**Leishmania major** possesses a unique HemG-type protoporphyrinogen IX oxidase

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**Synopsis**

*Leishmania major* was proposed to either utilize haem from its host or partially synthesize the tetrpyrrole from host provided precursors. However, only indirect evidence was available for this partial late haem biosynthetic pathway. Here, we demonstrate that the LMJF_06_1280 gene of *L. major* encodes a HemG-type PPO (protoporphyrinogen IX oxidase) catalysing the oxidation of protoporphyrinogen IX to protoporphyrin IX. Interestingly, trypanosomatids are currently the only known eukaryotes possessing HemG-type enzymes. The LMJF_06_1280 gene forms a potential transcriptional unit with LMJF_06_1270 encoding CPO (coproporphyrinogen III oxidase) and with LMJF_06_1290 for a cytochrome b₅₆. In vivo function of the *L. major* hemG gene was shown by the functional complementation of the *Escherichia coli* ΔhemG strain LG285. Restored haem formation in *E. coli* was observed using HPLC analyses. Purified recombinant *L. major* HemG revealed PPO activity *in vitro* using different ubiquinones and triphenyltetrazolium as electron acceptors. FMN was identified as the *L. major* HemG cofactor. Active site residues were found to be essential for HemG catalysis. These data in combination with the solved crystal structures of *L. major* CPO and the physiological proof of a ferrochelatase activity provide clear-cut evidence for a partial haem biosynthetic pathway in *L. major*.

**Key words:** amastigote partial haem biosynthesis, HemG, *Leishmania major*, parasite, protoporphyrinogen IX oxidase, tetrpyrrole


**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 haematophagy 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 b₅₆ 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 (proto IX) [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 Enterobacteriacea 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 respira-

tion. 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.

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 (Ultrascop 500 Pro, Amersham 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 OD 578 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...
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 OD<sub>578</sub> 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 redissolved in 10 ml harvesting buffer 100 mM Tris–HCl, pH 8.0, 500 mM NaCl, 0.2 % (w/v) Triton X-100. 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 protoporphyrin 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*

**Figure 5** HPLC analysis for the identification of the flavin cofactor of *L. major* HemG.

The retention time for the cofactor FAD was 13.28 min and 15.55 min for FMN. The purified cofactor from the *L. major* HemG fraction was detected at 15.55 min and thus identified as FMN.

**Figure 6** Protein model of *L. major* HemG.

The HemG model was created by the open access Swiss Model. The predicted active site is displayed in cyan and the locations of the mutated amino acid residues for the HemG variants are indicated in red.

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**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 (*Halogranaeum*). 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 synthesised 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 ΔhemG 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, tetrapyrroles 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 tetrapyrrole 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).
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 synthetic 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

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fumarate allowing electron transfer from protogen to fumarate via menaquinone containing fumarate reductase. Residual background 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*<sub>r</sub> 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 donor TTC (triphenyltetrazolium chloride). Under all tested conditions electron transfer from protogen to the electron 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* HemG 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<sup>α</sup> of the HemY and the HemG classes are utilizing flavins for the six electron oxidation of protogen. Eukaryotic HemY PPOs<sup>α</sup> possesses 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* AhemG 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; proto, protoporphyrinogen IX; proto, protoporphyrin IX; cyt b5, cytochrome b5; cyt P-450a, 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 Zworschke 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.

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