Leishmania major possesses a unique HemG-type protoporphyrinogen IX oxidase

Dagmar ZWERSCHKE*, Simone KARRIE†, Dieter JAHN* and Martina JAHN*1

*Institute of Microbiology, University Braunschweig, Spielmannstr. 7, D-38106 Braunschweig, Germany
†Cellular and Molecular Neurobiology, Institute of Cell Biology, University Braunschweig, Spielmannstr. 7, D-38106 Braunschweig, Germany

INTRODUCTION

The trypanosomatid of the Leishmania-type are flagellated protozoan parasites. These ancient eukaryotes cause leishmaniasis in 350 million people in 88 countries worldwide with up to 2 million new cases and up to 30000 deaths annually [1]. The disease patterns vary between innocuous cutaneous lesions and lethal visceral forms [1]. Traditional medication is associated with severe adverse effects [1,2]. Leishmania spp. have a digenetic life cycle, where they switch between the promastigote life form in the gut of haematophage insect vectors and the amastigote form residing inside phagolysosomes of macrophages [3]. In both forms, they utilize haemoproteins such as cytochrome $a$, $b$ and $c$ for their electron transport chain-dependent energy conservation [4]. Haem peroxidase, haem-containing protein kinases, flavohaemoglobin, cytochrome $b_5$ for fatty acid desaturation, enzymes of sulphite oxidation and nitrate reduction as well as multiple cytochrome $P-450$ enzymes constitute further haemoproteins of these organisms [4,5].

In 2012, a Leishmania haem uptake transporter for the promastigote state of Leishmania amazonensis was described, indicating a direct haem uptake from the gut of the blood-feeding host [6]. Concerning the haem source of the amastigotes only sparse information is available. Early experiments showed that Leishmania species were able to grow not only in the presence of haem, but also on defined media supplemented with protoporphyrin IX ($\text{proto}_x$) [7–9]. This led to the prediction of a functional ferrochelatase (FeCH$^*$) [10,11]. Later on, the corresponding gene (LMJF_17_1480) was discovered [12]. Additionally, one potential CPO$^*$ (coproporphyrinogen III oxidase) LMJF_06_1270 and a potential PPO$^*$ (protoporphyrinogen IX oxidase) (LMJF_06_1280) were annotated (Figure 1A) [13].

Recently, high-throughput structural biology projects yielded...
several *Leishmania* spp. CPO crystal structures (PDB # 1VJU, PDB # 2QT8, PDB # 3E8J and PDB # 3EJO, respectively). The overall structure and the bound ligand of the LMJF_06_1270 encoded protein finally identified it as oxygen-dependent CPO (PDB # 3DWR, PDB # 3DWS) [14,15]. Transcriptome analyses identified transcripts from the LMJF_06_1270 and LMJF_06_1280 genes indicating their functional expression [16]. The proposal was that the *Leishmania* spp. may take up copro$^a$ (coproporphyrinogen III) from their macrophage host which then is converted into haem [4]. However, no experimental evidence was available for the function of the potential PPO protein.

In nature the six electron oxidation of protoporphyrinogen IX (protogen$^a$) to the first coloured intermediate of haem biosynthesis proto is catalysed by a set of highly diverse, even partially completely unknown enzymes. Very recently the novel HemJ-type of PPO was discovered in cyanobacteria and *Acinetobacter* spp. [17,18]. Almost all haem synthesizing eukaryotes and some bacteria utilize the oxygen-dependent FAD enzyme of the HemY-type [19]. Database searches revealed that the only exceptions to this rule are trypanosomatid protozoa of the *Leishmania* class, which were proposed to possess a HemG-type PPO.

In addition, only a few bacteria, including several Enterobacteriaceae employ the flavodoxin-like FMN enzyme HemG [20,21]. The enzyme transfers six abstracted electrons via quinones to various terminal oxidases of the respiratory electron transport chains, which use oxygen or nitrate and fumarate under anaerobic conditions as electron acceptors. This allows the coupling of anaerobic and aerobic haem biosynthesis to cellular respiration. Thus, the reaction contributes to proton gradient formation [21].

Here, we demonstrate for the first time the activity of a eukaryotic HemG-type PPO. Thus, partial haem biosynthesis from phagocyte-derived haem precursors in *L. major* is highly probable. It serves most probably to haemoprotein formation during the amastigotic state in the macrophage [4].

### EXPERIMENTAL

**Bacterial strains and constructed plasmids**

The *Leishmania major* hemG cDNA of LMJF_06_1280 optimized for *E. coli* codon usage (http://www.jcat.de) was cloned into the BamHI and NotI sites of either the glutathione S-transferase tag encoding vector pGEX 6P-1 (Amersham Biosciences Little Chalfont/GB) resulting in plasmid pGEXhemGL or in the His tag encoding vector pET32a (Novagen) resulting in plasmid...
Partial haem biosynthesis in amastigote phenotype of Leishmania major

Figure 3 PPO activities of E. coli cell-free extract containing L. major HemG
PPO activities were obtained as described in Experimental. Proto formation was tested with fumarate, nitrate or ubiquinone-1 as electron acceptor. Arbitrary absorbance units related to the relative fluorescence after 60 min of enzyme assay are given. The T-bar indicates the standard deviation for n = 3.

| E. coli C43 | pGEX-6P-1 |
|-------------|-----------|
| +           | -         | +         | -         | +         | -         |

pET32ahemGL. For complementation studies, pGEXhemGL was transformed into the E. coli LG285 ΔhemG mutant cell line [hemG::KmR, supE44, supF58, HisDR14, galK2, galT22, metB1, trpR55, lacY1] [22]. E. coli LE392 [glnV44, supF58 (lacY1 or ΔlacZY), galK2, galT22, metB1, trpR55, hisDR14 (rKmK+)] (Promega) was used in complementation studies as wild-type strain. Cell-free extracts for enzyme activity analyses were prepared from E. coli OverExpress™ C43 (DE3) cells (Lucigen) for cells carrying the plasmid pGEXhemGL. E. coli BL21-CodonPlus®-RIL (Stratagene) carrying pET32ahemGL was used for protein production for subsequent purification and renaturation. The HemG variant genes were generated using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs) according to the manufacturer’s instructions. Correctness of introduced mutations in the genes was proven by DNA sequencing (GATC).

Complementation studies

E. coli LG285 ΔhemG cells carrying either the pGEXhemGL or pGEX 6P-1 as control were cultivated in 50 ml LB (Luria-Bertani) medium supplemented with 500 μM IPTG (isopropyl-β-d-thiogalactopyranoside) and 0.3 mM ampicillin at 37 °C with shaking at 180 rpm. The corresponding wild-type strain E. coli LE392 was cultivated in 50 ml LB supplemented with 500 μM IPTG at 37 °C at 180 rpm. Samples were taken every 2 h over 10 h and OD (optical density) was determined photometrically by 578 nm (Ultrspec 500 Pro, Amershams Biosciences). HPLC cultures were centrifuged by 2500×g, and cells were mechanically disrupted with glass beads (100 μm) in 50 mM Tris–HCl pH 8.0 containing 2% (v/v) Tween 80 using FastPrep®-24 Instrument (MP Biomedicals). Tetrapyrroles were extracted from cell-free extracts with a HCl/acetone (2:5:97.5) solution as described before [23]. Isolated tetrapyrroles were separated by reversed phase chromatography using an HPLC-system 2000 series (Jasco) and an Equisil BDS-C18 reversed phase column (Dr Maisch, Ammerbuch-Entringen, Germany) using a modified method of Lim [23].

Production of cell-free E. coli extracts harbouring L. major HemG

A 50 ml culture of E. coli OverExpress™ C43 (DE3) carrying pGEXhemGL was grown in LB medium supplemented with 0.3 mM ampicillin at 37 °C with shaking at 180 rpm. When the culture reached an OD578 of 0.9, protein production was induced with the addition of 500 μM IPTG. The cells were further cultivated at 25 °C and 180 rpm for 4 h, harvested and disrupted as described above. The suspension was centrifuged for 1 h by
D. Zwerschke and others

Figure 4  Recombinant production, purification and proto formation of L. major HemG

(A) L. major HemG after recombinant production in E. coli, purification from inclusion bodies and renaturation to solubility was subjected to SDS–PAGE (12 % gel) and visualized by Coomassie Brilliant Blue staining. Lane M: molecular weight standard; lane 1: affinity-tagged L. major HemG. (B) PPO activities of purified L. major HemG. PPO activities were obtained as described in Experimental. Proto formation was measured with addition of TTC, ubiquinone-O or ubiquinone-1 as electron acceptors and purified enzyme as shown in lane 1. Arbitrary absorbance units: relative fluorescence units with t = 60 min. The T-bar indicates the standard deviation for n = 3.

2500 × g and the supernatant was used as cell-free extract for PPO activity assays.

Production and purification of L. major HemG

Two litres of E. coli BL21-CodonPlus®-RIL cells carrying pET32ahemGL were grown in LB medium supplemented with 0.3 mM ampicillin at 37 °C with shaking at 180 rpm. When the culture reached an OD578 of 0.6, recombinant protein production was induced by the addition of 500 μM IPTG. The E. coli cells were further cultivated at 37 °C, with shaking at 180 rpm for 3.5 h and subsequently harvested. For isolation of the HemG-containing inclusion bodies the cell pellet was redisolved in 10 ml harvesting buffer 100 mM Tris–HCl, pH 8.0, lysozyme 0.2 mg/ml incubated for 5 min at room temperature and disrupted by sonication (0.5 s pulse, 0.5 s pause, 70 % amplitude; KE76; Sonoplus HD 2070). The resulting cell-free suspension was centrifuged (125000 × g, 4 °C) and the resulting pellet was washed twice in 6 ml isolation buffer 2 M urea, 20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 0.2 % (w/v) Triton X-100. The proteins were solubilized by sonication (0.5 s pulse, 0.5 s pause, 70 % amplitude; KE76; Sonoplus HD 2070) and residual debris was removed by centrifugation (125000 × g at 4 °C). Subsequently, the pellet was washed in 7 ml 100 mM Tris–HCl (pH 8.0). The purified inclusion bodies were redissolved in 10 ml buffer S containing 6 M guanidinium–HCl, 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 5 mM β-mercaptoethanol [24] and dialysed against buffer S without guanidinium–HCl twice for 24 h.

PPO activity assays

Approximately 2 mg of protoporphyin IX (Sigma-Aldrich) were dissolved in 7 ml of 10 mM KOH in 20% (v/v) ethanol and stirred 20 min in the dark. From this stock solution 2 ml were diluted into 3 ml of 10 mM KOH in 20% ethanol to create the working solution. The concentration of the working solution was determined by diluting 30 μl of it with 3 ml of 2.7 M HCl and measuring the absorbance at 408 nm, using the millimolar extinction coefficient value of 297 cm/mM (Ultrospec 500 Pro, Amersham Biosciences). Three millilitres of the working solution were reduced using 6 g freshly prepared pulverized 3 % (w/v) sodium mercury amalgam under nitrogen atmosphere [25]. The colourless reduction product protogen was filtered and used for the activity assay. Assay conditions were used as previously reported [21] with 0.62 mg/ml cell-free extract or 0.4 mg/ml pure protein.
Partial haem biosynthesis in amastigote phenotype of *Leishmania major*

**RESULTS AND DISCUSSION**

The *L. major* LMJF_06_1280 encoded protein reveals high amino acid sequence similarity to the HemG-type PPO

In order to answer the question for the existence of a partial haem biosynthetic pathway in *L. major* during its amastigotic, macrophage-associated life stage, the function of the LMJF_06_1280 gene encoded potential PPO was analysed. The amino acid sequence deduced from the corresponding cDNA showed approximately 50% amino acid sequence identity to the HemG-type PPO of *E. coli*. Interestingly, *L. major* HemF-type CPO (LMJF_06_1270) and FeCH (LMJF_17_1470) displayed 64 and 53% amino acid sequence identity to their *E. coli* counterparts, respectively [13]. These high amino acid sequence homologies are astounding because until now only Betaproteobacteria where found to be symbionts in the family of kinetoplastids [13]. Thus, horizontal gene transfer from Gammaproteobacteria might provide an explanation. In addition to Gammaproteobacteria, HemG-type PPOs were also found in selected species of Alphaproteobacteria (*Pseudovibrio*), Betaproteobacteria (*Thauera*), Cyanobacteria (*Prochlorococcus*) and even Archaea (*Halograneum*). The principle of this unique distribution and its underlying selection pressure remains to be elucidated.
**L. major** HemG serves as PPO in *E. coli* haem biosynthesis

In order to test for the PPO function of the *L. major* LMJF_06_1280 encoded HemG *in vivo*, the haem auxotrophic *E. coli* ΔhemG mutant LG285 strain [22] was complemented with the corresponding *L. major* cDNA synthesized in *E. coli* codon usage and cloned into an appropriate vector. Growth experiments of the wild-type *E. coli* strain LE392, the ΔhemG mutant LG285 and the complemented hemG mutant strains showed wild-type-like growth of the complemented *E. coli* ΔhemG mutant, whereas the not complemented mutant strain LG285 almost failed to grow because of its haem auxotrophy (Figure 1). In detail, the growth rates of the corresponding *E. coli* wild-type strain LE392 and the complemented *E. coli* ΔhemG strain were approximately the same ($k = 2.281$ and $2.186$, respectively). The non-complemented mutant showed a much weaker growth ($k = 0.238$) (Figure 1) and the reddish colour of the culture indicated an accumulation of haem precursor molecules as described earlier [26].

In order to prove that the observed growth of the *E. coli* ΔhemG mutant complemented with the *L. major* LMJF_06_1280 cDNA was due to restored haem biosynthesis, HPLC analyses for haem and its biosynthetic precursors were performed. For this purpose, tetrpyrroles were extracted from the various *E. coli* strains and separated using reversed phase chromatography as described in the Experimental section. UV/Vis spectra were recorded for the identification of haem and fluorescence spectra for the identification of the porphyrins. During tetrpyrrole extraction the unstable porphyrinogens get converted into porphyrins. Consequently, protogen and proto cannot be distinguished by this method. As expected, in wild-type *E. coli* LE392 only haem and no biosynthetic intermediates were identified (Figure 2A). In contrast in the mutant strain LG285, the haem precursors proto/protogen, and mainly coprogen were found (Figure 2B).

---

**Figure 7** PPO activities of *E. coli* cell-free extract containing *L. major* HemG mutants

PPO activities were obtained as described in the Experimental section. Proto formation was depicted with fumarate as electron acceptor. Arbitrary absorbance units: relative fluorescence units with $t = 60$ min. The T-bar indicates the standard deviation for $n = 3$. 

|       | Y137A | Y137F | Y137S | Y134F | R142A |
|-------|-------|-------|-------|-------|-------|
|       | +     | -     | -     | -     | -     |
|       |       |       | -     | -     | -     |
|       |       | +     | -     | -     | -     |
|       |       |       |       | +     | -     |
|       |       |       |       |       | +     |

**Table 1** Activity of *L. major* HemG mutants.
Partial haem biosynthesis in amastigote phenotype of Leishmania major

Figure 8 Spectral analysis of the influence of L. major HemG mutants on haem biosynthesis

Spectral analysis of the influence of L. major HemG mutants on haem biosynthesis of mutant LQ285 using HPLC analysis for the identification of haem, proto and copro. UV/Vis spectra (red) and fluorescence spectra (grey) at 409 nm were recorded simultaneously. The retention time for haem was 37.3 min, for proto 40.7 and 25.6 min for copro. Assayed HemG variants are indicated.

The low amount of haem in the ΔhemG background is most likely derived from the chemical interconversion of protogen into proto with the subsequent enzymatic iron insertion. However, this chemical process allows only for limited growth (see Figure 1). The accumulation of copro was already observed in the 1960s and 1970s after supplementation of bacterial cell cultures with 5-aminolevulinic acid [27], indicating a general limitation at the CPO-catalysed step in haem biosynthesis. In agreement, mutations in the late steps of haem synthesis often result in the accumulation of mainly coprogen in addition to the substrate to be metabolized by the inactivated enzyme [27]. Therefore coprogen accumulation is typical for most mutants of the late haem biosynthetic pathway. In the complemented E. coli, ΔhemG mutant rescued by the L. major hemG, the amount of proto/protogen and copro was found drastically reduced compared with the ΔhemG mutant strain, whereas the haem level was found increased (Figure 2C). Together with the growth experiments, these data clearly demonstrate PPO activity for the L. major LMJF_06_1280 gene product in vivo. The gene and the enzyme will be referred to as L. major hemG and L. major HemG, respectively.

L. major HemG transfers electrons from protoporphyrinogen IX to the fumarate reductase system of E. coli

Subsequently to the confirmation of the in vivo activity of L. major HemG, classical HemG assays were employed to demonstrate PPO activity in vitro. In the late 1970s it was already observed using E. coli cell-free extracts, that the tested PPO activity required ubiquinones or the menaquinone containing the respiratory fumarate system as electron acceptors [25]. For cell-free extract preparation the respective E. coli cells were grown under anaerobic conditions without nitrate addition in order to solely induce fumarate reductase formation. Under anaerobic growth conditions, fumarate reductase constitutes an alternative electron acceptor system to the oxygen respiratory machinery. Testing for nitrate respiration as electron accepting system for L. major HemG served as negative control, since the anaerobic onset for the production of the corresponding enzyme system requires the presence of nitrate in the growth medium [21]. As expected, the L. major HemG activity in E. coli cell-free extracts was solely seen in the presence of electron accepting ubiquinone-1 or of...
fumarate allowing electron transfer from protogen to fumarate via menaquinone containing fumarate reductase. Residual back- ground reactivity of ubiquinone with protogen was observed.

Thus, a dependency of *L. major* HemG catalysis on respiratory electron transport (Figure 3) was demonstrated. Furthermore, it was shown that ubiquinone-1 can act as direct electron acceptor.

**Purified *L. major* HemG has PPO activity in vitro**

To ultimatively demonstrate PPO activity for *L. major* HemG, purified recombinant protein was analysed in vitro. *L. major* HemG fused to a His-tag was produced in *E. coli*. The protein found misfolded in inclusion bodies was isolated, denatured and refolded, yielding up to 2 mg/l apparently homogeneous protein. A protein with a *M*ₐ of 42,000 (± 5000) was observed on SDS–PAGE. This is in good agreement with the calculated molecular mass deduced from the amino acid sequence of the *L. major* HemG fusion protein. Removal of the His-tag did not change the enzymatic behaviour, consequently further assays were performed with the fusion proteins. The purified HemG was tested with two ubiquinones and the artificial electron TTC (triphenyltetrazolium chloride). Under all tested conditions electron transfer from protogen to the protein acceptors with the formation of proto was detected (Figure 4). The observed fluorescence is direct proportional to proto formation. The PPO activity differed for the different tested electron acceptors between 593 nM proto/mg protein/h for ubiquinone-1, 572 nM proto/mg protein/h for ubiquinone-0 and 211 nM proto/mg protein/h for TTC, respectively. Again, residual reactivity of ubiquinone with protogen was observed. Observed enzyme activities for the *L. major* PPO were approximately ten times higher compared with the values obtained for *E. coli* HemG [21]. Thus, *L. major* HemG was finally directly identified as a PPO.

**FMN is the cofactor of *L. major* HemG**

PPO’s of the HemY and the HemG classes are utilizing flavins for the six electron oxidation of protogen. Eukaryotic HemY PPOs possess FAD as cofactor, whereas *E. coli* HemG is known to employ FMN. In order to reveal the cofactor of *L. major* HemG, the purified protein was denatured using perchloroacetic acid and the resulting supernatant was analysed using HPLC separation. The obtained UV/Vis spectrum of the respective chromatography was compared with the corresponding spectra from commercial standards of FAD and FMN. The retention times of both flavines were used for the identification of the *L. major* cofactor. Figure 5 presents the recorded spectra. The flavin extracted from *L. major* HemG was identified as FMN (Figure 5). The FMN was obviously non-covalently bound, based on the extraction method.

**Analysis of the active site of *L. major* HemG**

HemG proteins are highly related to flavodoxin proteins [20]. Based on the solved crystal structure of a related flavodoxin the structure of *E. coli* HemG was modelled [20,21]. Boynton et al. proposed a putative active site between amino acid residues 124 and 149 of *E. coli* HemG (Figure 6). This long loop represents an insertion into the flavodoxin backbone. Deletion of this loop of HemG resulted in the inactivation of the enzyme [20]. Here we analysed the corresponded active site of *L. major* HemG. Two tyrosines at positions 134 and 137 as well as the arginine at position 142 are highly conserved among HemG analogues (Figure 6). Tyrosine residues are known for their electron transport capacity through proteins because of their delocalized π -electron systems [28]. Arginines are often involved in tetrapyrrole binding via ring substituents coordination [29]. To investigate the contribution of these conserved amino acid residues to the *L. major* HemG activity a site directed mutagenesis approach was pursued. We exchanged tyrosine 134 against phenylalanine leading to the HemG variant Y134F. Tyrosine at position 137 was exchanged against phenylalanine, alanine or serine residues leading to the HemG variant Y137F, Y137A and Y137S, respectively (Figure 6). The arginine at position 142 was mutated to alanine leading to R142A. Phenylalanine differs from tyrosine only by the lack of the hydroxyl group in the ortho position on the benzene ring, Therefore an influence on the protein shape is unlikely. The serine hydroxyl group might be necessary for catalysis. For all variants the PPO activity was examined using cell-free extracts of *E. coli* expressing *L. major* HemG variants as shown in Figure 7. An *E. coli* cell-free extract without *L. major* HemG was used as control. Fumarate was used as electron acceptor (Figure 7).

To further verify these *in vitro* data the *hemG*-deficient strain LG285 was complemented with the various mutated *hemG* genes. Subsequent HPLC analyses for the detection of haem and its precursors were performed. Figure 8 shows HPLC analyses of the five tested *L. major* HemG variants. The grey line represents the fluorescence spectra at 409 nm used for the identification of copro and proto. UV/Vis spectra are shown in red revealing the signal for haem.

An activity assay of HemG variant R142A showed a 50 times lower activity compared with the wild-type *L. major* HemG; however, still slight enzyme activity was visible (Figure 7). HPLC analyses revealed almost no precursor molecules but low haem amounts (Figure 8). These results indicate a retarded reaction of the R142A. These results suggest a role of HemG residue R142 in substrate binding. Moreover, the HemG variant Y134F revealed residual PPO activity (Figure 7). In agreement, residual haem formation was observed with the typical accumulation of haem precursor molecules. Obviously, this residue is important but not essential to *L. major* HemG activity. In contrast, amino acid exchanges at position Y137 led to complete inactivation of the enzyme (Figure 7). Similar to the *E. coli* *hemG* mutant control, significant reduction of haem formation with the parallel accumulation of the haem precursors was observed. The strongest phenotype was detected for HemG variant Y137S. Obviously, residue Y137 is essential to *L. major* HemG activity suggesting a crucial role in electron transfer from the substrate.
Partial haem biosynthesis in amastigote phenotype of Leishmania major

Figure 9 Model for the intracellular localization of haem synthetic enzymes and haem trafficking in L. major amastigotes

Illustrated is a mammalian macrophage in which the haem biosynthesis starts within the mitochondrion (right side). Haem precursors are then transported into the cytosol (grey) and further processed to copro. Finally, copro is transported via membrane-bound transporters into L. major (cyan) located in the polysomes (left side), where partial haem biosynthesis via the activity of the last three enzymes yield in haem for integration into multiple haemoproteins. The abbreviated enzyme names are: HemF, aerobic coproporphyrinogen III oxidase; HemG, FMN-containing protoporphyrinogen IX oxidase; HemH, FeCH. The abbreviated haem precursor molecules and hemoproteins are: ALA, δ-aminolevulinic acid; PBG, porphobilinogen; HMB, hydroxymethylbilane; uro, uroporphyrinogen III; copro, coproporphyrinogen III; protogen, protoporphyrinogen IX; proto, protoporphyrin IX; cyt b5, cytochrome b5; cyt P-450α, cytochrome P-450, respectively.

Conclusions

L. major is a dangerous pathogen responsible for the deaths of approximately 30000 people/year. The organism requires haem for its multiple essential haemoproteins. However, L. major does not possess a complete haem biosynthesis. Obviously, the promastigote form of the pathogen acquires haem via haem transporter mediated import [6]. Here, PPO activity for the HemG-type L. major protein encoded by LMJF_06_1280 was demonstrated using in vivo as well as in vitro analyses. Structural biology identified the protein encoded by LMJF_06_1270 as CPO (see the Introduction section). Corresponding genes are expressed in L. major [16]. Finally, physiological evidence and a potential gene coding for FeCH (LMJF_17_1480) are available [7–9]. Our data suggest that the amastigote form localized in the macrophages utilizes coprogen from the host to produce haem (Figure 9). Corresponding coprogen transporters remain to be identified. Since trypanosomatids are the only eukaryotes possessing a HemG-type PPO, the L. major enzyme represents a perfect drug target for the treatment of Leishmaniasis most probably without the detrimental side effects of today’s treatment.

AUTHOR CONTRIBUTION

Dagmar Zwerschke has made substantial contributions to the acquisition and analysis of the data. Simone Karrie has made contribution of generating the plasmids used in this study. Overall supervision of the presented study as well as involvement in revising the manuscript critically for important intellectual content and final approval of the version to be published was undertaken by Martina Jahn and Dieter Jahn.

ACKNOWLEDGEMENTS

We thank Dr Gunhild Layer for support. We also thank the members of NBRP-E. coli at NIG for supporting with the strain LG285.

FUNDING

This work was supported by the Deutsche Forschungsgesellschaft [grant number DFG-Ja470/101].
REFERENCES

1. WHO (2010) Control of the Leishmaniases-report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22–26 March 2010. pp. 1–5, World Health Organization, Geneva

2. Davis, A. J. and Kedzierski, L. (2005) Recent advances in antileishmanial drug development. Curr. Opin. Investig. Drugs 6, 163–169 CrossRef PubMed

3. Handman, E. and Bullen, D. V. (2002) Interaction of Leishmania with the host macrophage. Trends Parasitol. 18, 332–334 CrossRef PubMed

4. Koreny, L., Obornik, M. and Lukes, J. (2013) Make it, take it, or leave it: heme metabolism of parasites. PLoS Pathog. 9, e1003088 CrossRef PubMed

5. Tripodi, K. E., Menendez Bravo, S. M. and Circo, J. A. (2011) Role of heme and heme-proteins in trypanosomatid essential metabolic pathways. Enzyme Res. 2011, 873230 CrossRef PubMed

6. Huynh, C., Yuan, X., Miguel, D. C., Renberg, R. L., Protchenko, O., Philpott, C. C., Hamza, I. and Andrews, N. W. (2012) Heme uptake by Leishmania amazonensis is mediated by the transmembrane protein LHR1. PLoS Pathog. 8, e1002795 CrossRef PubMed

7. Chang, K. P., Chang, C. S. and Sassa, S. (1975) Heme biosynthesis in bacterium-protozoon symbioses: enzyme defects in host hemoglobin and complemental role of their intracellular symbiotes. Proc. Natl. Acad. Sci. U.S.A. 72, 2979–2983 CrossRef PubMed

8. Sah, J. F., Ito, H., Kolli, B. K., Peterson, D. A., Sassa, S. and Chang, K. P. (2002) Genetic rescue of Leishmania deficiency in porphyrin biosynthesis creates mutants suitable for analysis of cellular events in uroporphyrina and for photodynamic therapy. J. Biol. Chem. 277, 14902–14909 CrossRef PubMed

9. Akiyama, O., Kosaka, S., O’Riordan, K. and Hasan, T. (2007) Parasiticidal effect of delta-aminolevulinic acid-based photodynamic therapy for cutaneous leishmaniasis is indirect and mediated through the killing of the host cells. Exp. Dermatol. 16, 651–660 CrossRef PubMed

10. Chang, C. S. and Chang, K. P. (1985) Heme requirement and acquisition by extracellular and intracellular stages of Leishmania mexicana amazonensis. Mol. Biochem. Parasitol. 18, 267–276 CrossRef PubMed

11. Fred, R. and Oppendoes, P. A. M. (2008) The metabolic repertoire of Leishmania and implications for drug discovery. Leishmania: After the Genome (Peter John Myler, N. F. C., ed.), pp. 137–140, Caister Academic Press, Norfolk

12. Ivens, A. C., Peacock, C. S., Worthy, E. A., Murphy, L., Aggarwal, G., Berriman, M., Sisk, E., Rajandream, M. A., Adlem, E., Aert, R. et al. (2005) The genome of the kinetoplastid parasite, Leishmania major. Science 309, 436–442 CrossRef PubMed

13. Koreny, L., Lukes, J. and Obornik, M. (2010) Evolution of the haem syntactic pathway in kinetoplastid flagellates: an essential pathway that is not essential after all? Int. J. Parasitol. 40, 149–156 CrossRef PubMed

14. Lee, D. S., Flachsova, E., Bodnarova, M., Demeler, B., Martasek, P. and Raman, C. S. (2005) Structural basis of hereditary coproporphyria. Proc. Natl. Acad. Sci. U.S.A. 102, 14232–14237 CrossRef PubMed

15. Phillips, J. D., Whitby, F. G., Warby, C. A., Labbe, P., Yang, C., Pflugrath, J. W., Ferrara, J. D., Robinson, H., Kushner, J. P. and Hill, C. P. (2004) Crystal structure of the oxygen-dependent coproporphyrinogen oxidase (Hem13p) of Saccharomyces cerevisiae. J. Biol. Chem. 279, 38960–38968 CrossRef PubMed

16. Rastrojo, A., Carrasco-Ramiro, F., Martin, D., Crespillo, A., Reguera, R. M., Aguado, B. and Requena, J. M. (2013) The transcriptome of Leishmania major in the axenic promastigote stage: transcript annotation and relative expression levels by RNA-seq. BMC Genomics 14, 223 CrossRef PubMed

17. Kato, K., Tanaka, R., Sano, S., Tanaka, A. and Hosaka, H. (2010) Identification of a gene essential for protoporphyrinogen IX oxidase activity in the cyano bacterium Synechoystis sp. PCC6803. Proc. Natl. Acad. Sci. U.S.A. 107, 16649–16654 CrossRef PubMed

18. Boynton, T. O., Gerdes, S., Craven, S. H., Neidle, E. L., Phillips, J. D. and Dailey, H. A. (2011) Discovery of a gene involved in a third bacterial protoporphyrinogen oxidase activity through comparative genomic analysis and functional complementation. Appl. Environ. Microbiol. 77, 4795–4801 CrossRef PubMed

19. Heinemann, I. U., Jahn, M. and Jahn, D. (2008) The biochemistry of heme biosynthesis. Arch. Biochem. Biophys. 474, 238–251 CrossRef PubMed

20. Boynton, T. O., Daugherty, L. E., Dailey, T. A. and Dailey, H. A. (2009) Identification of Escherichia coli HemG as a novel, menadione-dependent flavodoxin with protoporphyrinogen oxidase activity. Biochemistry 48, 6705–6711 CrossRef PubMed

21. Mobius, K., Arias-Cartin, R., Breckau, D., Hannig, A. L., Riedmann, K., Biedendieck, R., Schroder, S., Becher, D., Magalon, A., Moser, J. et al. (2010) Heme biosynthesis is coupled to electron transport chains for energy generation. Proc. Natl. Acad. Sci. U.S.A. 107, 10436–10441 CrossRef PubMed

22. Narita, S., Taketani, S. and Inokuchi, H. (1999) Oxidation of protoporphyrinogen IX in Escherichia coli is mediated by the aerobic coproporphyrinogen oxidase. Mol. Gen. Genet. 261, 1012–1020 CrossRef PubMed

23. Lim, C. K. (2009) High-Performance Liquid Chromatography and Mass Spectrometry of Porphyrins, Chlorophylls and Bilins (Methods in Chromatography), World Scientific Pub Co., London

24. Schauer, S., Luer, C. and Moser, J. (2003) Large scale production of biologically active Escherichia coli glutamy-RNA reductase from inclusion bodies. Protein Expr. Purif. 31, 271–275 CrossRef PubMed

25. Jacobs, J. M. and Jacobs, N. J. (2001) Measurement of protoporphyrinogen oxidase activity. Curr. Protoc. Toxicol. Chapter 8, Unit 8 5

26. Cox, R. and Charles, H. P. (1973) Porphyrin-accumulating mutants of Escherichia coli. J. Bacteriol. 113, 122–132 CrossRef PubMed

27. Doss, M. and Philipp-Dormston, W. K. (1971) Porphyrin and heme biosynthesis from Endogenous and exogenous delta-aminolevulinic acid in Escherichia coli, Pseudomonas aeruginosa, and Achromobacter metalcaligenes. Hoppe Seylers Z. Physiol. Chem. 352, 725–733 CrossRef PubMed

28. Biskup, T., Paulus, B., Okafuji, A., Hitomi, K., Getzoff, E. D., Weber, S. and Schleicher, E. (2013) Variable electron transfer pathways in an amphibian cryptochrome: tryptophan versus tyrosine-based radical pairs. J. Biol. Chem. 288, 9249–9260 CrossRef PubMed

29. Storbeck, S., Saha, S., Krause, J., Klink, B. U., Heinz, D. W. and Layer, G. (2011) Crystal structure of the heme d1 biosynthesis enzyme NirE in complex with its substrate reveals new insights into the catalytic mechanism of Sadenosyl-L-methionine-dependent uroporphyrinogen III methyltransferases. J. Biol. Chem. 286, 26754–26767 CrossRef PubMed

Received 13 May 2014/5 June 2014; accepted 6 June 2014
Published as Immediate Publication 25 June 2014, doi 10.1042/BSR20140081

© 2014 The Author(s). This is an Open Access article distributed under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.