Chapter 2

*De novo heme biosynthetic pathway in malaria parasite*
2.1 Heme

Heme or iron protoporphyrin IX, a modified tetrapyrrole is a prosthetic group in many regulatory proteins involved in fundamental biological processes like electron transport chain driving aerobic and anaerobic respiration and photosynthesis, the metabolism and transport of oxygen, signal transduction, microRNA processing and circadian clock control and in enzymes such as catalases, peroxidases, or cytochrome P450 as essential cofactor and binding of heme by transcription factors to regulate gene expression thus playing a vital role in almost all living organisms (Storbeck et al., 2010; Layer et al., 2010; Hamza and Dailey, 2012; Sigala and Goldberg, 2014). Heme consists of an iron (Fe) atom bound to four nitrogen atoms of the pyrrole ring of protoporphyrin IX (Figure 2.1). Fe is a transitional metal, that exists in either a ferrous (Fe2+) or a ferric (Fe3+) forms and this ability imparts the catalytic nature to many compounds that contains Fe, whilst porphyrin is an organic compound capable of producing singlet oxygen (1O2) in its excited state and its hydrophobic nature facilitates the interaction with lipophilic molecules, including proteins and lipids. This reactive nature of both Fe and porphyrin make heme an essential molecule for which, all the cells have evolved robust pathways to either biosynthesize heme de novo or to scavenge it from their environment (Toh et al., 2010; Sigala and Goldberg, 2014).

![Figure 2.1: Chemical structure of Heme. (adapted from Sigala and Goldberg, 2014)](image-url)
2.2 Heme Biosynthesis: Shemin and C₅-Pathway

2.2.1 Synthesis of δ-aminolevulinate ‘the committed precursor’

δ-Aminolevulinate (ALA), the first committed and universally used precursor for the biosynthesis of heme and other tetrapyrroles, is the sole source of all carbon and nitrogen atoms present in the tetrapyrrole backbone. The ALA is synthesized in nature by two different known pathways. The first one, “Shemin pathway”, named after one of the two discoverers, where in ALA is formed via the condensation of succinyl-coA and glycine by pyridoxal-5’-phosphate (PLP)-dependent ALA synthase (ALAS) resulting in the elimination of CO₂ and occurs in mammals, fungi and α proteobacteria. It is noteworthy that the C1-carboxyl group of glycine undergoes decarboxylation in this reaction and therefore, only 2¹⁴C-glycine but not 1¹⁴C-glycine can radiolabel heme. In an alternative second pathway that occurs in Plants, algae and cyanobacteria termed as “C₅-pathway”, the C₅-skeleton of glutamate is converted to ALA involving tRNA-bound glutamate in a three steps reaction.

In the presence of ATP and Mg (II), glutamyl-tRNA synthetase (GluRS) catalyzes the first step in which the α-carboxyl group of glutamate is activated by its ligation to t-RNA-Glu. The second and committed enzyme of this pathway, glutamyl-tRNA reductase (GluTR) catalyzes the reduction of glutamyl-tRNA in a NADH/NADPH dependent reaction to glutamate-1-semialdehyde (GSA). In the following step, the amino group of GSA is transferred to its terminal carbon atom to yield ALA, in a PLP-dependent intramolecular transamination reaction catalyzed by GSA aminotransferase (GSA-AT). Based on available information, the photosynthetic phytoflagellate *Euglena gracilis* is the only known organism that utilizes both the Shemin and the C₅-pathways for tetrapyrrole biosynthesis. Here, ALA derived from the Shemin pathway is incorporated into mitochondrial tetrapyrroles, whereas ALA synthesized from the C₅-pathway is incorporated into plastidic tetrapyrroles (reviewed by Padmanaban et al., 2013; Nagaraj et al., 2013).

2.2.2 Conversion of ALA into Heme

Once ALA is synthesized, the subsequent steps that lead to the formation of heme are common for both Shemin and C₅-pathways. ALA dehydratase (ALAD), also known as porphobilinogen synthase, catalyzes the asymmetric condensation of two molecules of ALA to yield the first pyrrole derivative porphobilinogen (PBG). The enzyme reaction is initiated by the formation of Schiff base bonds between the two ALA substrate molecules and two
conserved lysine residues present in the active site. Despite the structural conservation of a TIM-barrel fold and an octomeric assembly in ALADs of various species, considerable diversity in the use of metal ion cofactors [Zn (II)- or Mg (II)-dependent] has been reported. PBG is converted into protoheme (heme b) by the sequential action of six downstream enzymes, namely PBG deaminase (PBGD), uroporphyrinogen III synthase (UROS), uroporphyrinogen III decarboxylase (UROD), coproporphyrinogen III oxidase (CPO), protoporphyrinogen IX oxidase (PPO), and ferrochelatase (FC) (Figure 2.2) (reviewed by Padmanaban et al., 2013; Nagaraj et al., 2013b).

2.3 Compartmentalization of Heme-Biosynthetic Pathway

![Heme-biosynthetic pathway](image)

**Figure 2.2**: The heme-biosynthetic pathway in animals and plants. Although the biosynthetic machinery is partitioned between the mitochondria and cytosol in animals, the present understanding is that, the entire pathway is operational in the chloroplast of plants. Animals and plants make ALA through different routes (adapted with modification from Ralph et al. 2004).
In bacteria, the enzymes of the heme biosynthetic pathway leading to the formation of heme are cytosolic, with the exception of the last two enzymes, PPO and FC, which are localized to the cytoplasmic membrane. In animals, fungi, and yeast, ALA synthesized in the mitochondrion by Shemin pathway is transported to the cytoplasm, where it is sequentially acted upon by ALAD, PBGD, UROS, and UROD to give rise to coproporphyrinogen III. While CPO catalyzing the next step is localized to the outer mitochondrial membrane of animals, yeast and fungi CPOs are cytosolic. The last two enzymes of this pathway, PPO and FC, are localized to the inner mitochondrial membrane, leading to the formation of heme in the mitochondrion. In plants and algae, ALA is synthesized in the chloroplast by C5 pathway. The subsequent steps leading to the formation of heme are also localized to the chloroplast. The chloroplast is the major site for heme synthesis in both photosynthetic and non-photosynthetic cells of higher plants, and the significance of minor heme production in plant mitochondria, if any, needs to be assessed (Figure 2.2) (reviewed by Padmanaban et al., 2013; Nagaraj et al., 2013).

2.4 Heme biosynthetic pathway in malaria parasite

*Plasmodium* essentially follows the Shemin pathway to make ALA from succinyl-CoA and glycine. *In vitro* studies carried out with *P. falciparum* cultures revealed that the parasite heme is radiolabeled with 214C-glycine and inhibition of this pathway with a specific inhibitor such as succinylacetone led to death of the parasite in culture, indicating its potential as a drug target (Surolia and Padmanaban, 1992; Bonday et al., 1997; reviewed by Padamanban et al., 2013; Nagaraj et al., 2013). An unusual hybrid pathway for heme biosynthesis involving three different compartments of the parasites, namely the apicoplast, mitochondria and cytosol have been predicted based on the presence or absence of targeting sequences using the Bioinformatics approach (Ralph et al., 2004; Sato et al., 2004; reviewed by Nagaraj et al., 2013b; Padmanaban et al., 2013). Earlier studies have completely characterized all the enzymes in *P. falciparum* heme biosynthetic pathway. The parasite enzymes are unique in terms of their localization and catalytic efficiencies. Experimental evidences from immunofluorescence studies performed with antibodies raised against the recombinant enzymes, and the co-localization of native parasite enzymes with organellar markers together with GFP-reporter studies in transfected parasites revealed that PfALAS is localized in the mitochondrion (Varadharajan et al., 2002; Sato et al. 2004).
The three enzymes subsequent to \textit{PfALAS–PfALAD} (Dhanasekaran \textit{et al.}, 2004; Sato \textit{et al.} 2004), \textit{PfPBGD} (Nagaraj \textit{et al.}, 2008; Sato \textit{et al.}, 2004), and \textit{PfUROD} (Nagaraj \textit{et al.}, 2009a) are localized in the apicoplast, a chloroplast relic that is unique to apicomplexan parasites. The next enzyme, \textit{PfCPO}, is localized in the parasite cytosol (Nagaraj \textit{et al.}, 2010a). \textit{PfPPO} (Nagaraj \textit{et al.}, 2010b) and \textit{PfFC} (Nagaraj \textit{et al.}, 2009b), the penultimate and the last enzymes of this pathway, are localized in the parasite mitochondrion. Thus, ALA synthesized in the mitochondrion has to be transported to the apicoplast to get converted into porphobilinogen, uroporphyrinogen III, and then to coproporphyrinogen III. The conversion of coproporphyrinogen III to protoporphyrinogen IX takes place in the cytosol, which in turn has to be transported back into the mitochondria to form protoporphyrin IX and eventually heme. \textbf{Figure 2.3} provides the schematic representation of the pathway. A close physical association between the mitochondrion and apicoplast has been proposed to facilitate the transport of ALA from the mitochondrion to the apicoplast. The transporters involved in the shuttling of other intermediates are yet to be identified (reviewed by Nagaraj \textit{et al.}, 2013b; Padmanaban \textit{et al.}, 2013).

\textbf{Figure 2.3}: De novo heme biosynthetic pathway of \textit{Plasmodium falciparum} (\textit{Pf}) (adapted with modification from Nagaraj \textit{et al.}, 2013a)
2.4.1 Features of Heme Biosynthetic Enzymes in Plasmodium

*Pf*ALAS, the first enzyme of the heme biosynthetic pathway is a PLP-dependent enzyme that catalyzes the condensation of glycine and succinyl-CoA to give δ-aminolevulinate. The recombinant *Pf*ALAS is expressed as 63kDa protein that is found to be active as a dimer. The *Pf*ALAS carries a mitochondria-targeting signal and the native enzyme for the same is shown to be localized in the parasite mitochondrion. The *in vitro*-translated *Pf*ALAS has been shown to be translocated into mitochondria, and the process is inhibited by hemin. Ethanolamine was found to inhibit the parasite enzyme at 160nM concentration and also the parasite heme synthesis suggesting that *Pf*ALAS could be a drug target (Varadharajan *et al.* 2002).

*Pf*ALAD, the second enzyme of the pathway catalyzes the dimerization of two molecules of ALA to give PBG and is localized in the apicoplast. The recombinant *Pf*ALAD is expressed as an octamer with a mass of 35kDa and does not require any divalent metal ion for activity unlike the red cell counterpart. However, its activity is stimulated by Mg²⁺ to an extent of 20–30 %. Also, the basal enzyme activity is not inhibited by EDTA, even at a concentration of 25 mM. There is evidence for the import of host ALAD into the parasite cytosol, the significance of which is not clear. Interestingly, the ALAD activity in the parasite lysate is inhibited by EDTA, indicating that bulk of the enzyme activity is due to the imported host enzyme (Dhanasekaran *et al.* 2004).

The conversion of PBG to uroporphyrinogen III formation is a two-step reaction involving PBGD and UROS activity. *Pf*PBGD have been shown to be localized in apicoplast (Sato *et al.*, 2004; Nagaraj *et al.*, 2008). Surprisingly, *Pf*PBGD is a bifunctional enzyme and catalyzes the conversion of PBG to uroporphyrinogen III, indicating that it also manifests the UROS activity (Nagaraj *et al.* 2008). A separate gene for *Pf*UROS has not been annotated so far in the *Plasmodium* genome (Ralph *et al.* 2004).

Further, UROD, the next enzyme of the pathway catalyzes the decarboxylation of four acetic chains uroporphyrinogen III to yield corresponding methyl groups of coproporphyrinogen III (Padmanaban *et al.*, 2013). Molecular modeling of *Pf*UROD based on the known crystal structure of the human enzyme indicated that the protein exhibits a distorted triose phosphate isomerase (TIM) barrel fold which is conserved in all the known structures of UROD. An interesting feature of *Pf*ALAD, *Pf*PBGD, and *Pf*UROD, all localized in the apicoplast, is their low catalytic efficiency compared to the corresponding red cell counterparts. This appears to be due to the key differences that exist in some of the amino acid residues. For example, *Pf*UROD shares all the conserved or invariant amino acid residues at the active and substrate binding sites, but is rich in lysine residues compared with the host enzyme (Nagaraj
et al. 2009a). Mutation of specific lysine residues corresponding to residues at the dimer interface in human UROD enhanced the catalytic efficiency of the enzyme and dimer stability indicating that the lysine-rich nature and weak dimer interface of the wild-type PfUROD are responsible for its low catalytic efficiency. Coproporphyrinogen III Oxidase catalyzes the sequential decarboxylation of the propionate groups of pyrrole rings A and B of coproporphyrinogen III via monovinyl intermediate harderoporphyrinogen to form protoporphyrinogen IX, a divinyl product (Padmanaban et al., 2013). Two structurally unrelated CPOs, aerobic and anaerobic, are known to exist in nature. In facultative organisms such as E. coli and S. cerevisiae, both forms of CPO are present, and their expression is subject to strict oxygen regulation. PfCPO exists as a 58–61 kDa monomer in the parasite cytosol and is aerobic (Nagaraj et al., 2010a). The cytosolic localization of PfCPO provides conclusive evidence for the hybrid pathway proposed earlier that heme biosynthesis in the malaria parasite involves enzymes of three different compartments, namely, apicoplast, mitochondrion, and cytosol. PfPPO catalyzing the penultimate step in the heme biosynthetic enzyme has been found to be an anaerobic mitochondrial enzyme, requiring the parasite ETC for its activity (Nagaraj et al., 2010b). It has all along been assumed that the parasite ETC is only meant to sustain a single reaction catalyzed by the enzyme dihydroorotate dehydrogenase (DHOD) in the pyrimidine pathway. Thus, the parasite ETC function is required for heme biosynthesis and has a role beyond sustaining just one reaction in the pyrimidine pathway.

The discovery of a new function for the parasite ETC provides additional drug targets and suggests that although the parasite derives energy through glycolysis in the intraerythrocytic stages, the mitochondrion may have functions that still need to be evaluated (Nagaraj et al., 2013b; Padmanaban et al., 2013). The final step in the heme biosynthetic pathway is catalyzed by the enzyme ferrochelatase (FC) which incorporates iron into protoporphyrin IX. PfFC gene encodes multiple transcripts of which the one encoding the full length functional protein has been cloned and the recombinant protein is overexpressed and purified from E. coli. PfFC shows maximum activity with iron, and its unique features are that zinc is a poor substrate and copper, which shows a sigmoidal response with mouse FC, gives a hyperbolic curve. Immunofluorescence studies with antibodies to functional ferrochelatase reveal that the native enzyme is localized to the mitochondrion of the parasite indicating that this organelle is the ultimate site of heme synthesis (Nagaraj et al. 2009b). All these results establish that the heme biosynthetic pathway in P. falciparum involves the participation of
enzymes localized in the mitochondrion, apicoplast, and cytosol (Padmanaban et al. 2013). Table-1 summarizes the kinetic properties of the recombinant heme biosynthetic enzymes of *P. falciparum*.

**Table 1:** Kinetic properties of recombinant *P. falciparum* heme biosynthetic enzymes (adapted from Nagaraj et al., 2013b)

<table>
<thead>
<tr>
<th>Heme biosynthetic enzymes of <em>P. falciparum</em></th>
<th>PlasmoDB gene ID</th>
<th>Subcellular localization</th>
<th>K_m for substrate</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ-aminolevulinate synthase (ALAS)</td>
<td>PF3D7_1246100</td>
<td>Mitochondrion</td>
<td>50 mM (glycine) 100 μM (succinyl-CoA)</td>
<td>1.2 μmol/mg/h</td>
</tr>
<tr>
<td>δ-aminolevulinate dehydratase (ALAD)</td>
<td>PF3D7_1440300</td>
<td>Apicoplast</td>
<td>0.21 mM</td>
<td>4 μmol/mg/h</td>
</tr>
<tr>
<td>Porphobilinogen deaminase (PBGD)</td>
<td>PF3D7_1209600</td>
<td>Apicoplast</td>
<td>1.3 mM</td>
<td>0.5 nmol/mg/h</td>
</tr>
<tr>
<td>Uroporphyrinogen III decarboxylase</td>
<td>PF3D7_0607300</td>
<td>Apicoplast</td>
<td>10.9 μM</td>
<td>0.14 μmol/mg/h</td>
</tr>
<tr>
<td>Coproporphyrinogen III oxidase (CPO)</td>
<td>PF3D7_1142400</td>
<td>Cytosol</td>
<td>9.67 μM</td>
<td>110 nmol/mg/h</td>
</tr>
<tr>
<td>Protoporphyrinogen IX oxidase (PPO)</td>
<td>PF3D7_1028100</td>
<td>Mitochondrion</td>
<td>47.6 μM</td>
<td>170.80 nmol/mg/h</td>
</tr>
<tr>
<td>Ferrochelatase (FC)</td>
<td>PF3D7_1364900</td>
<td>Mitochondrion</td>
<td>0.50 μM</td>
<td>0.47 μmol/mg/min</td>
</tr>
</tbody>
</table>

*a*PBGD is a bifunctional enzyme and it has uroporphyrinogen III synthase (UROS) activity as well. All the three apicoplast-localized enzymes – PfALAD, PfPBGD, and PfUROD – are catalytically less efficient when compared to their host counterparts. PfCPO is aerobic and PfPPO is anaerobic.