two successive rounds of gene targeting were required to generate null mutants [27]. The first PAH allele was replaced by the hygromycin B resistance marker (HYG) and the second was replaced with the blasticidin (BSD) resistance marker (Fig. 3A). Southern blot analysis
3.4. In vivo detection of PAH-dependent phenylalanine hydroxylase activity by metabolic labeling

*Leishmania* were grown to logarithmic phase, collected, washed twice, and inoculated into media containing radiolabeled phenylalanine, tyrosine or tryptophan. After 2 h, samples were collected, deproteinized, and separated by HPLC after addition of the appropriate standards (Fig. 4A–C). When labeled with [3H]-Phe, WT but not *pah*− extracts showed incorporation into a peak comigrating with the free Tyr standard (Fig. 4A). In contrast, no incorporation above background was seen using [3H]-Tyr or [3H]-Trp, into their predicted products DOPA or 5-hydroxy-tryptophan (Fig. 4B and C). Since limiting concentrations of radiolabeled essential amino acids were used in order to maximize the specific activity, and free amino acids are used in protein synthesis, in preliminary experiments we varied the time of labeling from 1 to 4 h. As before no further metabolism of radiolabeled Tyr or Trp was detected, while [3H]-Tyr formation from [3H]-Phe was maximal at 2 h. Other than Tyr formation, no differential was seen between WT and *pah*−, or between the *pah*−/+PAH overexpressor (data not shown). These data indicate that the *Leishmania* PAH is required for phenylalanine but not tyrosine or tryptophan hydroxylase activ-
Fig. 4. Metabolic labeling with aromatic amino acids and catecholamine analysis of L. major. Panels A–C, WT (black lines) or pah− (red line) were grown in the presence of [3H] amino acids, and incorporation into free Phe, Tyr or Trp in deproteinized cell lysates was determined by liquid scintillation counting following derivatization and HPLC separation. Panel D, Analysis of L. major for the presence of catecholamines. Deproteinized lysates were separated by HPLC following derivatization as described in Section 2. The fluorescence traces are shown for WT (blue) and WT mixed with norepinephrine (NE), epinephrine (E) and dopamine (DA) standards (red). Offset below these are traces (black) for separation of NE, E, and DA run individually.

3.5. PAH and aromatic amino acid growth requirements

To probe the consequences of PAH depletion or overexpression, parasites were inoculated into semidefined medium, or this lacking phenylalanine, tryptophan or tyrosine. The WT and pah− lines were incapable of sustained proliferation in media lacking any of the aromatic acids (Fig. 5A–C), consistent with the view that Leishmania are auxotrophic for aromatic amino acids [43]. Unlike WT cells which arrested at constant cell numbers, pah− showed a tendency towards decreased cell numbers over time (Fig. 5A–C).

After a substantial delay of ~8 days, the pah−/+PAH overexpressor was able to resume growth in Tyr-deficient media, although more slowly than in the presence of Tyr (Fig. 5C). These parasites were adapted further by passage in Tyr-deficient media (six passages of 1:100 serial dilutions), and then retested. These P6 pah−/+PAH parasites now exhibited growth in the absence of Tyr comparable to WT in the presence of Tyr (Fig. 5D). Adaptation was dependent on the presence of the episomal PAH gene, as WT parasites never increased in their rate of growth (not shown). The basis of adaptation was not studied further but its dependency on episomal PAH suggests that increased plasmid copy number and PAH expression may account for this finding, as seen originally in ‘superselections’ of L. major episomal vectors [21]. These data provide independent confirmation of the PAH activity of PAH, through its role in provision of Tyr under limiting conditions, and alleviation of this requirement when overexpressed.

3.6. Infectivity of pah− L. major in mouse or macrophage infections

The ability of WT and pah− Leishmania to survive within peritoneal macrophages was assessed following infection of complement-opsonized stationary phase parasites. Parasitemia was monitored by the percent infected macrophages (Fig. 6C) and...
the number of parasites per 100 macrophages (Fig. 6D). Again, no difference in parasite survival was seen.

3.7 Metacyclogenesis

Previously we showed that H4B levels were associated with increased differentiation into the infective metacyclic form, which occurs normally following entry into stationary phase [44]. Since PAH activity could affect H4B levels, we explored the effect of PAH depletion or overexpression on metacyclogenesis. Tests were performed in folate-depleted M199 media (fdM199) containing either minimal (0.001 µg ml⁻¹) or saturating (2 µg ml⁻¹) concentrations of bioppterin. As before, no differences in growth rate were observed, and metacyclogenesis was elevated under low-bioppterin conditions (10–11% vs 4.5–5%; Fig. S2, or data not shown). However, no differences were seen in metacyclogenesis between WT or pah⁻ under either condition (Fig. S2). Similar tests showed no effect of epinephrine (100 µM), norepinephrine (100 µM) or propanolol (10 µM) on either parasite growth or differentiation, for either WT or pah⁻ (Fig. S2). We conclude that neither PAH or the presence of potential downstream effectors arising from PAH activity have any effect on metacyclogenesis, even when grown in the minimum level of bioppterin required to support normal growth.

3.8 Reactivity with anti-melanin antibody MAb 6D

Melanin is a downstream metabolite of AAAH activity in many species [45,46] and we used reactivity with the melanin antibody MAb 6D2 to reveal its presence in Leishmania. While WT parasites showed no reactivity, comparable to isotypic MAb controls (Fig. 7A or data not shown), when grown in media containing 250 µM DOPA, strong reactivity was observed (Fig. 7C). In contrast, pah⁻ parasites showed no reactivity when grown in the presence of DOPA (Fig. 7B). Restoration of PAH expression in the pah⁻/+PAH line restored MAb 6D2 reactivity (Fig. 7D). We observed in some but not all experiments that the cells were pigmented in PAH-expressing

### Table 1
Metabolic labeling studies of L. major with [3H] Phe.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>WT</th>
<th>pah⁻</th>
<th>pah⁻/+PAH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Phe uptake/ incorporation</strong></td>
<td>2433 ± 251 (n=3)</td>
<td>1820 ± 276* (n=5)</td>
<td>3140 ± 190* (n=4)</td>
</tr>
<tr>
<td>Free phenylalanine</td>
<td>129 ± 49</td>
<td>101 ± 11</td>
<td>34.5 ± 14.4*</td>
</tr>
<tr>
<td>Free tyrosine</td>
<td>6.5 ± 1.7</td>
<td>0 (not detectable)**</td>
<td>15.3 ± 10.7</td>
</tr>
</tbody>
</table>

The values shown are pmol [3H]-Phe or Tyr/2.5 × 10⁷ cells).

* Statistical significance from WT is indicated as P < 0.1.
** Statistical significance from WT is indicated as P < 0.05.
*** Statistical significance from WT is indicated as P < 0.01.

![Fig. 5](image-url) Growth of L. major WT, pah⁻ and pah⁻/+PAH lines in aromatic amino acid-deficient media. The growth of parasites was measured following inoculation at 2 × 10⁵/ml on day 1; all studies were performed at least three times, with a representative experiment shown. Panel A, growth in media containing (dark symbols) or lacking Trp (light symbols). Panel B, growth in media containing (dark symbols) or lacking (open symbols) Phe. Panel C, growth in media containing (dark symbols) or lacking (open symbols) Tyr. Panel D, growth in media lacking Tyr. Cell lines were WT (●, ○), pah⁻ (■, □) or pah⁻/+PAH (▲, △), or pah⁻/+PAH adapted by six serial passages of growth in media lacking Tyr (♦).
cells grown in the presence DOPA. We sought to confirm that the reactive material was indeed melanin, using a protocol which solubilizes most cellular components but leaves insoluble melanin intact, but were unsuccessful with WT cells grown in the presence of DOPA (not shown).

4. Discussion

In this work we identified and explored potential roles for _L. major_ PAH. As we were unable to express active PAH for biochemical characterization, we relied primarily on the predicted properties based on the PAH protein sequence, and the characterization of both a _pah−_ null mutant and a PAH overexpressor obtained following transfection of a multicopy episomal _PAH_ expression vector. Collectively the aromatic amino acid hydroxylase family shares many physical, structural and catalytic properties [4,5,16]. The predicted _L. major_ PAH protein showed a typical mammalian-type AAH domain structure, with a highly conserved central catalytic core flanked by less conserved N terminal and C terminal regions, implicated in regulatory and oligomerization roles in other AAHs (Fig. 1). The _L. major_ PAH central catalytic core showed good conservation of catalytic residues and motifs seen in other AAHs. Importantly, these residues include those responsible for binding H4-biopterin.

Several other protozoans bear AAH genes, including the apicomplexan parasite _Toxoplasma_ which has two genes encoding potentially secreted Phe and Trp hydroxylase activity [36], and the cellular slime mold _Dictyostelium_ [47]. Phenylalanine hydroxylases also occur sporadically throughout prokaryotes [4,18]. Evolution-
arily, *L. major* PAH falls as expected near the base of the eukaryotic radiation ([Fig. S3]). While all *Leishmania* species genomes reported to date encode a PAH ortholog, it is absent from the genomes of both African and South American trypanosomes. Since a PAH gene occurs in the free-living Kinetoplastid relative *Bodo saltans* ([Family Bodonidae; Fig. S3]), we infer that PAH was lost in the common ancestor of trypanosomes following their divergence from *Leishmania*. However, the genome of African trypanosomes predicts a *PCD* gene and trypanosomes express high levels of QDPR activity [13]. This would be consistent with the idea that H4Bt is required for essential processes in *Leishmania* and trypanosomes, other than as a cofactor for AAAH activity.

Characterization of the *L. major* pah− null mutant showed phenotypes firmly establishing the requirement for PAH for phenylalanine hydroxylase activity. While WT parasites converted radiolabeled Phe to Tyr, the pah− mutant was unable to do so ([Fig. 4A, Table 1]), and correspondingly, the pah−/+PAH overexpressor showed an elevated ability to do so (Table 1). Despite this activity, WT *L. major* were auxotrophic for Tyr as well as Phe and Trp ([Fig. 5A–C]), suggesting that WT PAH levels were insufficient for metabolic needs. Notably, WT and PAH overexpressing parasites produced reproductively higher susceptibility to Phe or Trp deprivation than for Tyr deprivation ([Fig. 5C]). Moreover, the pah−/+PAH episomal overexpression showed only partial growth inhibition, and could be readily adapted for normal growth without Tyr ([Fig. 5C and D]). These data suggest that WT PAH activity can only partially alleviate Tyr insufficiency, and is able to facilitate cell survival but not full growth. The ability of PAH overexpression to rescue Tyr auxotrophy of the pah− mutant firmly establishes the PAH activity of *LmPAH*.

In contrast to the PAH-dependent Phe hydroxylase activity, Tyr or Trp hydroxylase activity was not detected by in vivo radiolabeling, using either WT, pah− or the pah−/+PAH overexpressor. This is consistent with *LmPAH* amino acid sequence, which shows residues more typical of AAAs with Phe rather than Tyr hydroxylase activity (Fig. 1). Correspondingly, PAH expression levels did not alter growth under Phe or Trp deprivation ([Fig. 5A and B]). These data thus suggest that *Leishmania PAH* is specific for Phe.

4.1. A melanin-like substance in *Leishmania*?

Curiously, we were able to show reactivity of *Leishmania* against an antibody to melanin, which was dependent on the presence of both PAH and exogenous DOPA ([Fig. 7]), and in some experiments the *Leishmania* appeared pigmented. However, we failed to recover insoluble acid-resistant pigment characteristic of melanin [30], suggesting that the reactive substance was unlikely to be authentic melanin. A number of melanin-like substances have been described, arising from a variety of diphenolic or other precursors [48]. In the fungus *Cryptococcus neoformans*, melanin plays important roles in virulence [45]. However, since *Leishmania* requires PAH to make the melanin-like substance, yet does not require PAH for differentiation or infectivity in mice or macrophages ([Fig. 7]), a role for the melanin-like substance in *Leishmania* virulence seems unlikely.

4.2. PAH null mutants remain viable and virulent

Given the importance of H4Bt to *Leishmania* viability and virulence, and that melanin-like substances have been implicated in host-pathogen interactions in several species [36,46,49], we asked whether PAH-dependent metabolites played a role in the *Leishmania* infectious cycle. First we examined the ability of the parasite to differentiate to infectious metacyclic parasites in vitro, a process known to be up-regulated by low H4Bt levels in *L. major* [44]. We confirmed this result, virulent *L. major* FV1 line studied here, but the percent metacyclics was identical between WT and pah− lines when grown in either high or minimal bipterin levels, and nor was it altered in the presence of downstream metabolites including catecholamines or inhibitors ([Fig. S2]). Similarly, the pah− line behaved identically to WT following infections of primary macrophages in vitro, or in infections of susceptible and resistant mice in vivo ([Fig. 6 or data not shown]). Finally, in preliminary studies the pah− line survived well within sand flies, the insect vector of leishmaniasis (D. Sacks, L-FL and SMB, unpublished data).

Thus, while under some circumstances PAH can partially mitigate the Tyr growth requirement of *L. major*, it is not essential for normal growth or virulence across the *Leishmania* infective cycle. Similarly, the PAH gene is absent in trypanosomes, which like *Leishmania* require H4Bt [50]. Future studies may consider other metabolic roles for PAH, perhaps in Phe anabolism, drug resistance or in other metabolic pathways such as theeothero metabolism [45,51]. However, since the pah− mutant grows normally in vitro and in vivo, it seems unlikely that PAH activity or PAH-dependent metabolites can account completely for the strong bipterin growth requirement of *Leishmania*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.molbiopara.2010.09.004.

References


