The Peculiarities and Paradoxes of *Plasmodium* Heme Metabolism

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Abstract

For over a century, heme metabolism has been recognized to play a central role during intraerythrocytic infection by *Plasmodium* parasites, the causative agent of malaria. Parasites liberate vast quantities of potentially cytotoxic heme as a by-product of hemoglobin catabolism within the digestive vacuole, where heme is predominantly sequestered as inert crystalline hemozoin. *Plasmodium* spp. also utilize heme as a metabolic cofactor. Despite access to abundant host-derived heme, parasites paradoxically maintain a biosynthetic pathway. This pathway has been assumed to produce the heme incorporated into mitochondrial cytochromes that support electron transport. In this review, we assess our current understanding of the love-hate relationship between *Plasmodium* parasites and heme, we discuss recent studies that clarify several long-standing riddles about heme production and utilization by parasites, and we consider remaining challenges and opportunities for understanding and targeting heme metabolism within parasites.

Contents

INTRODUCTION

Heme, or iron protoporphyrin IX, is a ubiquitous biological cofactor required by nearly all organisms for growth and viability (**Figure 1**) (69). Heme can lose or regain an electron to toggle between reduced ferrous (Fe^{2+}) and oxidized ferric (Fe^{3+}) states, and this ability is exploited and tuned by assorted heme-binding proteins to carry out diverse and fundamental reactions of oxidative cellular metabolism. These reactions include cellular respiration via the electron transport chain and diverse redox conversions that are mediated by cytochromes, the neutralization of oxygen radicals by catalase, oxygen binding and transport by globins, diatomic gas sensing and signal transduction by soluble guanylate cyclase, and heme binding by transcription factors to regulate gene expression (99). To satisfy their critical need for heme, virtually all cells have evolved robust pathways to either biosynthesize heme de novo or scavenge it from their environment.

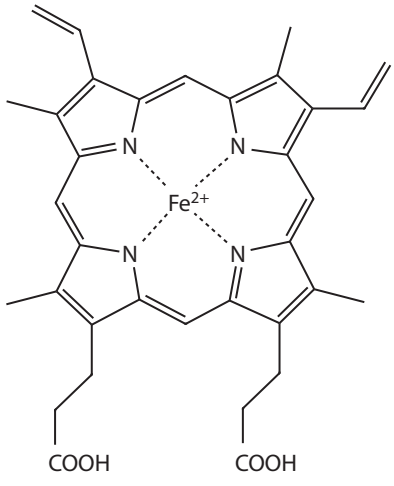

Figure 1

Chemical structure of heme.

Protozoan parasites of the genus *Plasmodium*, the causative agents of malaria, have evolved a life cycle with intimate and seemingly paradoxical connections to heme. Asexual parasites invade and develop within erythrocytes, the most heme-rich cells in the human body, and all of the clinical symptoms of malaria arise during this blood-stage infection. Indeed, the visualization of crystalline heme aggregates, or malaria pigment, within the blood of infected patients was recognized as a hallmark of malaria even before discovery of the *Plasmodium* parasitic origin of the disease (121). On the basis of their rapid, energy-intensive multiplication during intraerythrocytic infection, parasites might be expected to have evolved an energy metabolism that exploits this heme- and oxygen-rich environment for lucrative ATP production via heme-dependent oxidative phosphorylation. However, genomic and functional evidence suggests that parasites have a limited metabolic need for heme during growth within red blood cells. In contrast, recent studies suggest enhanced roles for heme-related processes within the parasite as it develops within comparatively heme-barren environments during exoerythrocytic growth inside human hepatocytes or the mosquito vector. In this review, we discuss these paradoxes and the idiosyncrasies of *Plasmodium* heme metabolism.

METABOLISM OF HOST-DERIVED HEME

Liberation of Heme During Hemoglobin Digestion and Incorporation into Hemozoin

During its 48-hour residence within an infected red blood cell, an asexual *Plasmodium* parasite imports up to 80% of host hemoglobin into the acidic digestive vacuole, where the collaborative action of multiple aspartic, cysteine, and metal-dependent proteases hydrolyzes the globin polypeptide into diverse small peptides and amino acids (**Figure 2**) (46, 102). This massive catabolic process liberates copious amounts of the noncovalently associated heme prosthetic group. Unlike the proteolytic products of hemoglobin digestion, which are transported into the parasite cytoplasm and further metabolized, the vast majority of host-derived heme remains within the food vacuole and accumulates to an estimated concentration that would approach 0.5 M if homogeneously distributed (9, 41).

In most species, intracellular heme levels are stringently regulated, and heme is found tightly associated with cognate hemoproteins or various heme-trafficking proteins that minimize accumulation of unbound heme (99). Free, dissociated heme, especially at high concentration, can have a variety of cytotoxic properties based on its ability to bind and inhibit protein function, to partition into and disrupt membrane bilayers, and to undergo electron transfer reactions with molecular oxygen to produce diverse species of reactive oxygen (41, 59, 71). Within the parasite digestive vacuole, the copious heme liberated during hemoglobin proteolysis is expected (and commonly assumed) to be cytotoxic based on its potential to destabilize the vacuolar membrane, to inhibit the function of proteases and other enzymes, and to spontaneously generate reactive oxygen molecules either within this compartment or upon protonation and passive diffusion into the reduced environment of the parasite cytoplasm (9, 41). However, the cellular mechanisms of heme toxicity to parasites remain poorly defined in vivo. There are previous reports that exogenously added free heme can lyse parasite-infected erythrocytes (39, 91), but the physiological relevance of these prior studies is unclear. Nevertheless, the general ability of the parasite to tolerate and indeed proliferate despite generation of high local concentrations of heme suggests the presence of protective mechanisms to attenuate heme toxicity.

Many organisms express heme oxygenase enzymes that, in concert with other enzymes, cleave and degrade heme to less toxic metabolites that can be readily excreted for disposal. Indeed, the

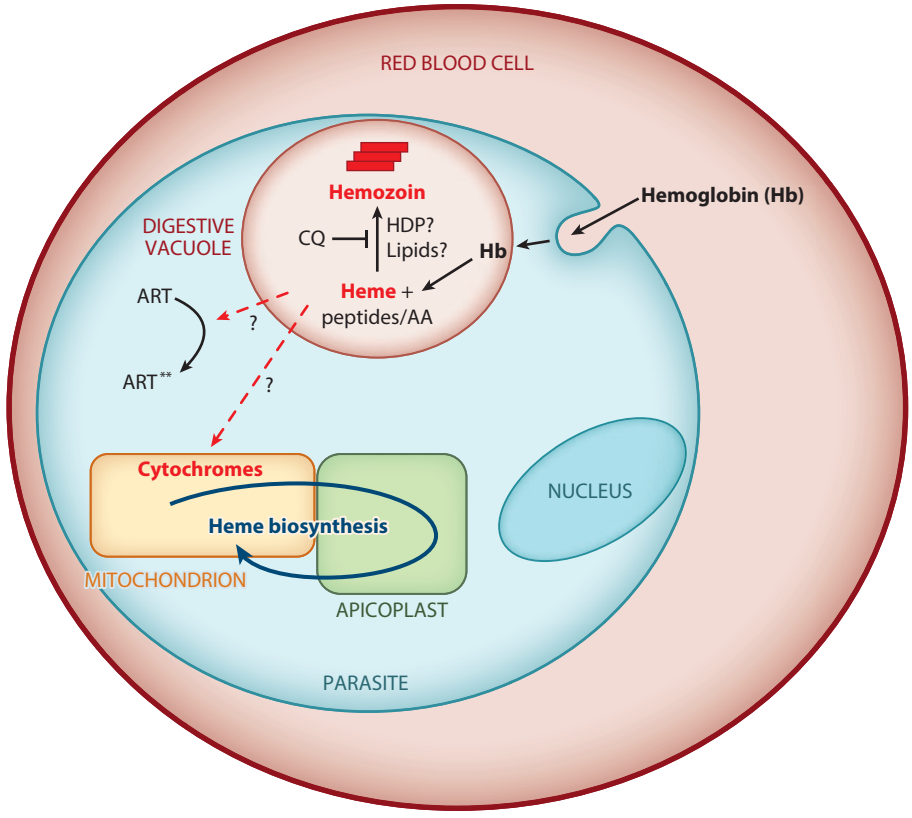

Figure 2

Schematic depiction of heme metabolism within blood-stage *Plasmodium* parasites. For simplicity, organelles are depicted with a single membrane. Abbreviations: AA, amino acids; ART, artemisinin; ART**, activated artemisinin; CQ, chloroquine; Hb, hemoglobin; HDP, heme detoxification protein. Adapted from Reference 113.

massive amount of heme that is released in the mammalian spleen during macrophage-mediated destruction of senescent or damaged red blood cells is detoxified by efficient enzymatic cleavage by heme oxygenase-1, derivatization and soluble excretion of the tetrapyrrole backbone, and reuse of heme-bound iron (42, 99). Based on the amount of heme encountered during erythrocytic infection and on the dietary need to scavenge host iron to support growth, *Plasmodium* parasites might be expected to have evolved a heme oxygenase pathway to degrade and detoxify heme. Although recent works have identified a heme oxygenase ortholog, PfHO, encoded by the parasite genome (87, 105), this protein lacks the key residues expected for activity and does not degrade heme in vitro or in vivo (113), and direct metabolic tests fail to identify breakdown products from enzymatic heme degradation in parasites (113). Thus, *Plasmodium* parasites appear to lack a heme oxygenase pathway for heme detoxification and disposal. This absence, and the apparent functional divergence of PfHO, may reflect evolutionary pressures that have guided parasites away from large-scale heme degradation and the associated challenge of disposing of the copious iron and other breakdown products that would be released.

Rather than degrading heme, *Plasmodium* parasites sequester host heme into crystals of insoluble hemozoin in the digestive vacuole (**Figures 2** and **3*a***) (41, 121). In this regard, parasites

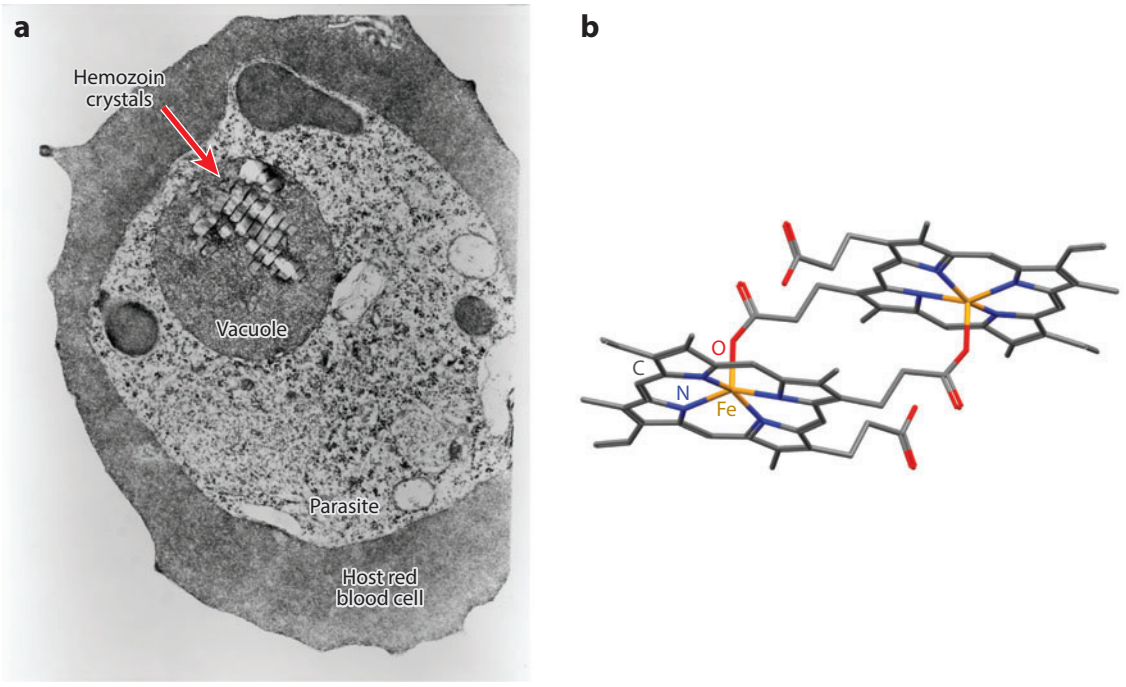

Figure 3

Hemozoin formation within parasites. (*a*) Electron micrograph of an asexual parasite within a host red blood cell, reproduced from Reference 47. The red arrow indicates hemozoin crystals in the digestive vacuole. (*b*) Heme-heme reciprocal dimer that forms a repeating subunit within hemozoin crystals, based on the structural coordinates from Reference 14. Atoms are colored by element: gray, carbon; blue, nitrogen; red, oxygen; yellow, iron.

are similar to other hematophagous organisms that primarily sequester heme rather than enzymatically degrade it (126). Like *Plasmodium* spp., the blood fluke *Schistosoma mansoni*, the kissing bug *Rhodnius prolixus*, and the related apicomplexan parasite *Haemoproteus colombae* biomineralize host-derived heme into crystalline hemozoin (89), although hemozoin morphology in these organisms differs from that of hemozoin in *Plasmodium* spp. (24, 89). *Aedes aegypti* mosquitoes sequester blood-meal heme in association with the peritrophic matrix surrounding the midgut but do not biomineralize it (97). Hemozoin crystals, which feature repeating reciprocal dimers of ferric (Fe^{3+}) protoporphyrin IX in which the carboxylate moiety of a propionate group on one monomer coordinates the iron atom in a second monomer and vice versa (**Figure 3*b***) (93, 116), are chemically inert. Their formation thus neutralizes the oxidative, cytotoxic properties of unassociated heme in situ without a requirement for further chemical processing.

Hemozoin formation within parasites commences upon initiation of hemoglobin import and digestion and becomes detectable in mid- to late-ring-stage parasites (41, 64). Although the structure of hemozoin crystals has been characterized in detail (93, 114, 121), the specific mechanisms that nucleate and propagate hemozoin crystallization within the digestive vacuole remain unresolved. Distinct hypotheses have proposed that nucleation and growth occur (*a*) within the digestive vacuole itself or within hemoglobin-containing vesicles en route to the digestive vacuole (1, 53, 121), (*b*) within lipid droplets suspended in vacuolar compartments or at the membrane lipid–aqueous interface (2, 11, 60), or (*c*) via a spontaneous or protein-assisted process (32, 36, 115, 122). Although hemozoin formation occurs in vitro in the absence of protein

catalysts under conditions that mimic the acidic environment with the digestive vacuole (32, 36), several *Plasmodium* proteins known to be present within the food vacuole, including histidine-rich protein II (HRPII) and heme detoxification protein (HDP), have been shown to accelerate heme crystallization in vitro, suggesting a role in hemozoin formation in vivo (**Figure 2**) (58, 96, 122).

HRPII does not appear to be required by parasites for hemozoin formation, as HRPII is unique to *P. falciparum*, yet other *Plasmodium* species also make hemozoin (122). Furthermore, parasites that lack the HRPII gene show no perturbation to hemozoin growth or heme crystallization (121, 122, 130). Although HDP potently accelerates hemozoin formation in vitro, its genomic locus has so far been refractory to integration, including attempted modifications that would either retain or truncate the open reading frame (58), precluding direct tests or strong conclusions regarding the importance of its role in hemozoin formation in vivo. Recently, several food vacuole proteases and HDP were copurified as a complex from parasites, suggesting that these proteins interact in vivo and form associations that may facilitate hemoglobin proteolysis and the transfer of the liberated heme group to HDP for assembly into hemozoin (25). Cooperative action of the proteins isolated in this complex has not been demonstrated, and the importance of complexation for hemozoin formation in vivo remains to be tested.

The physical and chemical properties of hemozoin crystals have been extensively investigated in fixed parasites, in isolated hemozoin purified from parasites, and in the chemically analogous β-hematin prepared synthetically in vitro (10, 13, 35, 60, 93, 116, 121). In their native environment within live parasites, hemozoin crystals are frequently observed to be dynamically moving within the digestive vacuole (**Supplemental Video 1**; follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**). Although ubiquitous, this phenomenon is rarely mentioned in the literature, and the few studies that mention it describe this motion as Brownian in nature (104, 133). Crude calculations (using Fick's laws of diffusion, estimated viscosities for the digestive vacuole matrix, and a simplified model of hemozoin motion as rodlike rotation about a minor axis), however, suggest that the observed motion is one to two orders of magnitude faster than expected for Brownian diffusion (P. Sigala and D. Goldberg, unpublished results). These observations may suggest that the motion of hemozoin crystals, which are paramagnetic owing to the Fe^{3+} oxidation state of the coordinated iron atoms (12, 18), is stimulated by specific electrochemical properties of the digestive vacuole rather than being the random, diffusive motion of particles suspended in a liquid matrix. Ultimately, the physical nature and origin of hemozoin motion and its functional significance for hemoglobin digestion or heme crystallization remain uncertain. Advancing technologies for time-lapse, super-resolution imaging with fluorescence or electron microscopy and specific in vivo functional tests may enable a more incisive physical understanding of this fascinating motion and its significance for parasite physiology.

Supplemental Material

Distribution and Fate of Host-Derived Heme Within Parasites

There is general consensus that the majority of heme liberated during hemoglobin digestion is sequestered in hemozoin crystals within the digestive vacuole, although discrete approaches have given differing estimates for this conversion (29, 35, 74, 135). It remains less clear, however, whether a fraction of host-derived heme escapes mineralization and either remains within the digestive vacuole matrix (free or in association with lipids or proteins) or effluxes out of the vacuole and into the parasite cytoplasm or endomembrane milieu (**Figure 2**). The propionic acid side chains ($pK_a \sim 5$) of heme molecules not sequestered into hemozoin may be protonated within the acidic (pH ~ 5) environment of the vacuole (**Figure 1**). These neutral heme molecules might then partition into the digestive vacuole membrane and diffuse out into the parasite cytoplasm,

whose neutral pH would favor the ionization of these side chains (9). Alternatively, heme molecules escaping mineralization could be exported from the digestive vacuole via specific membrane transporters. These mechanisms are speculative, however, and specific tests will be required to identify labile, unsequestered heme, its distribution within parasites, and the pathways by which it may egress from the digestive vacuole. Such tests will need to surmount the formidable challenge of identifying and distinguishing low levels of dispersed heme from the concentrated heme present within hemozoin. This daunting experimental task might be approachable via genetically encoded biosensors or other emerging spectroscopic technologies for in situ metabolite detection in intact cells (16, 22, 29, 33, 88).

Heme that escapes mineralization into hemozoin has the potential to disrupt membranes, inhibit protein function, and generate cytotoxic reactive oxygen, as discussed previously. Parasites appear to lack a heme oxygenase pathway to degrade heme (113), but two alternative mechanisms have been proposed for nonenzymatic degradation of unassociated heme that accumulates within the digestive vacuole or that diffuses into the parasite cytoplasm. These mechanisms, based on either the auto- or glutathione-catalyzed peroxidative cleavage of the porphyrin macrocycle within the respective compartments of the digestive vacuole or cytosol (5, 74, 135), have been shown to operate in vitro, but it remains unknown whether nonenzymatic degradation of heme occurs inside parasites.

Interactions of Anti-Malarial Drugs with Heme

Host-derived heme has been implicated as a likely activator of the potent antimalarial artemisinin. This drug has an endoperoxide moiety that is required for antiparasitic activity and is thought to be reductively cleaved within parasites to form a reactive radical that broadly modifies lipids, proteins, and other metabolites to elicit pleiotropic cytotoxic effects (51, 61, 77, 90). A number of studies have implicated reduced heme as the likely electron donor in this activation, but the compartment in which activation occurs and its mechanism remain uncertain. Recent work has shown that genetic or chemical inhibition of hemoglobin digestion reduces parasite sensitivity to artemisinin (65), suggesting an important role for hemoglobin-derived heme in drug activation. Does artemisinin diffuse into the digestive vacuole and get activated by heme within this compartment, or does activation occur elsewhere? Heme is oxidized from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state upon release from hemoglobin, which would not be competent to activate artemisinin. Heme that escapes from the digestive vacuole into the reduced intracellular environment of the parasite, however, would be expected to be in the ferrous (Fe^{2+}) state and may therefore be the more likely candidate to activate artemisinin (**Figure 2**). Deeper understanding of the activation mechanism of artemisinin remains a key challenge given its critical role in antimalarial chemotherapy and emerging reports of clinical resistance (4, 23, 31, 78, 125).

Chloroquine and other quinoline drugs, despite widespread resistance in recent decades, have historically been some of the most potent and successful antiparasitic drugs. These compounds are thought to act primarily within the digestive vacuole and to interfere with the process of hemozoin formation (**Figure 2**), by binding either to free heme or to the exposed faces of nascent hemozoin crystals. Such interactions, which have been demonstrated in vitro (49, 123), are expected to reduce the amount of heme sequestered and neutralized as hemozoin within the digestive vacuole, leading to an increase in chemically labile free heme that could kill parasites via oxidative and inhibitory mechanisms discussed above. In support of this model, several studies have shown that chloroquine treatment decreases the amount of detectable hemozoin within parasites (29, 115, 135). This treatment appears to be accompanied by a concomitant increase in unassociated heme, and electron imaging of iron atoms distributed within parasites suggests that a portion of this increased,

unbound heme effluxes from the food vacuole into the cytoplasm or endomembrane system via unknown pathways (29). Although it is clear that chloroquine and heme can interact, the specific mechanisms and processes by which chloroquine treatment results in parasite death are complex and only partially understood (121). For example, recent studies suggest that chloroquine killing of parasites does not correlate with its inhibition of hemozoin formation and that parasite death may result from effects more complex than just accumulation of unassociated heme (48, 49). A full mechanistic understanding of the antiparasitic properties of chloroquine remains a future challenge.

Host Response to Parasite Liberation of Hemoglobin-Bound Heme

At the end of their 48-hour developmental cycle within an infected red blood cell, asexual parasites lyse the erythrocyte to release merozoites that rapidly invade new host cells. This lytic process also releases the contents of the infected blood cell into the host serum, including undigested hemoglobin, free heme, and hemozoin. These products, ordinarily not released by circulating healthy erythrocytes, are sensed by the host immune system and activate an inflammatory response that affects disease progression and pathology, including the onset of cerebral malaria (3, 26, 37). Some of the mechanisms involved in this activation have been identified (27, 109, 112), but the physiological consequences of the resulting immune response remain only partially understood.

Expression of host heme oxygenase-1 (HO1) is increased as part of the host immune response. This inducible enzyme can degrade the circulating free heme to more benign tetrapyrrole products, and this activity appears to play a key role in host protection against both cerebral and noncerebral manifestations of malaria (95, 110). Protection appears to be based, in part, on the ability of carbon monoxide produced by HO1-catalyzed heme degradation to bind and stabilize hemoglobin released into the bloodstream by rupturing schizonts, preventing further release of free heme into circulation (37). A similar mechanism involving host HO1 also appears to contribute to the observed resistance to severe malaria in individuals expressing sickle hemoglobin, which releases heme at low levels and activates HO1 expression to confer protection against *Plasmodium* infection (38, 52).

HEME UTILIZATION

Heme is an ancient and ubiquitous biological cofactor that is required by nearly all organisms to carry out diverse reactions of oxidative metabolism (99). Relative to the genomes of other eukaryotic parasites that grow within aerobic environments, the genomes of *Plasmodium* spp. appear to encode few orthologs of common heme proteins (44). Cytochrome components of the mitochondrial electron transport chain are readily identified, but few additional heme proteins are apparent in the genome (67, 127). *Plasmodium* parasites retain a single mitochondrion with multiple cytochromes that function as part of an active electron transport chain that accepts electrons from ubiquinone and contributes to an inner membrane potential (127, 128). The essential role of cytochrome function in mitochondrial electron transport is underscored by the ability of atovaquone to kill parasites by competitively binding to cytochrome b (63, 120). This mechanistic model is supported by observations that mutations in cytochrome b confer resistance to atovaquone (7, 76, 119).

In most aerobic organisms, function of the citric acid (TCA) cycle provides a dominant route of electron flux into the mitochondrial electron transport chain to maintain an inner membrane potential that drives ATP production via the ATP synthase complex. Despite access to abundant oxygen and host heme within erythrocytes, asexual *Plasmodium* parasites rely primarily on glycolysis

rather than mitochondrial oxidative phosphorylation to produce the ATP required to support their rapid blood-stage growth (43). Indeed, less than 7% of the carbon atoms derived from glucose imported by parasites appears to be oxidized within the mitochondrion (75). This curious reliance on glycolysis for energy production despite the presence of a functional mitochondrial electron transport chain appears to be due, in full or in part, to the absence of a mitochondrial pyruvate dehydrogenase (PDH) to convert the pyruvate produced by glycolysis into acetyl CoA for entry into the TCA cycle. Residual PDH activity, proposed to be provided by a branched-chain α-keto acid dehydrogenase, has been detected in the mitochondrion, but the modest level of this activity results in low overall flux of glucose-derived carbon through the TCA cycle in asexual blood-stage parasites (28, 75).

TCA cycle flux of glucose carbons, however, appears to be upregulated in sexual gametocytes, suggesting enhanced roles for energy production involving the electron transport chain and for other TCA cycle-dependent processes in this and possibly other stages. Consistent with these differential TCA cycle activities, treatment of parasites with an aconitase inhibitor had no detectable effect on asexual parasite growth but strongly attenuated gametocyte maturation (75). Similar effects were reported from targeted gene disruption of succinate dehydrogenase in *P. berghei*, which impaired the development of mosquito-stage but not blood-stage parasites (54). These results may suggest greater utilization of heme in the gametocyte and exoerythrocyte stages to support upregulated energy metabolism within the mitochondrion during these periods of the parasite life cycle. The evolutionary pressures that have led to differing energy metabolisms in discrete life cycle stages remain unknown, but these differences may be influenced by variations in glucose availability in distinct host environments.

Rather than accepting electrons from the TCA cycle, the critical role of electron transport by mitochondrial cytochromes in blood-stage parasites appears to be recycling the ubiquinone cofactor used by the mitochondrial dihydroorotate dehydrogenase (DHOD). This enzyme catalyzes a key step during pyrimidine biosynthesis, which the parasite maintains because it lacks an ability to scavenge pyrimidines (56). Parasites that express a cytosolic version of DHOD derived from yeast that does not utilize ubiquinone as an electron acceptor are resistant to atovaquone and develop normally, despite loss of mitochondrial electron transport (94). This result strongly suggests that the sole essential function of cytochrome-mediated electron transport in the mitochondrion of a blood-stage parasite is to support DHOD activity and pyrimidine biosynthesis, although different strains of *P. falciparum* expressing the yeast DHOD show different atovaquone resistance profiles (62).

Other than mitochondrial cytochromes, the only bioinformatically identifiable heme proteins in the parasite genome are several cytochrome b5 orthologs of unknown function (127). Multiple additional *Plasmodium* proteins present within parasites have been shown to bind heme in vitro (20, 57, 58, 86, 107, 113, 118), but it remains unclear whether these associations with heme represent cognate or promiscuous interactions. Many organisms express a heme oxygenase to enzymatically derivatize heme for downstream metabolic utilization in signaling or light-harvesting complexes or to scavenge iron (8, 131), but as noted above *P. falciparum* parasites lack detectable HO activity and appear to only utilize heme as a metabolic end product (113). Cytochrome function during mitochondrial electron transport is thus the only known essential role for heme in parasites. However, nearly half of the parasite genome encodes hypothetical proteins whose sequences are sufficiently divergent from known proteins such that biological roles cannot be predicted (6), and one or more of these ~2,500 proteins may function as a noncanonical heme protein. A full understanding of heme utilization by *Plasmodium* parasites during discrete developmental stages and in different host environments remains an unmet challenge, and identification of additional essential roles could provide novel therapeutic targets.

HEME BIOSYNTHESIS

Nearly all organisms have robust mechanisms to acquire heme from their environment or to synthesize it de novo to satisfy metabolic requirements. *Plasmodium* parasites require heme for growth and have access to abundant host-derived heme within the infected erythrocyte that could, in principle, be scavenged and utilized. Parasites, however, have historically been assumed to sequester this heme within the digestive vacuole without further utilization. Rather, parasites express a functionally complete suite of heme biosynthesis enzymes (44, 124), and this pathway has been thought to produce the heme used by the parasite in mitochondrial cytochromes (**Figure 2**). This scenario is similar to some hematophagous organisms (126) but appears to contrast with the blood-feeding tick *Boophilus microplus*, which sequesters host heme like *Plasmodium* spp. but lacks a heme biosynthetic pathway and relies instead on scavenging a fraction of dietary heme (17, 72).

Pathway Structure and Activity in Blood Stages

Animal and fungal cells typically sprinkle their heme biosynthetic enzymes between the mitochondria and cytosol (50). In plants, a complete pathway is found within the photosynthetic plastid, where it branches off for chlorophyll biosynthesis, but the two terminal enzymes are functionally duplicated in mitochondria to produce the heme required for cellular respiration (79, 100, 127). The heme biosynthetic pathway of *Plasmodium* (and other apicomplexan) parasites combines features of both plant and animal cells in that it targets enzymes to the mitochondrion, the plant-like but nonphotosynthetic apicoplast, and the cytosol (**Figure 4**) in a complex arrangement that reflects the convoluted evolution of the pathway and the endosymbiotic origin of the organelles that it spans (73, 127).

Early metabolic tracing studies established that initiation of the parasite pathway is animal-like and not plant-like in that it incorporates glycine rather than glutamate into the first committed pathway intermediate, δ-aminolevulinic acid (ALA) (124), utilizing a mitochondrion-localized

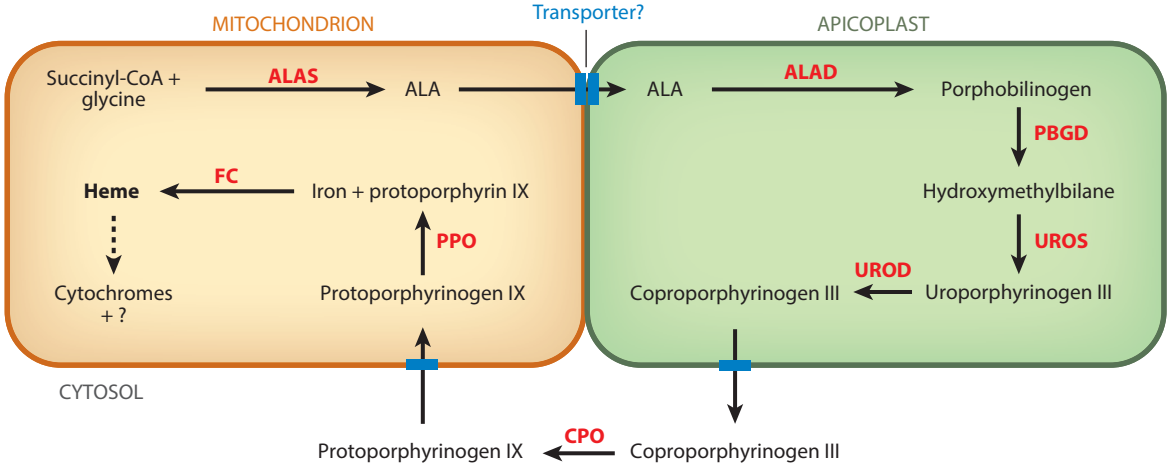

Figure 4

Schematic model of the heme biosynthesis pathway in *Plasmodium* parasites. For simplicity, organelles are depicted with a single membrane. Blue rectangles depict putative transporters of heme intermediates. Abbreviations: ALA, δ-aminolevulinic acid; ALAD, aminolevulinic acid dehydratase; ALAS, aminolevulinic acid synthase; CPO, coproporphyrinogen oxidase; FC, ferrochelatase; PBGD, porphobilinogen deaminase; PPO, protoporphyrinogen oxidase; UROD, uroporphyrinogen decarboxylase; UROS, uroporphyrinogen synthase.

ALA synthase that was identified and localized in subsequent studies (106, 129, 132). In this regard parasites are similar to other nonphotosynthetic eukaryotes (50). The parasite enzymes that catalyze the next four pathway steps (condensation of ALA into porphobilinogen and then hydroxymethylbilane, cyclization of the tetrapyrrole backbone to form uroporphyrinogen III, and decarboxylation to make coproporphyrinogen III) are all targeted to the apicoplast (**Figure 4**), based on studies of the ALA dehydratase, porphobilinogen deaminase, and uroporphyrinogen III decarboxylase enzymes encoded by the *P. falciparum* genome (80, 106). An obvious ortholog of uroporphyrinogen III synthase (UROS), which catalyzes cyclization of hydroxymethylbilane, has not been identified in the parasite genome, but this activity has been reported in vitro as a dual function of the porphobilinogen deaminase (81). An alternative origin of this activity is suggested by a recent bioinformatic analysis that identified a UROS ortholog in the parasite genome with low-level sequence similarity to annotated UROS proteins (68), although the function and localization of this protein remain untested.

Conversion of coproporphyrinogen III to protoporphyrinogen IX appears to occur in the parasite cytosol, based on localization studies of the coproporphyrinogen III oxidase (83). This cytosolic localization is observed in fungi but contrasts with the mitochondrial localization in animal cells (19, 127). The final two pathway steps involving oxidation to protoporphyrin IX and iron insertion to form heme are catalyzed by protoporphyrinogen IX oxidase and ferrochelatase, respectively. These enzymes have been localized to the mitochondrion, identifying this organelle as the terminus of the parasite's heme biosynthetic pathway (**Figure 4**) (82, 84).

The linear tetrapyrrole and macrocyclic porphyrin intermediates in heme biosynthesis do not readily diffuse across membranes (111). The complex distribution of the parasite enzymes across three subcellular compartments, including two organelles surrounded by multiple membranes, suggests the existence of specific transporters or binding proteins that may facilitate and coordinate the trafficking of intermediates between enzymes in discrete compartments. The mitochondrion and apicoplast appear to be in close contact during a portion of the parasite life cycle (55), and this proximity may facilitate metabolic flux between these two organelles. A few candidate transporters and carrier proteins of heme intermediates have been identified in other organisms (50, 70, 108), but proteins that may carry out analogous roles in *Plasmodium* parasites have not been identified (73).

All of the parasite enzymes appear to be expressed during the asexual blood stages, based on transcriptional (microarray and RNA-seq) and translational (mass spectrometry and Western blot) studies of individual components. The RNA-seq data further indicate that the expression of all pathway genes is synchronous and peaks 30–35 hours after invasion (6), as expected for a complete biosynthetic pathway. However, the minimal blood-stage activity of the TCA cycle (28, 75) and limited production of the early heme precursor, succinyl CoA (**Figure 4**), lead to some uncertainty about pathway activity. Nevertheless, the parasite pathway appears to be complete and functional. Isotopic labeling studies using ^{14}C-glycine or ^{14}C-ALA have reported incorporation of these labels into heme (85, 124). A recent study has also provided evidence that this ^{14}C-labeled heme is loaded into mitochondrial cytochromes (85), which has been assumed but not previously demonstrated. Additional evidence for heme biosynthetic activity in *P. falciparum* is provided by observations that parasites become photosensitive when grown in exogenous ALA (66, 117), as expected for stimulated biosynthetic flux and accumulation of phototoxic protoporphyrin IX under these culture conditions (21). Based on these studies, it is clear that parasites are capable of de novo heme synthesis, but how much heme biosynthesis occurs under normal growth conditions remains unresolved.

Earlier work suggested that the parasite, in addition to expressing its own heme biosynthesis enzymes, might import some or all of the host enzymes that remain in the red cell after maturation

of the heme-synthesizing reticulocyte precursor and that these imported enzymes might constitute a parallel route of heme production within parasites (15, 92). However, the mature red blood cells parasitized by *P. falciparum* lack mitochondria and thus are missing the ALA synthase and the three terminal pathway enzymes required to produce heme (85, 98, 103). Whether the four cytosolic host enzymes (corresponding to the four apicoplast-localized parasite enzymes, **Figure 4**) that remain in the mature red blood cells contribute to basal or ALA-stimulated heme biosynthesis in parasites remains unclear. Recently published chemical and genetic disruptions of *Plasmodium* enzymes (discussed below) have begun to address this question (85), and additional gene knockouts in progress in several labs can be expected to further clarify contributions by host versus parasite enzymes to observed heme biosynthetic activity.

Is De Novo Heme Synthesis Critical for Parasite Growth?

Identification of a complete parasite heme biosynthetic pathway despite the presence of abundant host heme during intraerythrocytic infection suggested early on that parasites might depend entirely on de novo synthesis to meet critical metabolic needs rather than utilize host heme liberated within the digestive vacuole. This view seemed to be supported by initial reports that succinylacetone, which is known to be a specific inhibitor of ALA dehydratase in other organisms (34), could inhibit de novo synthesis and kill asexual parasites, albeit at concentrations of several hundred micromolar to a few millimolar (101, 124). Recent observations, however, suggest that de novo heme synthesis is dispensable in blood-stage parasites. In the following three paragraphs, we review these recent studies and their implications.

First, activity of the penultimate pathway enzyme, protoporphyrinogen IX oxidase, was shown in vitro to require an electron acceptor molecule that is likely coupled in vivo to the mitochondrial electron transport chain, given that treatment with the cytochrome b inhibitor, atovaquone, attenuated de novo heme synthesis in parasites (82). Based on the model discussed above that cytochrome function is only required by blood-stage parasites to support DHOD activity (94), this apparent inhibition of heme synthesis by atovaquone suggested that either heme biosynthesis was dispensable entirely or heme was only required to support cytochrome function (82).

The following year, it was reported that exogenously supplied isopentenyl pyrophosphate (IPP), could prevent parasite death from antibiotics such as doxycycline that inhibit replication of the apicoplast and cause apicoplast loss (134). In the presence of IPP (which allows parasites to make isoprenoids), parasites could then be cultured indefinitely in the absence of an intact apicoplast organelle or the small apicoplast genome. Given that four of the heme biosynthetic steps occur within the apicoplast, organelle disruption might also be expected to perturb their function and therefore to ablate de novo heme synthesis entirely. The ability to rescue the growth of these apicoplast-disrupted parasites with just IPP would then suggest that de novo heme synthesis by intraerythrocytic parasites is dispensable. A caveat, however, is that doxycycline treatment does not ablate expression of nuclear-encoded genes (30), including those that encode the relevant heme biosynthetic enzymes as well as the vast majority of the apicoplast proteome (40, 136). In apicoplast-disrupted parasites, nuclear-encoded proteins appear to accumulate within intracellular vesicles that may reflect orphaned trafficking intermediates (45, 134). The composition of these putative vesicles and the functional status of proteins within them, including the apicoplast-targeted heme biosynthesis enzymes, remain uncertain. Indeed, apicoplast-disrupted parasites retain their capacity for de novo heme synthesis (P. Sigala and D. Goldberg, unpublished results), and future direct tests will be required to unravel and to fully understand which functions of apicoplast-targeted proteins have or have not been disrupted in doxycycline-treated, IPP-rescued parasites.

The most recent study has provided direct genetic and chemical evidence that *Plasmodium* parasites do not require a functional heme biosynthetic pathway for intraerythrocytic growth (85). In this work, the ALA synthase (ALAS) and ferrochelatase (FC) genes in *P. berghei* were disrupted via double-crossover homologous recombination, and asexual blood-stage growth was shown to be unaffected by these deletions, suggesting that parasite growth does not depend on pathway function. De novo heme synthesis was assessed with autoradiography to monitor ^{14}C-ALA incorporation into heme, but the authors were unable to show that these mutations ablated parasite heme biosynthesis because *P. berghei* preferentially invades metabolically active reticulocytes, which also synthesize heme. The authors therefore revisited succinylacetone inhibition of ALA dehydratase activity in *P. berghei* and in *P. falciparum* as an alternative strategy. Whereas prior reports had indicated that high micromolar concentrations of the inhibitor ablated heme biosynthesis and killed parasites (124), the new study indicates that parasites from both species remain viable and display no significant growth defect in 50 μM succinylacetone despite the absence of detectable heme synthesis. This chemical inhibition provides the most direct and compelling data that de novo heme synthesis is indeed dispensable for blood-stage parasite growth, clarifying that inhibition of pathway activity is unlikely to be a successful therapeutic strategy for intraerythrocytic malaria and that parasite death at higher drug concentrations presumably reflects off-target toxicity.

In the absence of heme biosynthesis, how do parasites obtain the heme required for cytochrome function and growth? Although the vast majority of hemoglobin-derived heme is sequestered as hemozoin in the food vacuole, parasites may metabolically scavenge a trace amount of the unassociated vacuolar heme that escapes mineralization and transits into the parasite cytoplasm via unknown mechanisms (**Figure 2**). The prior study presented data that suggest that scavenging of hemoglobin-derived heme may occur in parasites (85), but much more follow-up will be necessary to identify and dissect the trafficking pathways that enable intraerythrocytic parasites to obtain heme without making it.

If blood-stage parasites do not require de novo heme synthesis, why have parasites retained this biosynthetic pathway? The answer appears to be that heme biosynthesis becomes essential for parasite growth in the mosquito and liver stages, when parasites develop within host environments in which heme is less abundant and accessible. Indeed, *P. berghei* parasites with a disrupted ALAS or FC gene were unable to develop into sporozoites within mosquitos (85). Addition of exogenous ALA to the mosquito feeding solution enabled the ALAS knockout parasites to mature to sporozoites, but subsequent infection of naive mice failed to result in an asexual blood-stage infection.

The enhanced role for heme biosynthesis in exoerythrocytic parasites fits with observations that glucose flux through the TCA cycle, which supplies the succinyl-CoA used by ALAS to initiate heme synthesis, is upregulated in sexual gametocytes and is of greater importance during mosquito stages (54, 75). Pathway activity in asexual blood-stage parasites may be restricted under normal growth conditions by the low availability of succinyl-CoA, a limited need for heme, and the ability to scavenge host heme. Nevertheless, a complete heme biosynthetic pathway is maintained in these parasites. This metabolic scenario may prime them for rapid development into sexual gametocytes and for the environmental transition into the mosquito vector.

CONCLUSIONS AND OUTLOOK

Heme metabolism plays a central role in the life cycle of *Plasmodium* parasites across all developmental stages, especially during intraerythrocytic infection, where heme serves both as a toxic by-product of hemoglobin catabolism and as an essential metabolic cofactor. Recent studies have

demonstrated that heme metabolism in blood-stage parasites is highly manipulable. De novo heme synthesis can be ablated without developmental consequences, the heme utilized by parasites can be provided by discrete sources, and the distribution of labile heme within parasites can be altered by drug treatment. This metabolic and functional plasticity is exceptional and may provide unique opportunities in *Plasmodium* parasites for dissecting and understanding general principles in heme trafficking and utilization, ubiquitous cellular processes that remain poorly understood in most organisms. Although inhibition of heme biosynthesis appears ill suited as a therapeutic strategy to combat blood-stage malaria, the enhanced reliance on pathway function in exoerythrocytic stages may open doors to target other stages for antimalarial prophylaxis. Deeper understanding of the routes and mechanisms of heme trafficking in parasites may also reveal novel therapeutic targets and strategies. Finally, the regulatory mechanisms that govern heme metabolism and indeed much of the developmental progression of parasites remain poorly understood and are a frontier for future discovery.

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LITERATURE CITED

1. Abu Bakar N, Klonis N, Hanssen E, Chan C, Tilley L. 2010. Digestive-vacuole genesis and endocytic processes in the early intraerythrocytic stages of *Plasmodium falciparum*. *J. Cell Sci.* 123:441–50
2. Ambele MA, Egan TJ. 2012. Neutral lipids associated with haemozoin mediate efficient and rapid β-haematin formation at physiological pH, temperature and ionic composition. *Malar. J.* 11:337–49
3. Arese P, Schwarzer E. 1997. Malarial pigment (haemozoin): a very active 'inert' substance. *Ann. Trop. Med. Parasitol.* 91:501–16
4. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, et al. 2013. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature* 505:50–55
5. Atamna H, Ginsburg H. 1995. Heme degradation in the presence of glutathione: a proposed mechanism to account for the high levels of non-heme iron found in the membranes of hemoglobinopathic red blood cells. *J. Biol. Chem.* 270:24876–83
6. Aurrecoechea C, Brestelli J, Brunk BP, Dommer J, Fischer S, et al. 2009. PlasmoDB: A functional genomic database for malaria parasites. *Nucleic Acids Res.* 37:D539–43
7. Barton V, Fisher N, Biagini GA, Ward SA, O'Neill PM. 2010. Inhibiting *Plasmodium* cytochrome bc_1: a complex issue. *Curr. Opin. Chem. Biol.* 14:440–46
8. Beale SI. 1993. Biosynthesis of phycobilins. *Chem. Rev.* 93:785–802
9. Becker K, Tilley L, Vennerstrom JL, Roberts D, Rogerson S, Ginsburg H. 2004. Oxidative stress in malaria parasite-infected erythrocytes: host–parasite interactions. *Int. J. Parasitol.* 34:163–89
10. Bellemare MJ, Bohle DS, Brosseau CN, Georges E, Godbout M, et al. 2009. Autofluorescence of condensed heme aggregates in malaria pigment and its synthetic equivalent hematin anhydride (β-hematin). *J. Phys. Chem. B* 113:8391–401
11. Bendrat K, Berger BJ, Cerami A. 1995. Haem polymerization in malaria. *Nature* 378:138–39
12. Bohle DS, Debrunner P, Jordan PA, Madsen SK, Schulz CE. 1998. Aggregated heme detoxification byproducts in malarial trophozoites: β-hematin and malaria pigment have a single $s = 5/2$ iron environment in the bulk phase as determined by EPR and magnetic Mössbauer spectroscopy. *J. Am. Chem. Soc.* 120:8255–56

13. Bohle DS, Dinnebier RE, Madsen SK, Stephens PW. 1997. Characterization of the products of the heme detoxification pathway in malarial late trophozoites by X-ray diffraction. *J. Biol. Chem.* 272:713–16
14. Bohle DS, Dodd EL, Stephens PW. 2012. Structure of malaria pigment and related propanoate-linked metalloporphyrin dimers. *Chem. Biodivers.* 9:1891–902
15. Bonday ZQ, Dhanasekaran S, Rangarajan PN, Padmanaban G. 2000. Import of host δ-aminolevulinate dehydratase into the malarial parasite: identification of a new drug target. *Nat. Med.* 6:898–903
16. Bonifacio A, Finaurini S, Krafft C, Parapini S, Taramelli D, Sergo V. 2008. Spatial distribution of heme species in erythrocytes infected with *Plasmodium falciparum* by use of resonance Raman imaging and multivariate analysis. *Anal. Bioanal. Chem.* 392:1277–82
17. Braz GR, Coelho HS, Masuda H, Oliveira PL. 1999. A missing metabolic pathway in the cattle tick *Boophilus microplus*. *Curr. Biol.* 9:703–6
18. Butykai A, Orban A, Kocsis V, Szaller D, Bordacs S, et al. 2013. Malaria pigment crystals as magnetic micro-rotors: key for high-sensitivity diagnosis. *Sci. Rep.* 3:1431–41
19. Camadro JM, Chambon H, Jolles J, Labbe P. 1986. Purification and properties of coproporphyrinogen oxidase from the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 156:579–87
20. Campanale N, Nickel C, Daubenberger CA, Wehlan DA, Gorman JJ, et al. 2003. Identification and characterization of heme-interacting proteins in the malaria parasite, *Plasmodium falciparum*. *J. Biol. Chem.* 278:27354–61
21. Celli JP, Spring BQ, Rizvi I, Evans CL, Samkoe KS, et al. 2010. Imaging and photodynamic therapy: mechanisms, monitoring, and optimization. *Chem. Rev.* 110:2795–838
22. Chaurand P. 2012. Imaging mass spectrometry of thin tissue sections: A decade of collective efforts. *J. Proteomics* 75:4883–92
23. Cheeseman IH, Miller BA, Nair S, Nkhoma S, Tan A, et al. 2012. A major genome region underlying artemisinin resistance in malaria. *Science* 336:79–82
24. Chen MM, Shi LR, Sullivan DJ. 2001. *Haemoproteus* and *Schistosoma* synthesize heme polymers similar to *Plasmodium* hemozoin and β-hematin. *Mol. Biochem. Parasitol.* 113:1–8
25. Chugh M, Sundararaman V, Kumar S, Reddy VS, Siddiqui WA, et al. 2013. Protein complex directs hemoglobin-to-hemozoin formation in *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA* 110:5392–97
26. Coban C, Ishii KJ, Horii T, Akira S. 2007. Manipulation of host innate immune responses by the malaria parasite. *Trends Microbiol.* 15:271–78
27. Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, et al. 2005. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J. Exp. Med.* 201:19–25
28. Cobbold SA, Vaughan AM, Lewis IA, Painter HJ, Camargo N, et al. 2013. Kinetic flux profiling elucidates two independent acetyl-CoA biosynthetic pathways in *Plasmodium falciparum*. *J. Biol. Chem.* 288:36338–50
29. Combrinck JM, Mabotha TE, Ncokazi KK, Ambele MA, Taylor D, et al. 2013. Insights into the role of heme in the mechanism of action of antimalarials. *ACS Chem. Biol.* 8:133–37
30. Dahl EL, Shock JL, Shenai BR, Gut J, DeRisi JL, Rosenthal PJ. 2006. Tetracyclines specifically target the apicoplast of the malaria parasite *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* 50:3124–31
31. Dondorp AM, Fairhurst RM, Slutsker L, Macarthur JR, Breman JG, et al. 2011. The threat of artemisinin-resistant malaria. *N. Engl. J. Med.* 365:1073–75
32. Dorn A, Stoffel R, Matile H, Bubendorf A, Ridley RG. 1995. Malarial haemozoin/β-hematin supports haem polymerization in the absence of protein. *Nature* 374:269–71
33. Dubar F, Bohic S, Slomianny C, Morin JC, Thomas P, et al. 2012. In situ nanochemical imaging of label-free drugs: a case study of antimalarials in *Plasmodium falciparum*-infected erythrocytes. *Chem. Commun.* 48:910–12
34. Ebert PS, Hess RA, Frykholm BC, Tschudy DP. 1979. Succinylacetone, a potent inhibitor of heme biosynthesis: effect on cell growth, heme content and δ-aminolevulinic acid dehydratase activity of malignant murine erythroleukemia cells. *Biochem. Biophys. Res. Commun.* 88:1382–90
35. Egan TJ, Combrinck JM, Egan J, Hearne GR, Marques HM, et al. 2002. Fate of haem iron in the malaria parasite *Plasmodium falciparum*. *Biochem. J.* 365:343–47
36. Egan TJ, Ross DC, Adams PA. 1994. Quinoline antimalarial-drugs inhibit spontaneous formation of β-hematin (malaria pigment). *FEBS Lett.* 352:54–57

37. Ferreira A, Balla J, Jeney V, Balla G, Soares MP. 2008. A central role for free heme in the pathogenesis of severe malaria: the missing link? *J. Mol. Med.* 86:1097–111
38. Ferreira A, Marguti I, Bechmann I, Jeney V, Chora A, et al. 2011. Sickle hemoglobin confers tolerance to *Plasmodium* infection. *Cell* 145:398–409
39. Fitch CD, Chevli R, Banyal HS, Phillips G, Pfaller MA, Krogstad DJ. 1982. Lysis of *Plasmodium falciparum* by ferriprotoporphyrin IX and a chloroquine-ferriprotoporphyrin IX complex. *Antimicrob. Agents Chemother.* 21:819–22
40. Foth BJ, Ralph SA, Tonkin CJ, Struck NS, Fraunholz M, et al. 2003. Dissecting apicoplast targeting in the malaria parasite *Plasmodium falciparum*. *Science* 299:705–8
41. Francis SE, Sullivan DJ Jr, Goldberg DE. 1997. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. *Annu. Rev. Microbiol.* 51:97–123
42. Fraser ST, Midwinter RG, Berger BS, Stocker R. 2011. Heme oxygenase-1: a critical link between iron metabolism, erythropoiesis, and development. *Adv. Hematol.* 2011:473709
43. Fry M, Webb E, Pudney M. 1990. Effect of mitochondrial inhibitors on adenosinetriphosphate levels in *Plasmodium falciparum*. *Comp. Biochem. Physiol. B* 96:775–82
44. Gardner MJ, Hall N, Fung E, White O, Berriman M, et al. 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 419:498–511
45. Gisselberg JE, Dellibovi-Ragheb TA, Matthews KA, Bosch G, Prigge ST. 2013. The Suf iron-sulfur cluster synthesis pathway is required for apicoplast maintenance in malaria parasites. *PLoS Pathog.* 9:e1003655
46. Goldberg DE. 2005. Hemoglobin degradation. *Curr. Top. Microbiol.* 295:275–91
47. Goldberg DE, Slater AF, Cerami A, Henderson GB. 1990. Hemoglobin degradation in the malaria parasite *Plasmodium falciparum*: An ordered process in a unique organelle. *Proc. Natl. Acad. Sci. USA* 87:2931–35
48. Gorka AP, Alumasa JN, Sherlach KS, Jacobs LM, Nickley KB, et al. 2013. Cytostatic versus cytocidal activities of chloroquine analogues and inhibition of hemozoin crystal growth. *Antimicrob. Agents Chemother.* 57:356–64
49. Gorka AP, Sherlach KS, de Dios AC, Roepe PD. 2013. Relative to quinine and quinidine, their 9-epimers exhibit decreased cytostatic activity and altered heme binding but similar cytocidal activity versus *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* 57:365–74
50. Hamza I, Dailey HA. 2012. One ring to rule them all: trafficking of heme and heme synthesis intermediates in the metazoans. *Biochim. Biophys. Acta* 1823:1617–32
51. Hartwig CL, Rosenthal AS, D'Angelo J, Griffin CE, Posner GH, Cooper RA. 2009. Accumulation of artemisinin trioxane derivatives within neutral lipids of *Plasmodium falciparum* malaria parasites is endoperoxide-dependent. *Biochem. Pharmacol.* 77:322–36
52. Hebbel RP, Morgan WT, Eaton JW, Hedlund BE. 1988. Accelerated autoxidation and heme loss due to instability of sickle hemoglobin. *Proc. Natl. Acad. Sci. USA* 85:237–41
53. Hempelmann E, Motta C, Hughes R, Ward SA, Bray PG. 2003. *Plasmodium falciparum*: sacrificing membrane to grow crystals? *Trends Parasitol.* 19:23–26
54. Hino A, Hirai M, Tanaka TQ, Watanabe Y, Matsuoka H, Kita K. 2012. Critical roles of the mitochondrial complex II in oocyst formation of rodent malaria parasite *Plasmodium berghei*. *J. Biochem.* 152:259–68
55. Hopkins J, Fowler R, Krishna S, Wilson I, Mitchell G, Bannister L. 1999. The plastid in *Plasmodium falciparum* asexual blood stages: A three-dimensional ultrastructural analysis. *Protist* 150:283–95
56. Hyde JE. 2007. Targeting purine and pyrimidine metabolism in human apicomplexan parasites. *Curr. Drug Targets* 8:31–47
57. Imam M, Singh S, Kaushik NK, Chauhan VS. 2014. *Plasmodium falciparum* merozoite surface protein 3: oligomerization, self-assembly and heme complex formation. *J. Biol. Chem.* 289:3856–68
58. Jani D, Nagarkatti R, Beatty W, Angel R, Slebodnick C, et al. 2008. HDP—a novel heme detoxification protein from the malaria parasite. *PLoS Pathog.* 4:e1000053
59. Jeney V, Balla J, Yachie A, Varga Z, Vercellotti GM, et al. 2002. Pro-oxidant and cytotoxic effects of circulating heme. *Blood* 100:879–87
60. Kapishnikov S, Weiner A, Shimoni E, Guttmann P, Schneider G, et al. 2012. Oriented nucleation of hemozoin at the digestive vacuole membrane in *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA* 109:11188–93

61. Kappe SH, Vaughan AM, Boddey JA, Cowman AF. 2010. That was then but this is now: malaria research in the time of an eradication agenda. *Science* 328:862–66
62. Ke HJ, Morrisey JM, Ganesan SM, Painter HJ, Mather MW, Vaidya AB. 2011. Variation among *Plasmodium falciparum* strains in their reliance on mitochondrial electron transport chain function. *Eukaryot. Cell* 10:1053–61
63. Kessl JJ, Lange BB, Merbitz-Zahradnik T, Zwicker K, Hill P, et al. 2003. Molecular basis for atovaquone binding to the cytochrome bc_1 complex. *J. Biol. Chem.* 278:31312–18
64. Klonis N, Creek DJ, Tilley L. 2013. Iron and heme metabolism in *Plasmodium falciparum* and the mechanism of action of artemisinins. *Curr. Opin. Microbiol.* 16:722–27
65. Klonis N, Crespo-Ortiz MP, Bottova I, Abu-Bakar N, Kenny S, et al. 2011. Artemisinin activity against *Plasmodium falciparum* requires hemoglobin uptake and digestion. *Proc. Natl. Acad. Sci. USA* 108:11405–10
66. Komatsuya K, Hata M, Balogun EO, Hikosaka K, Suzuki S, et al. 2013. Synergy of ferrous ion on 5-aminolevulinic acid-mediated growth inhibition of *Plasmodium falciparum*. *J. Biochem.* 154:501–4
67. Koreny L, Obornik M, Lukes J. 2013. Make it, take it, or leave it: heme metabolism of parasites. *PLoS Pathog.* 9:e1003088
68. Koreny L, Sobotka R, Janouskovec J, Keeling PJ, Obornik M. 2011. Tetrapyrrole synthesis of photosynthetic chromerids is likely homologous to the unusual pathway of apicomplexan parasites. *Plant Cell* 23:3454–62
69. Koreny L, Sobotka R, Kovarova J, Gnipova A, Flegontov P, et al. 2012. Aerobic kinetoplastid flagellate *Phytomonas* does not require heme for viability. *Proc. Natl. Acad. Sci. USA* 109:3808–13
70. Krishnamurthy PC, Du G, Fukuda Y, Sun D, Sampath J, et al. 2006. Identification of a mammalian mitochondrial porphyrin transporter. *Nature* 443:586–89
71. Kumar S, Bandyopadhyay U. 2005. Free heme toxicity and its detoxification systems in human. *Toxicol. Lett.* 157:175–88
72. Lara FA, Lins U, Paiva-Silva G, Almeida IC, Braga CM, et al. 2003. A new intracellular pathway of haem detoxification in the midgut of the cattle tick *Boophilus microplus*: aggregation inside a specialized organelle, the hemosome. *J. Exp. Biol.* 206:1707–15
73. Lim L, McFadden GI. 2010. The evolution, metabolism and functions of the apicoplast. *Philos. Trans. R. Soc. Lond. B* 365:749–63
74. Loria P, Miller S, Foley M, Tilley L. 1999. Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other quinoline antimalarials. *Biochem. J.* 339:363–70
75. MacRae JI, Dixon MWA, Dearnley MK, Chua HH, Chambers JM, et al. 2013. Mitochondrial metabolism of sexual and asexual blood stages of the malaria parasite *Plasmodium falciparum*. *BMC Biol.* 11:67–77
76. Mather MW, Darrouzet E, Valkova-Valchanova M, Cooley JW, McIntosh MT, et al. 2005. Uncovering the molecular mode of action of the antimalarial drug atovaquone using a bacterial system. *J. Biol. Chem.* 280:27458–65
77. Meshnick SR. 2002. Artemisinin: mechanisms of action, resistance and toxicity. *Int. J. Parasitol.* 32:1655–60
78. Miller LH, Ackerman HC, Su XZ, Wellems TE. 2013. Malaria biology and disease pathogenesis: insights for new treatments. *Nat. Med.* 19:156–67
79. Mochizuki N, Tanaka R, Grimm B, Masuda T, Moulin M, et al. 2010. The cell biology of tetrapyrroles: a life and death struggle. *Trends Plant Sci.* 15:488–98
80. Nagaraj VA, Arumugam R, Chandra NR, Prasad D, Rangarajan PN, Padmanaban G. 2009. Localisation of *Plasmodium falciparum* uroporphyrinogen III decarboxylase of the heme-biosynthetic pathway in the apicoplast and characterisation of its catalytic properties. *Int. J. Parasitol.* 39:559–68
81. Nagaraj VA, Arumugam R, Gopalakrishnan B, Jyothsna YS, Rangarajan PN, Padmanaban G. 2008. Unique properties of *Plasmodium falciparum* porphobilinogen deaminase. *J. Biol. Chem.* 283:437–44
82. Nagaraj VA, Arumugam R, Prasad D, Rangarajan PN, Padmanaban G. 2010. Protoporphyrinogen IX oxidase from *Plasmodium falciparum* is anaerobic and is localized to the mitochondrion. *Mol. Biochem. Parasitol.* 174:44–52

83. Nagaraj VA, Prasad D, Arumugam R, Rangarajan PN, Padmanaban G. 2010. Characterization of coproporphyrinogen III oxidase in *Plasmodium falciparum* cytosol. *Parasitol. Int.* 59:121–27
84. Nagaraj VA, Prasad D, Rangarajan PN, Padmanaban G. 2009. Mitochondrial localization of functional ferrochelatase from *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 168:109–12
85. Nagaraj VA, Sundaram B, Varadarajan NM, Subramani PA, Kalappa DM, et al. 2013. Malaria parasite-synthesized heme is essential in the mosquito and liver stages and complements host heme in the blood stages of infection. *PLoS Pathog.* 9:e1003522
86. Nakatani K, Ishikawa H, Aono S, Mizutani Y. 2013. Heme-binding properties of heme detoxification protein from *Plasmodium falciparum*. *Biochem. Biophys. Res. Commun.* 439:477–80
87. Okada K. 2009. The novel heme oxygenase-like protein from *Plasmodium falciparum* converts heme to bilirubin IXα in the apicoplast. *FEBS Lett.* 583:313–19
88. Okumoto S, Jones A, Frommer WB. 2012. Quantitative imaging with fluorescent biosensors. *Annu. Rev. Plant Biol.* 63:663–706
89. Oliveira MF, Kycia SW, Gomez A, Kosar AJ, Bohle DS, et al. 2005. Structural and morphological characterization of hemozoin produced by *Schistosoma mansoni* and *Rhodnius prolixus*. *FEBS Lett.* 579:6010–16
90. O'Neill PM, Posner GH. 2004. A medicinal chemistry perspective on artemisinin and related endoperoxides. *J. Med. Chem.* 47:2945–64
91. Orjih AU, Banyal HS, Chevli R, Fitch CD. 1981. Hemin lyses malaria parasites. *Science* 214:667–69
92. Padmanaban G, Nagaraj VA, Rangarajan PN. 2007. An alternative model for heme biosynthesis in the malarial parasite. *Trends Biochem. Sci.* 32:443–9
93. Pagola S, Stephens PW, Bohle DS, Kosar AD, Madsen SK. 2000. The structure of malaria pigment β-haematin. *Nature* 404:307–10
94. Painter HJ, Morrisey JM, Mather MW, Vaidya AB. 2007. Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*. *Nature* 446:88–91
95. Pamplona A, Ferreira A, Balla J, Jeney V, Balla G, et al. 2007. Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nat. Med.* 13:703–10
96. Papalexis V, Siomos MA, Campanale N, Guo XG, Kocak G, et al. 2001. Histidine-rich protein 2 of the malaria parasite, *Plasmodium falciparum*, is involved in detoxification of the by-products of haemoglobin degradation. *Mol. Biochem. Parasitol.* 115:77–86
97. Pascoa V, Oliveira PL, Dansa-Petretski M, Silva JR, Alvarenga PH, et al. 2002. *Aedes aegypti* peritrophic matrix and its interaction with heme during blood digestion. *Insect Biochem. Mol. Biol.* 32:517–23
98. Pasini EM, Kirkegaard M, Mortensen P, Lutz HU, Thomas AW, Mann M. 2006. In-depth analysis of the membrane and cytosolic proteome of red blood cells. *Blood* 108:791–801
99. Ponka P. 1999. Cell biology of heme. *Am. J. Med. Sci.* 318:241–56
100. Ralph SA, van Dooren GG, Waller RF, Crawford MJ, Fraunholz MJ, et al. 2004. Metabolic maps and functions of the *Plasmodium falciparum* apicoplast. *Nat. Rev. Microbiol.* 2:203–16
101. Ramya TN, Mishra S, Karmodiya K, Surolia N, Surolia A. 2007. Inhibitors of nonhousekeeping functions of the apicoplast defy delayed death in *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* 51:307–16
102. Rosenthal PJ, Sijwali PS, Singh A, Shenai BR. 2002. Cysteine proteases of malaria parasites: targets for chemotherapy. *Curr. Pharm. Des.* 8:1659–72
103. Roux-Dalvai F, Gonzalez de Peredo A, Simo C, Guerrier L, Bouyssie D, et al. 2008. Extensive analysis of the cytoplasmic proteome of human erythrocytes using the peptide ligand library technology and advanced mass spectrometry. *Mol. Cell. Proteomics* 7:2254–69
104. Sachanonta N, Chotivanich K, Chaisri U, Turner GD, Ferguson DJ, et al. 2011. Ultrastructural and real-time microscopic changes in *P. falciparum*-infected red blood cells following treatment with antimalarial drugs. *Ultrastruct. Pathol.* 35:214–25
105. Sartorello R, Budu A, Bagnaresi P, Fernandes CA, Sato PM, et al. 2010. In vivo uptake of a haem analogue Zn protoporphyrin IX by the human malaria parasite *P. falciparum*-infected red blood cells. *Cell Biol. Int.* 34:859–65
106. Sato S, Clough B, Coates L, Wilson RJM. 2004. Enzymes for heme biosynthesis are found in both the mitochondrion and plastid of the malaria parasite *Plasmodium falciparum*. *Protist* 155:117–25
107. Schneider EL, Marletta MA. 2005. Heme binding to the histidine-rich protein II from *Plasmodium falciparum*. *Biochemistry* 44:979–86

108. Schultz IJ, Chen C, Paw BH, Hamza I. 2010. Iron and porphyrin trafficking in heme biogenesis. *J. Biol. Chem.* 285:26753–59
109. Schwarzer E, Alessio M, Ulliers D, Arese P. 1998. Phagocytosis of the malarial pigment, hemozoin, impairs expression of major histocompatibility complex class II antigen, CD54, and CD11c in human monocytes. *Infect. Immun.* 66:1601–6
110. Seixas E, Gozzelino R, Chora A, Ferreira A, Silva G, et al. 2009. Heme oxygenase-1 affords protection against noncerebral forms of severe malaria. *Proc. Natl. Acad. Sci. USA* 106:15837–42
111. Severance S, Hamza I. 2009. Trafficking of heme and porphyrins in metazoa. *Chem. Rev.* 109:4596–616
112. Shio MT, Eisenbarth SC, Savaria M, Vinet AF, Bellemare MJ, et al. 2009. Malarial hemozoin activates the NLRP3 inflammasome through Lyn and Syk kinases. *PLoS Pathog.* 5:e1000559
113. Sigala PA, Crowley JR, Hsieh S, Henderson JP, Goldberg DE. 2012. Direct tests of enzymatic heme degradation by the malaria parasite *Plasmodium falciparum*. *J. Biol. Chem.* 287:37793–807
114. Slater AF, Swiggard WJ, Orton BR, Flitter WD, Goldberg DE, et al. 1991. An iron-carboxylate bond links the heme units of malaria pigment. *Proc. Natl. Acad. Sci. USA* 88:325–29
115. Slater AFG, Cerami A. 1992. Inhibition by chloroquine of a novel heme polymerase enzyme activity in malaria trophozoites. *Nature* 355:167–69
116. Slater AFG, Swiggard WJ, Orton BR, Flitter WD, Goldberg DE, et al. 1991. An iron carboxylate bond links the heme units of malaria pigment. *Proc. Natl. Acad. Sci. USA* 88:325–29
117. Smith TG, Kain KC. 2004. Inactivation of *Plasmodium falciparum* by photodynamic excitation of heme-cycle intermediates derived from δ-aminolevulinic acid. *J. Infect. Dis.* 190:184–91
118. Spycher C, Klonis N, Spielmann T, Kump E, Steiger S, et al. 2003. MAHRP-1, a novel *Plasmodium falciparum* histidine-rich protein, binds ferriprotoporphyrin IX and localizes to the Maurer's clefts. *J. Biol. Chem.* 278:35373–83
119. Srivastava IK, Morrisey JM, Darrouzet E, Daldal F, Vaidya AB. 1999. Resistance mutations reveal the atovaquone-binding domain of cytochrome *b* in malaria parasites. *Mol. Microbiol.* 33:704–11
120. Srivastava IK, Rottenberg H, Vaidya AB. 1997. Atovaquone, a broad spectrum antiparasitic drug, collapses mitochondrial membrane potential in a malarial parasite. *J. Biol. Chem.* 272:3961–66
121. Sullivan DJ. 2002. Theories on malarial pigment formation and quinoline action. *Int. J. Parasitol.* 32:1645–53
122. Sullivan DJ Jr, Gluzman IY, Goldberg DE. 1996. *Plasmodium* hemozoin formation mediated by histidine-rich proteins. *Science* 271:219–22
123. Sullivan DJ Jr, Matile H, Ridley RG, Goldberg DE. 1998. A common mechanism for blockade of heme polymerization by antimalarial quinolines. *J. Biol. Chem.* 273:31103–7
124. Surolia N, Padmanaban G. 1992. De novo biosynthesis of heme offers a new chemotherapeutic target in the human malarial parasite. *Biochem. Biophys. Res. Commun.* 187:744–50
125. Takala-Harrison S, Clark TG, Jacob CG, Cummings MP, Miotto O, et al. 2013. Genetic loci associated with delayed clearance of *Plasmodium falciparum* following artemisinin treatment in Southeast Asia. *Proc. Natl. Acad. Sci. USA* 110:240–45
126. Toh SQ, Glanfield A, Gobert GN, Jones MK. 2010. Heme and blood-feeding parasites: friends or foes? *Parasit. Vectors* 3:108–18
127. van Dooren GG, Kennedy AT, McFadden GI. 2012. The use and abuse of heme in apicomplexan parasites. *Antioxid. Redox Signal.* 17:634–56
128. van Dooren GG, Stimmler LM, McFadden GI. 2006. Metabolic maps and functions of the *Plasmodium* mitochondrion. *FEMS Microbiol. Rev.* 30:596–630
129. Varadharajan S, Dhanasekaran S, Bonday ZQ, Rangarajan PN, Padmanaban G. 2002. Involvement of δ-aminolaevulinate synthase encoded by the parasite gene in de novo haem synthesis by *Plasmodium falciparum*. *Biochem. J.* 367:321–27
130. Wellems TE, Walkerjonah A, Panton LJ. 1991. Genetic mapping of the chloroquine-resistance locus on *Plasmodium-falciparum* chromosome 7. *Proc. Natl. Acad. Sci. USA* 88:3382–86
131. Wilks A, Burkhard KA. 2007. Heme and virulence: how bacterial pathogens regulate, transport and utilize heme. *Nat. Prod. Rep.* 24:511–22
132. Wilson CM, Smith AB, Baylon RV. 1996. Characterization of the δ-aminolevulinate synthase gene homologue in *P. falciparum*. *Mol. Biochem. Parasitol.* 75:271–76. Erratum. 1996. 79:135–40

133. Wongtanachai J, Silamut K, Day NPJ, Dondorp A, Chaisri U. 2012. Effects of antimalarial drugs on movement of *Plasmodium falciparum*. *Southeast Asian J. Trop. Med. Public Health* 43:1–9
134. Yeh E, DeRisi JL. 2011. Chemical rescue of malaria parasites lacking an apicoplast defines organelle function in blood-stage *Plasmodium falciparum*. *PLoS Biol.* 9:e1001138
135. Zhang J, Krugliak M, Ginsburg H. 1999. The fate of ferriprotorphyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. *Mol. Biochem. Parasitol.* 99:129–41
136. Zuegge J, Ralph S, Schmuker M, McFadden GI, Schneider G. 2001. Deciphering apicoplast targeting signals—feature extraction from nuclear-encoded precursors of *Plasmodium falciparum* apicoplast proteins. *Gene* 280:19–26