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FORUM REVIEW ARTICLE

The Use and Abuse of Heme in Apicomplexan Parasites

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Abstract

Significance: Heme is an essential prosthetic group for most life on Earth. It functions in numerous cellular redox reactions, including in antioxidant defenses and at several stages of the electron transport chain in prokaryotes and eukaryotic mitochondria. Heme also functions as a sensor and transport molecule for gases such as oxygen. Heme is a complex organic molecule and can only be synthesized through a multienzyme pathway from simpler precursors. Most free-living organisms synthesize their own heme by a broadly conserved metabolic pathway. Parasites are adept at scavenging molecules from their hosts, and heme is no exception. ***Recent Advances:*** In this review we examine recent advances in understanding heme usage and acquisition in Apicomplexa, a group of parasites that include the causative agents of malaria, toxoplasmosis, and several major parasites of livestock. ***Critical Issues:*** Heme is critical to the survival of Apicomplexa, although the functions of heme in these organisms remain poorly understood. Some Apicomplexa likely scavenge heme from their host organisms, while others retain the ability to synthesize heme. Surprisingly, some Apicomplexa may be able to both synthesize and scavenge heme. Several Apicomplexa live in intracellular environments that contain high levels of heme. Since heme is toxic at high concentrations, parasites must carefully regulate intracellular heme levels and develop mechanisms to detoxify excess heme. Indeed, drugs interfering with heme detoxification serve as major antimalarials. ***Future Directions:*** Understanding heme requirements and regulation in apicomplexan parasites promises to reveal multiple targets for much-needed therapeutic intervention against these parasites. *Antioxid. Redox Signal*. 00, 000–000.

Introduction: Tetrapyrroles in Life, Legends, and Parasites

Tetrapyrroles are critical for energy production and consumption in most of Earth's biosphere. They are responsible for the red color of our blood, the greens in our salad, and maybe even the werewolves and vampires of folklore. Their ubiquity among all three domains of life (Bacteria, Archaea, and Eukarya) suggests that tetrapyrroles assumed a central function early in the evolution of life. Nevertheless, there are some critical variations in how different organisms synthesize their tetrapyrroles (9, 143). The acquisition by eukaryotes of the tetrapyrroles heme and chlorophyll was major drivers in two of the most important events in cellular evolution—the endosymbiotic origin of mitochondria and plastids (chloroplasts) from bacterial antecedents. In this review, we will examine heme, a tetrapyrrole responsible for numerous cellular functions, including oxygen sensing and electron transport. We will focus, in particular, on the acquisition, functions, and breakdown of heme in a group of single-celled, obligate intracellular parasites called the Apicomplexa. As we shall see, heme is required for the survival of these organisms, and interfering with heme acquisition, functions, and breakdown provides promising therapeutic targets against some of the world's major parasitic diseases.

Tetrapyrroles comprise four pyrrole molecules that typically fuse into a cyclic, planar structure (Fig. 1). Tetrapyrroles frequently contain a metal ion at their core that is central to their role in biological redox reactions. Perhaps the best-known, and biologically important, tetrapyrroles are chlorophyll and heme. Chlorophyll consists of a tetrapyrrole ring with a magnesium ion at its center (Fig. 1B); it is the major light-harvesting pigment of photosynthetic organisms, such as cyanobacteria, plants, and algae. Chlorophylls first evolved in Bacteria, where they are found in cyanobacteria, purple bacteria, and several other groups (51). Chlorophylls were transferred to eukaryotes with the acquisition of plastid organelles from cyanobacteria through the process of endosymbiosis. This enabled eukaryotes to become photoautotrophs, a major event in eukaryotic evolution that led to the evolution of plants and numerous lineages of algae. In cyanobacteria and plastids, chlorophyll is a major component of photosystem complexes. Chlorophyll absorbs light of blue

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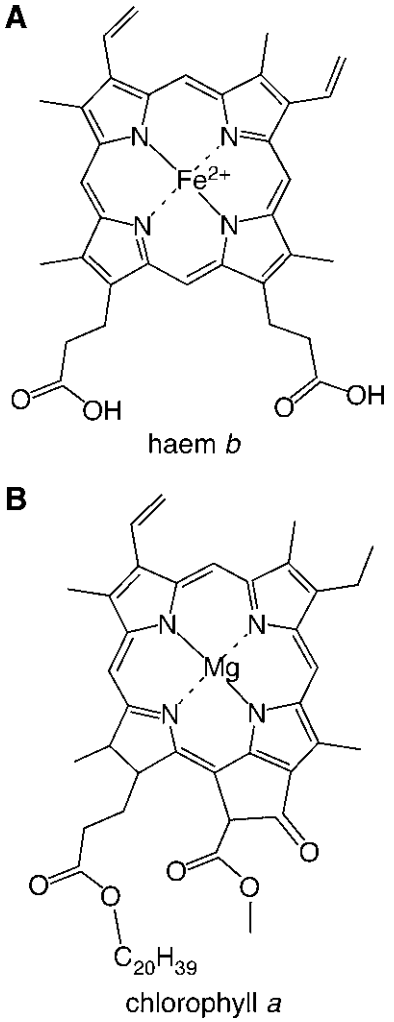

FIG. 1. The structure of tetrapyrroles. Tetrapyrroles consist of four pyrrole molecules arranged in a planar ring. **(A)** The structure of heme *b*, also known as protoheme, the simplest form of heme. Note the iron at the center of the ring that participates in redox reactions. **(B)** The structure of chlorophyll *a*. Note the magnesium ion at the center of the ring.

and red wavelengths (which gives the pigment its characteristic green color) and transfers this energy through to a central pair of chlorophyll molecules at the reaction center of the photosystem. The flow of energy into this reaction center results in the excitation of the chlorophyll pair and the release of an electron. The subsequent journey of this electron through an electron transport chain (ETC) results in the generation of adenosine triphosphate (ATP) and nicotinamine adenine dinucleotide phosphate (NADPH), which serve as energy currency to generate sugars in the so-called "dark" reactions of photosynthesis. Most of Earth's organic material is initially derived from photosynthesis. Photosynthesis also results in the generation of oxygen, which is formed through the oxidation of water to replenish the electrons lost at the reaction center chlorophylls. Indeed, virtually all of the oxygen found in Earth's atmosphere is a by-product of photosynthesis.

Heme is also adept at transferring electrons, but in this case they are derived from the iron at the center of the tetrapyrrole molecule (Fig. 1A). In certain proteinaceous environments, the iron in heme can also bind gases such as oxygen. Perhaps the best-known biological role of heme is in the vertebrate protein hemoglobin. Hemoglobin occurs in erythrocytes (red blood cells) and contains heme prosthetic groups that bind oxygen and transport it around the body. Heme belongs to a subset of tetrapyrrole molecules known as porphyrins. Porphyrins are distinguished by their purple color (porphyrin is derived from the Greek word for purple), and it is the heme in hemoglobin that gives blood its characteristic red color.

Given the structural similarities between chlorophyll and heme (Fig. 1), it is perhaps not surprising that these and other tetrapyrroles (such as vitamin B_{12}, a cobalt-containing prosthetic group found in numerous enzymes) are synthesized by a broadly similar metabolic pathway. Tetrapyrrole biosynthesis is critical for the survival of most organisms that utilize tetrapyrroles. In plants, compounds that inhibit tetrapyrrole synthesis (*e.g.*, acifluorfen) are potent herbicides. In humans, partial defects in heme synthesis lead to a set of medical conditions known as the porphyrias (7). Porphyrias result in the accumulation of various heme synthesis intermediates. Several of these intermediates produce free radicals upon exposure to light that result in (sometimes extreme) sensitivity to sunlight, skin blistering, and increased hair formation. Indeed, some hold that folkloric stories of werewolves and vampires stem from sufferers of porphyrias [*e.g.*, (55)]. The phototoxicity of porphyrins can also be utilized for medical benefit in a process known as photodynamic therapy (166). Patients can be treated with modified porphyrins, or with early intermediates of heme biosynthesis that result in accumulation of protoporphyrin IX, the immediate precursor of heme. The targeted exposure of a light source to desired tissue (*e.g.*, cancerous tumors) results in the production of free radicals that cause localized tissue death in a minimally invasive way.

Heme is essential to almost all living organisms and its synthesis is a core metabolic pathway. Parasites, by their very nature, are adept at acquiring nutrients, potentially including heme, from their host organisms. Nevertheless, heme is a toxic molecule, causing peroxidation of membrane lipids, and damage to DNA and proteins, all of which can lead to cell death (74). Host organisms must, therefore, limit their pool of free heme. This means that heme may not be readily available to parasites, suggesting that some may synthesize their own heme. Indeed, numerous apicomplexan parasites such as the causative agents of malaria and toxoplasmosis contain all the genes necessary for heme synthesis (41, 129, 160). We will examine the acquisition of heme in this group of intracellular parasites later in this review. We will first, however, examine the uses and synthesis of heme in nonparasitic organisms.

What Are the Functions of Heme?

Heme serves as a prosthetic group on a broad range of proteins, and is also a central component of ETCs. Perhaps the best-known role for heme is as a prosthetic group on globin proteins. Globins are broadly distributed in all three domains of life (39, 80). Globin proteins contain a distinctive "globin" fold consisting of numerous alpha helices, and a heme moiety that is co-ordinated through interaction with an invariant histidine residue. In this arrangement, the iron molecule in heme is able to reversibly bind to oxygen, nitric oxide, and other gases. Globins probably first evolved as oxygen-sensing

proteins in prokaryotes, enabling them to respond to local levels of oxygen (38, 167). Other globin functions are seen in vertebrate hemoglobins, which bind oxygen and transport it around the animal in the bloodstream. Leghemoglobins of legume plants sequester oxygen in root nodules, to prevent it from inhibiting the nitrogenase enzyme of symbiotic bacteria that fixes atmospheric nitrogen for the plant.

Heme also acts as a prosthetic group on catalases and some classes of peroxidases, both of which serve as critical antioxidant defenses in many organisms (179, 180). Hydrogen peroxide is a common reactive oxygen species generated by aerobic organisms as a by-product of processes such as electron transport in respiration and photosynthesis. Typically, these electron transport processes generate superoxide anions that are either spontaneously or catalytically converted to hydrogen peroxide. Hydrogen peroxide damages nucleic acids and proteins, and this oxidative stress can lead to cellular death. It is therefore critical that organisms have mechanisms to limit the abundance of hydrogen peroxide. Peroxidase and catalase enzymes catalyze the cleavage of hydrogen peroxide to form biologically inert molecules. In both catalases and heme-containing peroxidases, electrons from the central iron of heme reduce hydrogen peroxide to form water (48). Catalases also have the unique ability to oxidize hydrogen peroxide to form oxygen gas, with electrons donated to the heme iron. There are several classes of heme-containing peroxidases (48, 122, 180). These include cytochrome *c* peroxidases, which use cytochrome *c*—a component of ETCs—as a donor of electrons to reduce hydrogen peroxide. Ascorbate peroxidases use ascorbate as the electron donor in hydrogen peroxide reduction. Catalase-peroxidases, like catalases, catalyze both the oxidation and reduction of hydrogen peroxide to form water and oxygen gas, although there is no sequence similarity between the two.

In addition to their role in oxygen sensing, oxygen carrying, and antioxidant defenses, heme-containing proteins have several other key roles in eukaryotic cells. Cytochrome b_5 proteins are a family of heme *b*–containing proteins that function in donating electrons to a variety of biochemical reactions, for example, in desaturation of steroids and fatty acids (131). Similar roles are ascribed to the diverse cytochrome P450 family of enzymes, which are distributed in a broad range of prokaryotic and eukaryotic organisms (158).

As already alluded to, heme is a critical component at several stages of the ETCs central to respiration and photosynthesis. Since this review covers nonphotosynthetic organisms, we will not elaborate on the role of heme in photosynthesis, but it is worth noting that this process has many similarities to the ETC involved in respiration (51). Instead, we will focus on the ETC that occurs during respiration, a process that occurs at the inner membrane of the mitochondria of many eukaryotes, as well as on the inner or cytoplasmic membrane of many prokaryotes. Mitochondria were originally derived through the endosymbiotic acquisition of an α-proteobacterium, an event that occurred at the dawn of eukaryotic evolution. Eukaryotes therefore derived their mitochondrial ETC, and possibly also heme, from this symbiotic event.

The mitochondrial inner membrane contains numerous dehydrogenases that constitute the entry points of electrons into the ETC. Classically, these dehydrogenases include nicotinamine adenine dinucleotide (NADH) dehydrogenase (the so-called complex I of the ETC) and succinate dehydrogenase (complex II), in addition to various other dehydrogenases that we will cover later in this review. All of these dehydrogenases pass electrons to oxidized coenzyme Q (also called ubiquinone) to form reduced coenzyme Q (called ubiquinol). For example, succinate dehydrogenase is a multiprotein complex that catalyzes the oxidation of succinate to form fumarate. The electrons derived from succinate are transferred through several co-factors, including flavin adenine dinucleotide (FAD) and iron-sulfur clusters, to coenzyme Q [(127); Fig. 2]. Succinate dehydrogenase contains a heme prosthetic group, although the role of this heme in electron transport is unclear (127). The NADH dehydrogenase complex (also called NADH:ubiquinone reductase) accepts electrons from NADH and transfers them to coenzyme Q. During this process, NADH dehydrogenase translocates protons from the mitochondrial matrix to the intermembrane space. Along with two other proton-translocating components of the ETC (discussed later in this section), NADH dehydrogenase contributes to the generation of a proton gradient and membrane potential ($\Delta\Psi$) across the inner mitochondrial membrane. This proton gradient has numerous uses in the mitochondrion: (i) it can be harnessed by an F-type ATP synthase to generate ATP, (ii) it is critical for importing proteins into the organelle, and (iii) it has a crucial role in solute transport across the inner membrane (17, 115, 127).

Coenzyme Q is a hydrophobic molecule embedded in the inner mitochondrial membrane. Coenzyme Q shuttles electrons from the various inner membrane dehydrogenases to another complex in the inner membrane known as cytochrome *c* reductase (also called cytochrome bc_1 complex or complex III; Fig. 2). Reduced coenzyme Q carries two electrons. These electrons are donated to two separate electron acceptors within cytochrome *c* reductase (24). The first is donated to the iron-sulfur cluster of the so-called Rieske protein of the cytochrome *c* reductase complex. This electron is further donated to the heme group of cytochrome c_1, another protein in the cytochrome *c* reductase complex, which then passes it on to cytochrome *c*, a mobile protein peripherally associated with the complex. The second electron in coenzyme Q transfers through two heme groups of the cytochrome *b* protein of the complex, and is ultimately donated to oxidized coenzyme Q that docks at a second site within the complex (24). Two such transfers form fully reduced coenzyme Q, which can then again donate electrons at the first coenzyme Q docking site in the complex. The net reaction of cytochrome *c* reductase, therefore, results in one of the two electrons from reduced coenzyme Q being transferred to cytochrome *c*. A consequence of electron transfer in cytochrome *c* reductase is that protons are removed from the matrix side of the complex and added to the intermembrane space side of the complex (Fig. 2), resulting in a net movement of protons across the membrane that contributes to the proton gradient.

Cytochrome *c* reductase is the first point in the ETC where heme is essential for electron transport. The cytochrome *b* subunit of the complex contains two noncovalently bound heme molecules in the form of heme *b*, which represents the simplest form of heme (Fig. 1A). These heme *b* moieties are coordinated between two histidine residues each, which bind to the iron at the center of heme from above and below the planar molecule. Cytochrome c_1 contains a single heme molecule that, unlike in cytochrome *b*, is covalently bound to the

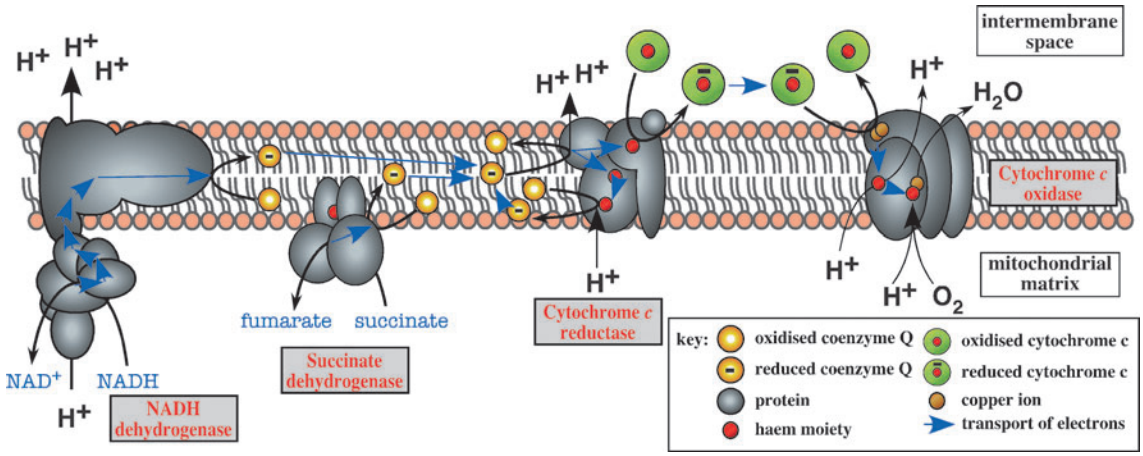

FIG. 2. The mitochondrial ETC that occurs in the inner mitochondrion membrane of most eukaryotes. The inner membrane contains several dehydrogenases, including NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II), which feed electrons from various sources to coenzyme Q (*yellow*), a molecule embedded within the membrane. Reduced coenzyme Q donates electrons to cytochrome *c* reductase (complex III). These electrons pass through several metal-based reaction centers, including heme (*red*), and ultimately reduce the heme prosthetic group of cytochrome *c* (*green*). Cytochrome *c* is a motile intermembrane space protein that donates electrons to cytochrome *c* oxidase (complex IV). Cytochrome *c* oxidase contains several metal centers, including two copper ions (*orange*) and two heme moieties, which ultimately donate electrons to oxygen, the terminal electron acceptor in the chain. The ETC generates a proton gradient and membrane potential across the inner membrane, which has several critical functions in the mitochondrion, including in the generation of ATP *via* an F-type ATP synthase. ATP, adenosine triphosphate; ETC, electron transport chain; NADH, nicotinamine adenine dinucleotide.

protein through two thioether bonds at conserved cysteine residues in a cysteine-X-X-cysteine-histidine amino acid motif (C-X-X-C-H motif) in the cytochrome protein (Fig. 5). In both cytochrome *b* and cytochrome c_1, the transfer of electrons to heme involves the reduction of the iron molecule at the center of heme from the ferric (Fe^{3+}) state to the ferrous (Fe^{2+}) state.

Cytochrome *c* localizes to the mitochondrial intermembrane space. In its oxidized form, cytochrome *c* docks at the cytochrome *c* reductase complex where it accepts electrons from cytochrome c_1 (54). Like cytochrome c_1, cytochrome *c* has a single covalently bound heme prosthetic group at a conserved C-X-X-C-H motif. Reduced cytochrome *c* migrates through the intermembrane space and docks with the cytochrome *c* oxidase complex (also called complex IV; Fig. 2). Cytochrome *c* donates electrons to a dinuclear copper site (called Cu_A) in cytochrome *c* oxidase. These electrons are transferred to a heme moiety and then onto a second heme moiety that sits in a complex with a second copper ion (called Cu_B) (35, 154, 177). The final electron transfer of the mitochondrial ETC involves the reduction of oxygen at the heme/Cu_B center, resulting in the formation of water. Indeed, the vast majority of oxygen in aerobic eukaryotes is utilized as the terminal electron acceptor in the mitochondrial ETC. Like complexes I and III, electron transport through cytochrome *c* oxidase drives the net transport of protons from the mitochondrial matrix to the intermembrane space (Fig. 2), contributing to the proton gradient across this membrane.

Both heme moieties in cytochrome *c* oxidase exist as heme *a* molecules. These differ from heme *b* by the presence of formyl and hydroxyethylfarnesyl side groups on the tetrapyrrole molecule (Fig. 5). The farnesyl group is a product of isoprenoid synthesis, comprising a chain of three isoprenoid units. It increases the hydrophobicity of heme and is thought to stabilize the moiety within the cytochrome *c* oxidase complex. The formyl group is thought to raise the redox potential of heme, a critical requirement for accepting lower energy electrons that are transferred at the terminal end of the ETC (181).

How Is Heme Made and Distributed Within Cells?

Heme is a structurally complex organic molecule that requires multiple enzymatic reactions to synthesize. The mechanism of heme synthesis was unraveled by pioneering studies in the 1940s and 1950s. The first of these examined the fate of heavy isotopes of glycine after human ingestion, where the authors noted that glycine was incorporated in heme (135). Subsequent studies found that succinyl-CoA, an intermediate of the tricarboxylic acid cycle in the mitochondrion, was also incorporated into heme (42). The condensation of glycine and succinyl-CoA forms a molecule known as δ-aminolevulinic acid (ALA), which represents the first committed precursor of tetrapyrrole biosynthesis. In α-proteobacteria, animals, fungi, and some other eukaryotes, ALA synthesis is catalyzed by an enzyme known as ALA synthase [ALAS; Fig. 3; (64)]. In eukaryotes, ALAS localizes to the mitochondrion and contains a pyridoxal 5′-phosphate (vitamin B_6) cofactor. ALAS is the major rate-limiting enzyme of heme synthesis in metazoa and many other organisms. Given the potential toxicity of heme, it is not surprising that ALA is tightly regulated at multiple levels, including transcription, messenger ribonucleic acid (mRNA) stability, protein stability, and even protein import into the mitochondrion (see section on Regulating Heme Metabolism).

After synthesis in the mitochondrion, ALA is exported from the organelle through unknown mechanisms (132). In the cytosol, two ALA molecules are condensed to form the pyrrole porphobilinogen, a reaction catalyzed by the enzyme porphobilinogen synthase (PBGS; also known as ALA dehydratase; Fig. 3). In animals, PBGS functions as an octamer and

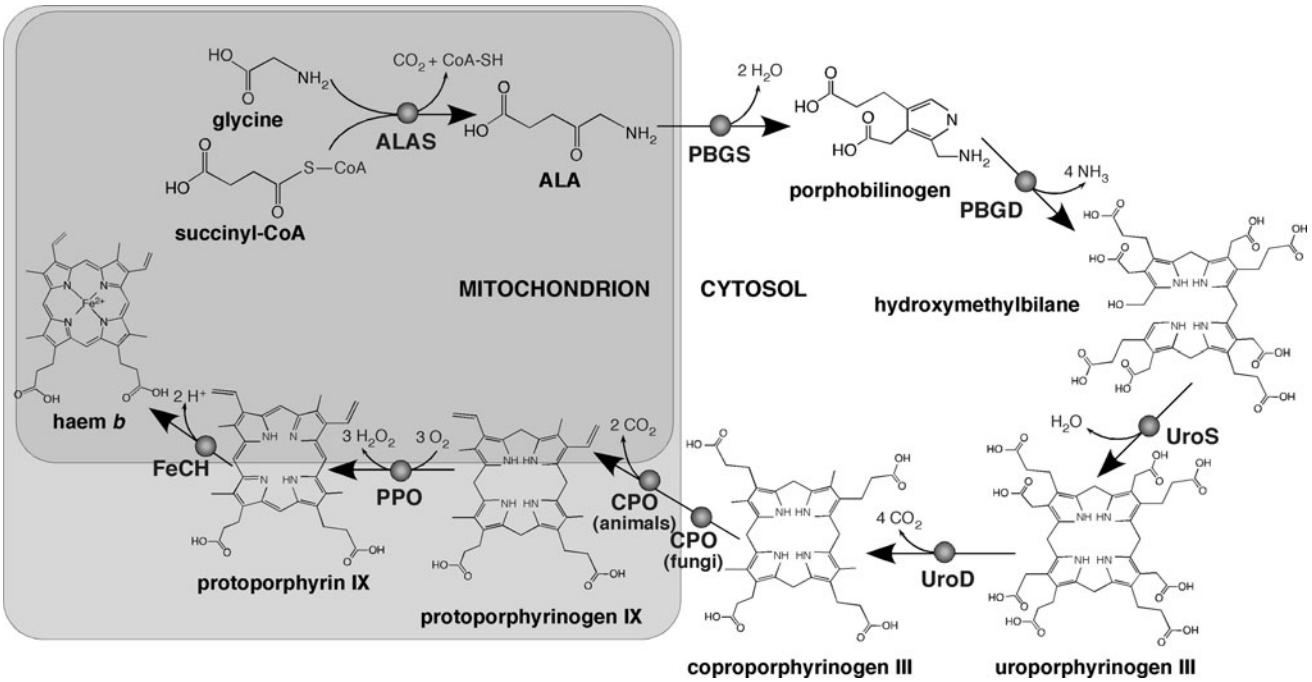

FIG. 3. Heme biosynthesis in animals and fungi. Heme synthesis is initiated in the mitochondrion, where ALAS reacts glycine and succinyl-CoA to form ALA. ALA is exported from the mitochondrion to the cytosol. Two molecules of ALA are condensed by PBGS to form the pyrrole porphobilinogen. Four porphobilinogen molecules are then condensed to form the tetrapyrrole hydroxymethylbilane, a reaction catalyzed by PBGD. Tetrapyrrole cyclization is catalyzed by UroS, forming uroporphyrinogen III. Uroporphyrinogen III is decarboxylated by UroD to form coproporphyrinogen III. Further decarboxylation generates protoporphyrinogen IX, a reaction catalyzed by CPO, which localizes to the cytosol in fungi and the mitochondrion in animals. Oxidation of protoporphyrinogen IX generates protoporphyrin IX, a reaction catalyzed by PPO at the intermembrane space side of the inner mitochondrion membrane. Finally, iron is inserted into the protoporphyrin ring to generate heme *b*, a reaction that is catalyzed by FeCH and which occurs on the matrix side of the inner membrane. ALA, *δ*-aminolevulinic acid; ALAS, *δ*-aminolevulinic acid synthase; CPO, coproporphyrinogen III oxidase; FeCH, ferrochelatase; PBGD, porphobilinogen deaminase; PBGS, porphobilinogen synthase; PPO, protoporphyrinogen IX oxidase; UroD, uroporphyrinogen III decarboxylase; UroS, uroporphyrinogen III synthase.

contains zinc ions in the catalytic sites (22). PBGS is exquisitely sensitive to lead, which displaces zinc in the catalytic site; many of the symptoms of lead poisoning result from impaired PBGS activity. The next two steps of heme synthesis involve the formation of the tetrapyrrole ring. First, porphobilinogen deaminase (PBGD) catalyzes the condensation of four porphobilinogen molecules to form the linear tetrapyrrole hydroxymethylbilane (Fig. 3). PBGD also localizes to the cytosol and contains an unusual cofactor called dipyrromethane. Dipyrromethane structurally resembles two molecules of porphobilinogen and acts as a template for condensing more porphobilinogen molecules to form the tetrapyrrole (22). Next, the linear tetrapyrrole is cyclized to form uroporphyrinogen III, a reaction catalyzed by the cytosolic enzyme uroporphyrinogen III synthase [UroS; Fig. 3; (22)].

The remainder of heme synthesis involves various modifications to the tetrapyrrole ring. First, the cytosolic enzyme uroporphyrinogen III decarboxylase (UroD) catalyzes the decarboxylation of the four acetate side groups of uroporphyrinogen III to generate coproporphyrinogen III (CPIII) [Fig. 3; (22)]. Next, coproporphyrinogen III oxidase (CPO) decarboxylates two of the four propionate side groups of CPIII to form protoporphyrinogen IX. CPO localizes to the mitochondrion in animal cells, where it is associated with the intermembrane face of the inner mitochondrial membrane, but is localized to the cytosol in fungi [Fig. 3; (15, 34, 44)]. The remaining enzymes of heme synthesis localize to the mitochondrion, the major site of heme requirement in most eukaryotes. The penultimate step of heme synthesis involves the oxidation of protoporphyrinogen IX to form protoporphyrin IX (Fig. 3). This reaction is catalyzed by inner mitochondrial membrane enzyme protoporphyrinogen IX oxidase (PPO), with oxygen acting as the electron acceptor (22, 27). The conversion of protoporphyrinogen IX to protoporphyrin IX is energetically favorable and can also occur in the absence of a catalyst. The synthesis of heme *b* is completed when the enzyme ferrochelatase (FeCH) inserts a ferrous iron into the center of the protoporphyrin IX ring, a reaction that occurs on the matrix side of the inner mitochondrial membrane [Fig. 3; (25)].

The greatest need for tetrapyrroles in photosynthetic eukaryotes such as plants and algae is in the plastid. Chlorophylls are a central component of light-harvesting complexes in photosynthesis, and heme is also required for electron transport in thylakoid membranes of plastids during photosynthesis. Plants also require heme in the mitochondrion and elsewhere in the cell. Early studies established some critical differences between tetrapyrrole synthesis in plants compared to nonphotosynthetic eukaryotes. These studies noted that plants are unable to synthesize ALA using glycine

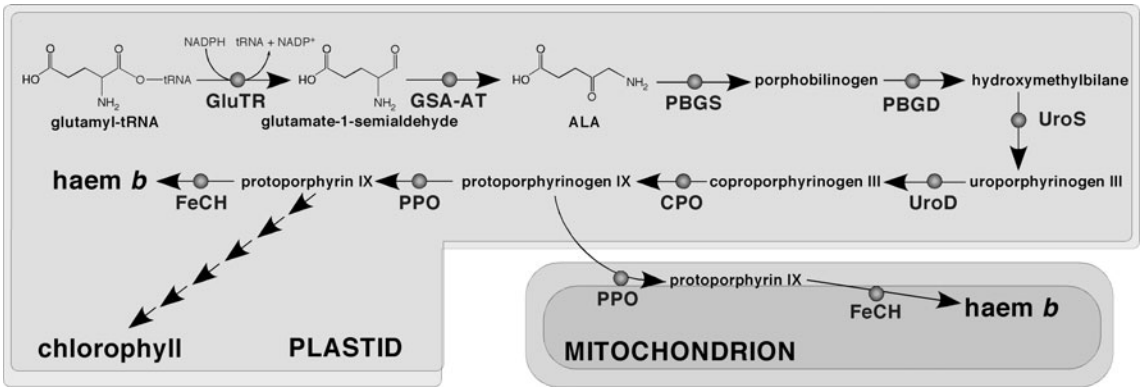

FIG. 4. Tetrapyrrole biosynthesis in plants commences with the synthesis of ALA by a two-step enzymatic process in the plastid. First, GluTR catalyzes the reduction of glutamyl-tRNA to form glutamate-1-semialdehyde. ALA is then formed from a transamination reaction catalyzed by GSA-AT. The remainder of the heme synthesis pathway features equivalent enzymes to those found in animals and fungi (see Fig. 3). The entire heme pathway occurs within the plastid, although the last two enzymes (PPO and FeCH) are also found in the mitochondrion, suggesting that plants have two sites of heme formation. The formation of protoporphyrin IX represents the branch point of the heme and chlorophyll synthesis pathways. GluTR, glutamyl-tRNA reductase; GSA-AT, glutamate semialdehyde aminotransferase.

and succinyl-CoA as precursors, but glutamate is readily incorporated (9). Further characterization of this pathway found that glutamyl-tRNA serves as the precursor for ALA synthesis. The enzyme glutamyl-tRNA reductase (GluTR) utilizes energy from NADPH to catalyze the formation of glutamate-1-semialdehyde (Fig. 4). Then, a second enzyme called glutamate semialdehyde aminotransferase (GSA-AT) converts glutamate-1-semialdehyde to ALA. Glutamate-derived ALA synthesis is common to virtually all photosynthetic eukaryotes, and also occurs in Archaea and most

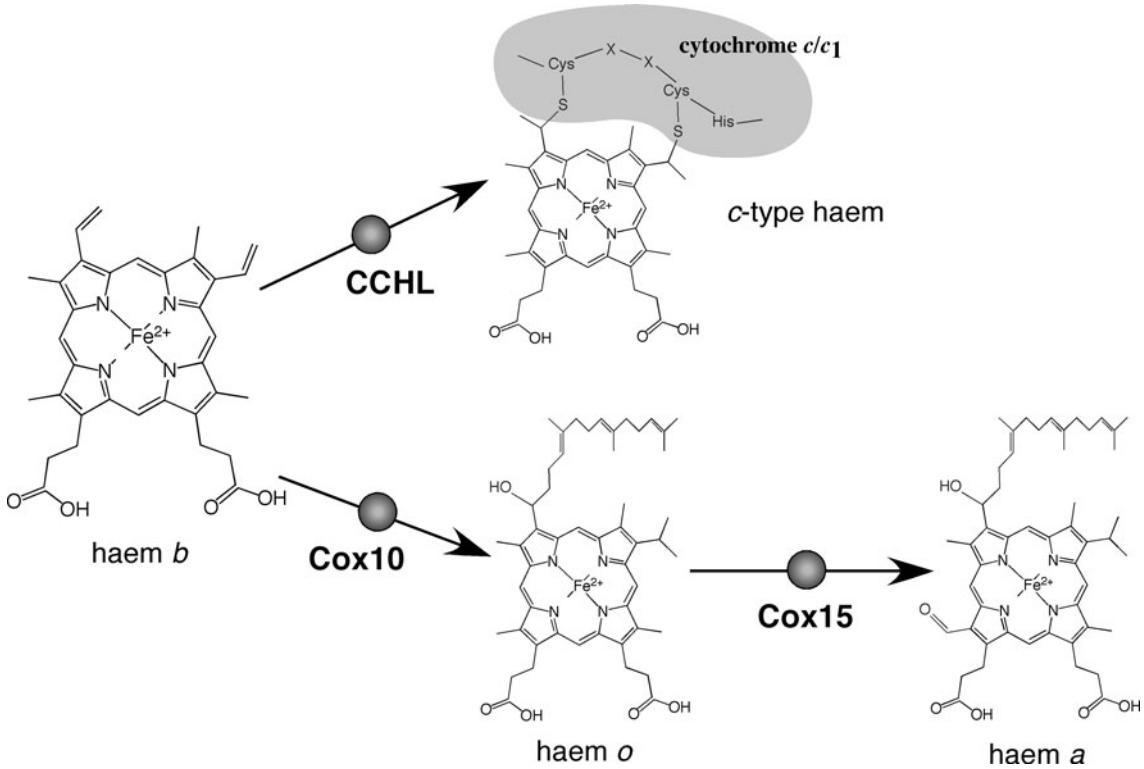

FIG. 5. Modifications of heme *b* in mitochondria. C-type cytochromes, such as cytochrome *c* and cytochrome c_1, are formed through the covalent attachment of heme *b* to two conserved cysteine residues in a C-X-X-C-H motif of the protein (*top*). The iron molecule at the center of heme is co-ordinated by the histidine of the motif. In animals, fungi, and Apicomplexa, heme insertion into C-type cytochromes is mediated by an enzyme called CCHL. Heme *a* is a critical component of the cytochrome *c* oxidase complex of the mitochondrial electron transport chain (Fig. 2). Heme *a* is formed through a two-step reaction from heme *b* (*bottom*). First, a farnesyl group is attached to heme *b* to form heme *o*, a reaction catalyzed by the enzyme Cox10. Cox15 then adds a formyl group to heme *o* to form heme *a*. CCHL, cytochrome c/c_1 heme lyase; C-X-X-C-H motif, cysteine-X-X-cysteine-histidine amino acid motif.

Bacteria. Indeed, glutamate-derived ALA synthesis has a considerably broader distribution than the ALAS-catalyzed reaction, which occurs only in α-proteobacteria and nonphotosynthetic eukaryotes.

Another key difference between plants and nonphotosynthetic eukaryotes is that ALA synthesis occurs in the plastid rather than in the mitochondrion. The remaining enzymes of heme synthesis in plants and algae are homologous to the enzymes found in other eukaryotes, but they too localize primarily to the plastid (Fig. 4). This likely reflects the major requirement for heme and chlorophyll in the plastid. Some smaller differences also exist between photosynthetic and nonphotosynthetic eukaryotes. For example, the metal co-factor in the PBGS protein is magnesium instead of zinc (22). Interestingly, plants have two isoforms of PPO and FeCH, the final two enzymes of the heme synthesis pathway. The two PPO genes have limited sequence identity, suggesting that they diverged very early in plant evolution (81). Interestingly, one PPO isoform localizes to the plastid, whereas the other localizes to the mitochondrion, or in some plants is dually targeted to the plastid and mitochondrion (Fig. 4). Similarly, one of the FeCH isoforms localizes to the plastid, while the other appears dually targeted to the plastid and the mitochondrion (22). This suggests that the final steps of heme synthesis can take place in both the plastids and mitochondria of plants (Fig. 4). This requires that protoporphyrinogen IX is exported from plastids and is imported into mitochondria, but mechanisms for this are unknown (56). The contribution of the two heme genesis pathways (mitochondrial and plastid) to heme required elsewhere in the cell is also unknown, although the amount of heme synthesized in plastids is considerably more than in mitochondria (21).

The synthesis of protoporphyrin IX is the last common step of heme and chlorophyll biosynthesis (Fig. 4). In chlorophyll biosynthesis, a Mg-chelatase inserts a magnesium ion into protoporphyrin, which is then modified in various ways to generate chlorophyll. The importance of tetrapyrroles for plant growth and viability is highlighted by the use of diphenyl ether herbicides (such as acifluorfen) that target the PPO enzyme.

Plastids were acquired by eukaryotes through a process of endosymbiosis from a cyanobacterial progenitor. Plastid acquisition postdates the acquisition of mitochondria, indicating that the glutamate-derived heme synthesis pathway was acquired with the cyanobacterial symbiont. It is therefore likely that the mitochondrial/cytosol and the plastid pathways for tetrapyrrole synthesis co-existed at some point in plant evolution. Plants apparently then streamlined their metabolism by discarding the mitochondrial/cytosol pathway and came to rely solely on the plastid-localized pathway. Interestingly, both the mitochondrial/cytosol and the plastid pathways co-exist in the protist *Euglena* (69, 171). *Euglena* is a photosynthetic eukaryote that acquired its plastid through a so-called secondary endosymbiotic event where a green alga became incorporated into a heterotrophic eukaryote. Instead of losing the mitochondrial/cytosol-localized pathway like in plants, the two pathways persist in *Euglena* (69, 171). The plastid-derived pathway probably provides all the heme and chlorophyll needed in the plastid, while the mitochondrial/cytosolic pathway presumably generates the heme needed in the mitochondrion and possibly the rest of the cell (69). Curiously, *Euglena* plastids are surrounded by three membranes rather than the two observed in plants. One possible reason for the retention of two independent heme pathways in *Euglena* is that the additional membrane never evolved the necessary transporter to export heme from the plastid to the rest of the cell, meaning that the plastid pathway would be unable to supply cellular heme. Interestingly, *Euglena* cells can lose their plastids entirely and live a heterotrophic lifestyle (108). The fact that *Euglena* can still synthesize heme in the absence of a plastid is likely crucial in permitting plastid loss, which never occurs in plants, even if they are nonphotosynthetic (69).

Heme *b* has several possible fates within cells. In the mitochondrion, it can be inserted into *b*-type cytochromes such as cytochrome *b* in complex III of the ETC. It can also be covalently bound to a C-X-X-C-H motif of *c*-type cytochromes, such as cytochrome *c* and c_1. The two cysteines of the motif bind to heme through thioether bonds, and the histidine residue co-ordinates the iron at the center of the ring (Fig. 5). Covalent insertion of heme into *c*-type cytochromes in fungal and animal mitochondria is mediated by a single-subunit, intermembrane space-localized enzyme known as cytochrome c/c_1 heme lyase [Fig. 5; (3, 71)]. In plant mitochondria, heme is also inserted into a C-X-X-C-H motif of cytochrome *c*, but this insertion requires a multiprotein pathway similar to that found in many prokaryotes. Heme *b* can also be modified to heme *a*, which inserts into the cytochrome oxidase complex of the respiratory chain. Heme *a* is formed through a two-step process. First, a farnesyl group (a product of isoprenoid synthesis) is added to heme *b* to form heme *o*, a reaction catalyzed by a farnesyl transferase protein called Cox10 (Fig. 5). Finally, a formyl group is generated on heme *o* by the Cox15 protein to form heme *a* (95).

Heme is required outside the mitochondrion, where it functions as a prosthetic group in proteins, such as globins, peroxidases, catalases, and cytochrome P450s. Despite its hydrophobic nature, heme cannot freely diffuse across cellular membranes, including the two membranes enclosing the mitochondrion (72). How heme is exported from mitochondria is unknown. Several studies have identified a possible role for ATP-binding cassette (ABC) transporters in the transport of heme and other porphyrins into and out of the mitochondrion (20, 73). ABC transporters are multimembrane-spanning proteins that utilize energy from the hydrolysis of ATP to transport solutes across membranes. Mammalian cells contain several mitochondrially localized ABC transporters. One such transporter, called ABCB6, occurs on the outer membrane. ABCB6 appears to have a key role in transporting CPIII—the final heme biosynthesis intermediate synthesized in the cytosol—into the mitochondrion, where the heme synthesis pathway concludes [Fig. 3; (73)]. The ABCB10 transporter localizes to the inner membrane where it interacts with FeCH, the terminal enzyme of heme synthesis (20). It is possible that ABCB10 is able to transport heme synthesized by FeCH out of the organelle. Although direct evidence for this is lacking, it is intriguing to note that an ABCB10 homologue is absent from the nematode worm *Caenorhabditis elegans*, which lacks the heme synthesis pathway, and does not need to export heme from the organelle (72).

Once in the cytosol, heme must be transported to various sites within the cell. The cytotoxic effects of heme mean that cells must keep intracellular free heme pools to a minimum. One way to do this is by binding free heme to heme-binding

proteins that mediate transport around the cell (132). Although cells contain numerous cytosolic proteins with a high affinity for heme, cytosolic heme carrier proteins (HCPs) have not yet been identified. In addition to heme synthesis, mammals are able to acquire dietary heme absorbed in the intestine. Intestinal epithelial cells contain several proteins that may mediate heme uptake, although the mechanism for heme uptake remains unclear. Candidates for heme uptake proteins include a low-affinity heme transporter called HCP1, whose major role is in folate transport (117, 134), and a so-called heme responsive gene (HRG) family that includes HRG-1 and HRG-4 (104, 119).

Heme Metabolism in Apicomplexan Parasites

Apicomplexa are a phylum of intracellular parasites that have major impacts on human populations throughout the world. The most notorious apicomplexan is the malaria-causing parasite *Plasmodium*, in particular *P. falciparum*, which causes cerebral malaria. *Plasmodium* parasites are transmitted to humans by mosquito bites. The parasites pass through a liver stage, and then infect erythrocytes, which constitutes the disease-causing part of the lifecycle. Over a third of the world's population is at risk from malaria, with more than 1 million people (predominantly children in sub-Saharan Africa) succumbing annually (45). The distribution of malaria has a strong correlation with poverty, and the medical, social, and economic impacts of the disease are profound in regions where it is prevalent. *Toxoplasma gondii* is a widespread apicomplexan parasite that infects virtually all nucleated mammalian cells and can cause encephalitis in immunocompromised individuals and ocular damage in healthy individuals. *Babesia* species and *Theileria* species belong to the Piroplasmid order of Apicomplexa. Both are major parasites of cattle and other livestock and are spread through tick bites. Like *Plasmodium*, *Babesia* infects erythrocytes, whereas *Theileria* sets up home inside leukocytes, co-opting the cell division machinery to proliferate, before differentiating into an erythrocyte-infecting form prior to transmission back into the tick (168). The final apicomplexan parasite we will consider in this review is *Cryptosporidium*, a gastrointestinal parasite that causes diarrhea symptoms and is responsible for several widespread outbreaks of disease.

The use of heme in Plasmodium *parasites*

Extensive genome sequencing and annotation in eight different *Plasmodium* species (collated and annotated at www.plasmodb.org) reveals the presence of multiple heme-containing proteins and protein complexes. Foremost among these are proteins involved in the mitochondrial ETC. The *P. falciparum* genome encodes proteins that, based on homology with components in other organisms, constitute the cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV) of the ETC (Fig. 6). These include the heme-containing proteins cytochrome *b* and cytochrome c_1 (Table 1). The *P. falciparum* genome also contains two homologues of cytochrome *c*, the mobile carrier of electrons between complexes III and IV (Fig. 6; Table 1). Why two cytochrome *c* homologues exist is unclear. The *P. falciparum* genome encodes two single-subunit cytochrome c/c_1 heme lyase genes, suggesting that insertion of heme into C-type cytochromes occurs through similar mechanisms as in fungi and animals (4, 160). *P. falciparum* encodes CoxI, the protein of cytochrome *c* reductase that co-ordinates the two heme *a* moieties of the complex (Table 1). The *P. falciparum* genome also encodes homologues of the Cox10 and Cox15 proteins required for heme *a* synthesis (Figs. 5 and 6).

The mitochondrial ETC is active in blood stages of *Plasmodium* parasites, and contributes to generating a $\Delta\Psi$ across the inner mitochondrial membrane (40, 141, 156, 157). Cytochrome *b* is the target for atovaquone, a major antimalarial drug. Atovaquone is thought to act as a competitive inhibitor of coenzyme Q binding to cytochrome *b*, and treating parasites with atovaquone results in a rapid loss of $\Delta\Psi$ across the inner membrane and subsequent inhibition of parasite growth (63, 140). Mutations in cytochrome *b* (a gene encoded on the mitochondrial genome of *Plasmodium*) can lead to atovaquone resistance, and such resistance has arisen in both laboratory and field settings, limiting the use of atovaquone in treating malaria.

The mitochondrial ETC is clearly critical for the survival of blood-stage *Plasmodium* parasites, but what is its role in cellular metabolism? At least five dehydrogenases can contribute electrons to the ETC *via* coenzyme Q [Fig. 6; (160)]. These include succinate dehydrogenase (complex II); malate:quinone oxidoreductase, which donates electrons acquired from the oxidation of malate; dihydroorotate dehydrogenase (DHOD), a pyrimidine biosynthesis enzyme that oxidizes dihydroorotate to form orotate; and a FAD-linked glycerol-3-phosphate dehydrogenase, which oxidizes glycerol-3-phosphate to form dihydroxyacetone phosphate. *Plasmodium* lacks a multisubunit complex I–type NADH dehydrogenase but does encode a single-subunit NADH dehydrogenase, which is able to oxidize NADH [Fig. 6; (10)]. The *Plasmodium* genome also contains most subunits of the ATP synthase complex that harvests the proton gradient formed by the ETC to generate ATP (8).

Interestingly, of all these functions, it appears that the only essential role of the ETC in *Plasmodium* blood-stage growth is as an electron sink for the DHOD reaction in pyrimidine synthesis (111). To demonstrate this, Painter *et al.* expressed the yeast DHOD enzyme in *P. falciparum* parasites. Yeast DHOD is unique in that it is a cytosolic enzyme that does not donate electrons to coenyzme Q. Expressing this cytosolic DHOD in *Plasmodium* will thus overcome the requirement for an active ETC in pyrimidine biosynthesis. When these parasites were treated with atovaquone to inhibit the ETC, the parasites survived, despite atovaquone collapsing the $\Delta\Psi$ across the inner membrane (111). These data suggested that the other dehydrogenases are dispensable for parasite growth in blood stages. In a separate approach to test for the role of the ETC in *P. falciparum*, Smilkstein *et al.* used drug selection to generate a *P. falciparum* strain resistant to cytochrome *c* reductase inhibitors such as atovaquone (139). Unlike previously characterized atovaquone-resistant mutants, cytochrome *c* reductase activity in these parasites remained sensitive to atovaquone. The authors concluded that, like in the yeast DHOD study, they had generated a parasite line that no longer required the mitochondrial ETC for parasite viability. This raises the question of what could function as the electron sink for the DHOD reaction in ETC-less parasites? One possibility is that instead of donating electrons to coenzyme Q, DHOD in these parasites may donate electrons to menaquinone, an electron carrier that can function in place of

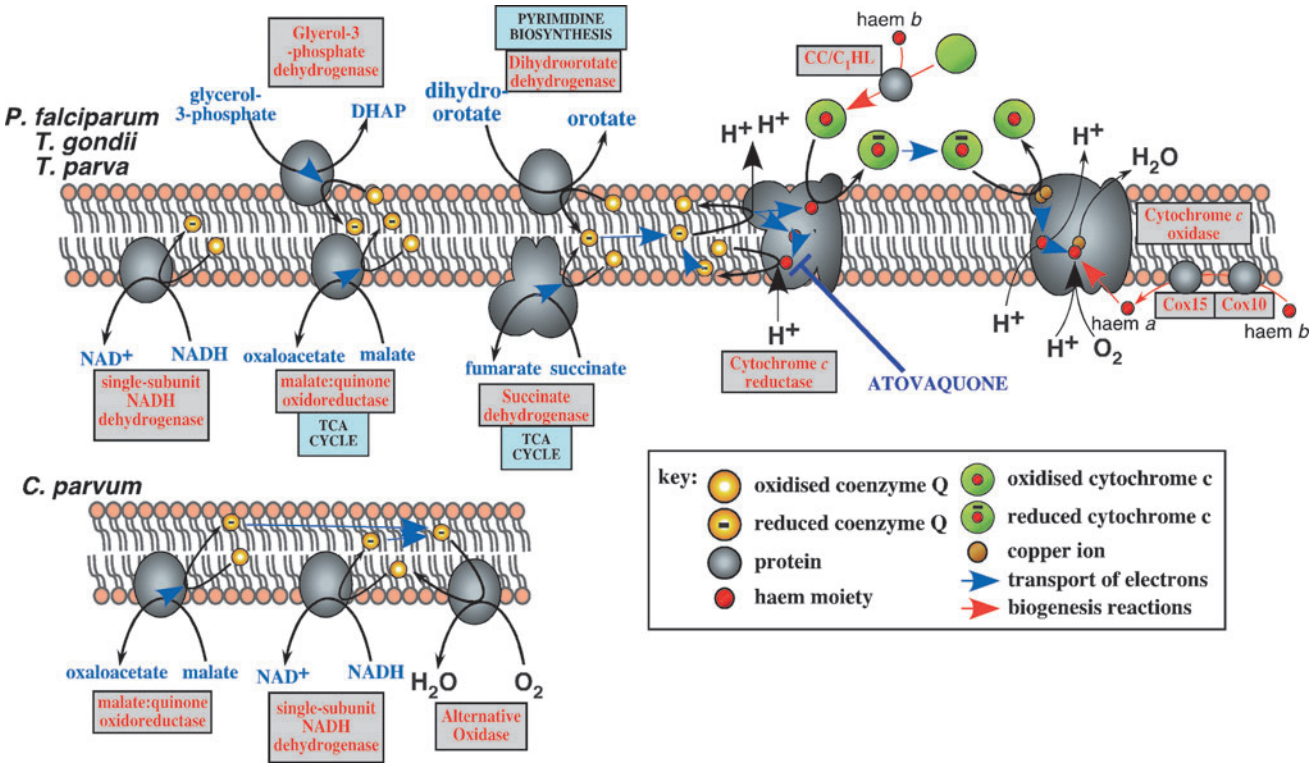

FIG. 6. The mitochondrial ETC in apicomplexan parasites. Five dehydrogenases feed electrons into the ETC of *Plasmodium*, *Toxoplasma*, and *Theileria* (*top*). These include the TCA cycle enzymes succinate dehydrogenase (complex II) and malate:quinone oxidoreductase, the pyrimidine synthesis enzyme dihydroorotate dehydrogenase, a single-subunit NADH dehydrogenase, and a glycerol-3-phosphate dehydrogenase. Note that apicomplexan succinate dehydrogenase appears to lack the conserved histidine residues that co-ordinate heme (93), suggesting that, unlike animal succinate dehydrogenase (Fig. 2), the apicomplexan complex does not bind heme. The remainder of the ETC resembles that found in "typical" eukaryotes. Cytochrome *c* reductase (complex III) accepts electrons from coenzyme Q and ultimately reduces cytochrome *c*. Notably, cytochrome *c* reductase is the target of the major antimalarial drug atovaquone. Electrons from cytochrome *c* are donated to cytochrome *c* reductase, where they ultimately reduce oxygen to form water. Proton transfer across the inner mitochondrial membrane occurs at both cytochrome *c* reductase and cytochrome *c* oxidase. The ETC in *Cryptosporidium* (*bottom*) differs considerably from that found in other Apicomplexa. Electrons are fed into the *Cryptosporidium* ETC by malate:quinone oxidoreductase and a single-subunit NADH dehydrogenase. *Cryptosporidium* lacks homologues of cytochrome *c* reductase, cytochrome *c*, and cytochrome *c* oxidase. Instead, reduced coenzyme Q donates electrons to an alternative oxidase, which reduces oxygen in the terminal step of the ETC. TCA, tricarboxylic acid.

coenzyme Q in several bacterial systems. It appears that menaquinone is present in blood-stage *P. falciparum* parasites, being upregulated in parasites grown in anaerobic conditions (*P. falciparum* usually grows as a microaerophile) (153). Menaquinone can act as an electron acceptor for the single-subunit NADH dehydrogenase in *P. falciparum*, although direct evidence for its role as an electron acceptor for DHOD is lacking.

Blood-stage *Plasmodium* parasites rely on glucose as their major energy source, meaning that if ATP synthase has a role in ATP generation, it is only minor during blood stages. As mentioned previously, the *Plasmodium* lifecycle consists of mosquito and liver stages in addition to the blood stages so far discussed. Glucose levels in the insect hemolymph are roughly equivalent to that found in mammalian blood (85), suggesting that insect stages of *Plasmodium* can derive similar amounts of glycolytic energy to blood stages. Curiously, a knockout mutant of the single-subunit NADH dehydrogenase in the mouse malaria *Plasmodium berghei* was viable in blood-stage parasites, but essential during midgut (oocyst) development of the parasites in the mosquito (14). This suggests that there is an increased role for the ETC in insect stages of the parasite. One possible explanation is that insect stages require more energy than blood stages, necessitating a more active ETC capable of synthesizing higher levels of ATP. It will now be of considerable interest to generate mutants in ATP synthase subunits to directly address this possibility. In the broader context of heme metabolism in *Plasmodium*, we can conclude that the parasite requires heme during both blood and insect stages for electron transport in the mitochondrion.

Outside the mitochondrion, *Plasmodium* contains few known heme-requiring proteins. The *P. falciparum* genome encodes three cytochrome b_5 homologues of unknown function and localization (Table 1; cytochrome b_5-1, -2, and -3). One of these (cytochrome b_5-1) contains a hydrophobic C-terminus that may function as a tail-anchor targeting motif for the endoplasmic reticulum or the outer mitochondrial membrane. *P. falciparum* appears to lack homologues of cytochrome P450 genes, and we were unable to identify obvious

Table 1. Heme-Containing Proteins in a Range of Apicomplexa

Protein	*Function*	*P. falciparum*	*T. gondii*	*T. parva*	*C. parvum*
Cytochrome *b*	Mitochondrial electron transport chain (cytochrome *c* reductase)	Mitochondrial genome	Mitochondrial genome	Mitochondrial genome	Absent
Cytochrome c_1	Mitochondrial electron transport chain (cytochrome *c* reductase)	PF14_0597	TGME49_046540	TP04_0384	Absent
Cytochrome c	Mitochondrial electron transport chain	PF14_0038	TGME49_019750	TP02_0396	Absent
Cytochrome *c*-2	Unknown	MAL13P1.55	TGME49_029420	TP04_0712	Absent
CoxI	Mitochondrial electron transport chain (cytochrome *c* oxidase)	Mitochondrial genome	Mitochondrial genome	Mitochondrial genome	Absent
Cytochrome b_5-1	Unknown	PFL1555w	TGME49_076110	Absent	Absent
Cytochrome b_5-2	Unknown	PFI0885w	TGME49_113580[a]	Absent	cgd2_1040
Cytochrome b_5-3	Unknown	PF14_0266	TGME49_054090	Absent	Absent
Cytochrome b_5-4	Hydroxylation of fatty acids—contains fatty acid hydroxylase domain in addition to cytochrome b_5 domain	Absent	TGME49_040770	Absent	Absent
Cytochrome b_5-5	Sulfite oxidation—contains molybdenum-binding sulfite oxidase domain	Absent	TGME49_095720	Absent	Absent
Cytochrome b_5-6	unknown	Absent	TGME49_073530	Absent	Absent
Cytochrome b_5-7	unknown	Absent	TGME49_076990	Absent	Absent
Cytochrome P450	unknown	Absent	TGME49_115770	Absent	Absent
Catalase	Antioxidant defense	Absent	TGME49_032250	Absent	Absent

[a]Gene model for TGME49_113580 is very likely incorrect. Correct gene model is probably similar to *Neospora caninum* gene model NCLIV_056850 (www.toxodb.org).

globin homologues in the *P. falciparum* genome. Antioxidant defenses in *P. falciparum* have been well characterized (62). They are required in the parasite cytosol, mitochondrion, and apicoplast, a nonphotosynthetic plastid organelle. *P. falciparum* contains several major types of antioxidant defenses: a glutathione-based system, a thioredoxin-based system, and two superoxide dismutases, but none of these systems require any heme-dependent proteins, such as catalases, ascorbate peroxidases, or cytochrome *c* peroxidases.

The abuse of heme in Plasmodium *parasites*

Blood-stage *Plasmodium* parasites live within hemoglobin-rich erythrocytes. Parasites engulf hemoglobin and other proteins from their host cell during intracellular growth by a process of endocytosis (46). Endocytic vesicles fuse with a lysosome-like compartment called the digestive vacuole where a suite of proteases digest hemoglobin to its constituent amino acids. These proteases include aspartic proteases (the plasmepsins), cysteine proteases (the falcipains in *P. falciparum*), the metalloprotease falcilysin, a dipeptidyl aminopeptidase, and aminopeptidases (33, 37, 65, 118). Plasmepsins likely perform the initial cleavage of hemoglobin, and then act in concert with falcipains to further degrade hemoglobin into shorter peptides. These are then cleaved by falcilysin into peptides ~4 to 10 amino acids long (33). A dipeptidyl aminopeptidase cleaves these short oligopeptides into dipeptides and aminopeptidases, then cleaves the final peptide bond to form the constituent amino acids (65, 118). Amino acids released from hemoglobin degradation are then transported into the parasite cytosol where they make up the bulk of amino acids utilized by the parasite in protein synthesis and other cellular functions (83).

The degradation of hemoglobin also releases heme into the digestive vacuole. Given the sheer volume of hemoglobin digested by the parasite (~70% of the total amount of hemoglobin in the erythrocyte), the large amount of heme released into the vacuole is potentially extremely toxic to the parasite. The parasite overcomes this by polymerizing heme into an insoluble pigment called hemozoin (110). Indeed, the vast majority of heme in the parasite (over 95%) is thought to be in the form of hemozoin (31).

How parasites polymerize heme is controversial. Hemozoin is known to associate with neutral lipid droplets found in the digestive vacuole (116). Neutral lipid droplets can catalyze the formation of hemozoin *in vitro*, suggesting that hemozoin formation can occur at the surface of neutral lipid droplets found in the digestive vacuole (49, 50, 116). Proteins have also been proposed to play a role in hemozoin formation, possibly mediating the initial nucleation event that precedes polymerization. Histidine-rich proteins (HRPs) found in *P. falciparum* can catalyze hemozoin formation *in vitro*, although at a low efficiency (57, 145). HRPs readily bind to heme, but HRP homologues are unique to *P. falciparum*, which perhaps suggests that other *Plasmodium* species can synthesize hemozoin without HRPs. Further, the majority of the best characterized HRP protein (HRP2) is secreted from the parasite into the host cell cytosol, suggesting that its major function may not be in the digestive vacuole (2, 53). Another recently identified protein candidate for hemozoin formation is the heme

detoxification protein (*Pf*HDP), which binds heme and can catalyze hemozoin formation *in vitro* at over 1000 times the efficiency of HRP2 and lipids (57). *Pf*HDP appears to be exported to the host cell cytosol before being internalized into the digestive vacuole during endocytosis of host cell proteins.

Regardless of how hemozoin is formed, it is clearly critical for parasite survival. The major antimalarial drug chloroquine interferes with heme polymerization, resulting in a reduction in hemozoin, swelling of the digestive vacuole membrane, and rapid parasite death (76, 86, 138, 170). Exactly how chloroquine interferes with hemozoin formation is still not fully understood. Chloroquine binds to heme and may prevent its incorporation into hemozoin (32). Chloroquine can also bind to hemozoin and may cap hemozoin crystals to prevent the addition of further heme molecules (146). The accumulation of free heme in chloroquine-treated parasites likely compromises membrane integrity (74, 107). Heme (and heme in a complex with chloroquine) can also inhibit some of the enzymes that degrade hemoglobin (43, 161). Effects on membrane integrity and hemoglobin digestion likely inhibit parasite growth in chloroquine-treated parasites. Curiously, a chloroquine-resistant strain of *P. berghei* generated in laboratory conditions appears to lack hemozoin (114). Exactly how these parasites overcome the toxic effects of free heme is unclear, although these chloroquine-resistant parasites revert to chloroquine sensitivity in the absence of drug pressure (47).

Heme may also play a critical role in the action of artemisinin and its derivatives, another major class of antimalarial drugs. The activity of artemisinin is based on the presence of an endoperoxide bridge in the molecule. It is believed that cleavage of this endoperoxide bridge leads to the formation of reactive intermediates that can ultimately inhibit parasite growth (105). The iron present in reduced heme is a prime candidate for mediating this cleavage event. Interestingly, artemisinins have been proposed to act in both the digestive vacuole and mitochondrion of *Plasmodium* (26, 169), the two major sites for heme in these parasites. Indeed, inhibiting the digestion of hemoglobin, and therefore inhibiting the release of free heme into the digestive vacuole, limits the efficacy of artemisinin (66). Exactly how artemisinin inhibits parasite growth is not fully understood. Reactive intermediates may alkylate proteins and other molecules, including heme itself, either inferring with their functions or generating cytotoxic compounds (91, 105). Alternatively, reactive artemisinin intermediates may lead to the formation of reactive oxygen species, for example, by lipid peroxidation, that inhibit proteins and other cellular processes, and affect the integrity of cellular membranes (13).

While polymerizing heme is critical for the survival of blood-stage *Plasmodium*, the generation of hemozoin has unanticipated effects on parasite interactions with the host. When *Plasmodium* parasites exit host red blood cells, they leave behind a so-called residual body that contains cellular material that is not packaged into the daughter cells. This includes hemozoin crystals. Egress of parasites from host erythrocytes corresponds to the release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), which in turn corresponds to the fever associated with malaria (92). During *Plasmodium* infection, considerable levels of hemozoin are released into the blood system of the host. Several lines of evidence point to a role of this hemozoin as a critical mediator of the innate host cell immune response to *Plasmodium*. Hemozoin can stimulate the production of TNF-α and other pro-inflammatory cytokines, as well as anti-inflammatory cytokines under certain conditions, through mechanisms not yet fully understood (136). Phagocytic cells of the immune system, such as monocytes, phagocytose hemozoin. The presence of hemozoin-containing monocytes correlates with reduced erythropoiesis (the generation of new erythrocytes), suggesting that hemozoin may also have a role in promoting anemia that is frequently associated with malaria (18, 113).

Mammalian cells detoxify heme through the action of heme oxygenase (HO). HO cleaves heme at a single point in the tetrapyrrole ring, linearizing the ring to produce a molecule called biliverdin, and releasing iron in the process (87). Biliverdin can be further catabolized and excreted from the organism. *P. falciparum* contains a single HO-like protein (*Pf*HO) that functions as a HO when assayed *in vitro* (106, 128). The role of *Pf*HO in parasite metabolism remains unclear, although its role in deactivating free heme is likely very minor compared to the generation of hemozoin. Although *Pf*HO was predicted to localize to the apicoplast based on a putative N-terminal targeting domain (106), heme seems unlikely to be required for apicoplast enzymes (Table 1). Experimental localization of *Pf*HO is therefore critical in determining its role. One possible function for *Pf*HO is iron scavenging. Iron released during the HO reaction can be incorporated into enzymes and other iron-requiring processes. Intriguingly, the apicoplast contains an iron-sulfur cluster synthesis pathway, and iron-sulfur clusters are essential for enzymes of several apicoplast metabolic pathways as well as the redox protein ferredoxin (120). One possibility we can propose is that heme serves as a molecule to transport iron into the apicoplast, with HO then releasing iron for use in iron-sulfur cluster synthesis. This scenario currently lacks any supportive evidence, and would require the existence of transporters able to transport heme across the four membranes that surround the apicoplast.

Heme metabolism in other apicomplexa

In addition to *Plasmodium*, we examined putative heme-binding proteins in a range of other Apicomplexa, including *T. gondii*, *Theileria parva*, and *Cryptosporidium parvum*. Mitochondria in *T. gondii* have an active ETC and parasites are highly sensitive to atovaquone (6, 165). The *T. gondii* genome encodes homologues of all the proteins that putatively comprise the ETC in *Plasmodium*, including the presence of all heme-binding proteins (Fig. 6). In addition to the five mitochondrial dehydrogenases found in *P. falciparum*, *T. gondii* contains a second NADH dehydrogenase homologue; although mutant studies indicate that neither protein is essential for parasite viability in the asexual tachyzoite life stage (82). Less is known about the mitochondrial ETC of *T. parva*, but genome mining indicates the presence of the same complement of genes as in *P. falciparum* (Fig. 6). Interestingly, like in *Plasmodium*, both *T. gondii* and *T. parva* encode two cytochrome *c* homologues, so two cytochrome *c* proteins could be a common feature of the mitochondrial ETC in Apicomplexa.

C. parvum contains a highly reduced mitochondrion whose major function is likely the synthesis of iron-sulfur clusters (75, 123). The *C. parvum* genome encodes two mitochondrial dehydrogenases (namely, malate:quinone oxidoreductase and a single-subunit NADH dehydrogenase), as well as the genes necessary for coenzyme Q synthesis. This suggests the

presence of a mitochondrial ETC. However, the genome lacks genes encoding any of the subunits for cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV) (Table 1). Instead, electrons from coenzyme Q are donated to a so-called alternative oxidase, a single-subunit protein that functions in place of complex IV to reduce oxygen [Fig. 6; (124, 148)].

Compared with *P. falciparum*, *T. gondii* contains several additional heme-containing proteins. These include a catalase protein that functions in antioxidant defense (29, 137), and a single cytochrome P450 homologue of unknown function (Table 1). In addition to putative orthologues of the three cytochrome b_5 proteins found in *P. falciparum*, *T. gondii* contains an additional four proteins with cytochrome b_5–containing domains (Table 1). These include one protein that likely functions in fatty acid hydroxylation. Another cytochrome b_5–containing protein has homology to sulfite oxidases from other organisms. Sulfite oxidase is a molybdenum-containing enzyme that oxidizes sulfite, for example, sulfite derived from the degradation sulfur-containing amino acids, to generate sulfate. Electrons from sulfite are donated to molybdenum, and then on to the heme group of the cytochrome b_5 domain of the enzyme to reoxidize the molybdenum (61). In mammalian cells the electrons from the cytochrome b_5 domain are then donated to cytochrome *c* in the mitochondrial ETC. In *T. parva*, we were unable to identify any further heme-containing proteins beyond those of the mitochondrial ETC. Interestingly, we did identify a homologue of one of the *P. falciparum* cytochrome b_5–containing proteins in *C. parvum*, the only heme-containing protein we could identify in this organism (Table 1).

With respect to heme detoxification, *T. gondii* and *T. parva* both have homologues of *Pf*HO (accession numbers TGME49_059190 and TP01_0873, respectively; www.eupathdb.org), suggesting that HO may be active in these parasites. Surprisingly, *T. gondii* and *T. parva* also have homologues of *Pf*HDP (TGME49_085280 and TP04_0891), even though there is no evidence for hemozoin formation in either parasite. Both proteins appear absent from *Cryptosporidium*.

Heme Acquisition in Apicomplexan Parasites

Apicomplexan parasites clearly require heme for mitochondrial electron transport and several other cellular reactions. In this section, we will examine the possible sources for heme in Apicomplexa. We will consider the ability of parasites to synthesize their own heme from simple metabolic precursors, and the possibility that Apicomplexa scavenge heme from their host cells.

Heme synthesis in apicomplexan parasites

Blood-stage *Plasmodium* parasites live in a very heme-rich environment, but most of the heme internalized by the parasite is sequestered as hemozoin. To determine whether *Plasmodium* is capable of synthesizing its own heme, Surolia and Padmanaban incubated parasites with radiolabeled ALA (147). They observed the incorporation of the radiolabel into heme, indicating that *P. falciparum* does indeed synthesize heme. To ascertain the route of ALA synthesis, they incubated parasites in either radiolabeled glycine or radiolabeled glutamate, and found that only glycine labeled effectively into heme. This indicates that the route of ALA synthesis occurs through ALAS, as is the case in other nonphotosynthetic eukaryotes. This biochemical finding was supported by the identification of an ALAS homologue in the *P. falciparum* genome (172).

The genomes of *P. falciparum* and other *Plasmodium* species encode an almost complete complement of enzymes for the heme synthesis pathway (41). The *T. gondii* genome also appears to encode all the enzymes necessary for heme biosynthesis (www.toxodb.org). As mentioned earlier, the presence of ALAS activity and the absence of GluTR and GSA-AT activity in *P. falciparum* suggest that the heme synthesis pathway may resemble that observed in nonphotosynthetic eukaryotes. Nevertheless, most Apicomplexa (including *Plasmodium*, *T. gondii*, *Babesia*, and *Theileria*, but excluding *Cryptosporidium*) retain a nonphotosynthetic, plastid-derived organelle called the apicoplast (67, 88, 173). When the apicoplast was first acquired, it was photosynthetic and had a plastid-localized tetrapyrrole synthesis pathway (58). Therefore, it is possible that some of these plastid-derived heme synthesis enzymes were retained by the parasites.

To test these possibilities, several groups set out to localize each of the heme synthesis enzymes in *P. falciparum* and *T. gondii*. As expected, the ALAS protein in both *P. falciparum* and *T. gondii* localizes to the mitochondrion [Fig. 7; (129, 163, 175); G.v.D. and G.I.M., unpublished]. Unexpectedly, PBGS, the next enzyme in the pathway, localizes to the apicoplast [Fig. 7; (28, 129)]. Like plastid-localized PBGS, the apicomplexan protein has an affinity for magnesium ions instead of zinc; although unlike the plant enzyme it is not dependent on magnesium for activity. Phylogenetic analyses suggest that apicomplexan PBGS is derived from the endosymbiont that gave rise to the apicoplast (70, 130, 133). The different subcellular localizations and phylogenetic ancestry of ALAS and PBGS suggest that heme synthesis in Apicomplexa combines elements of both the mitochondrial/cytosolic and the

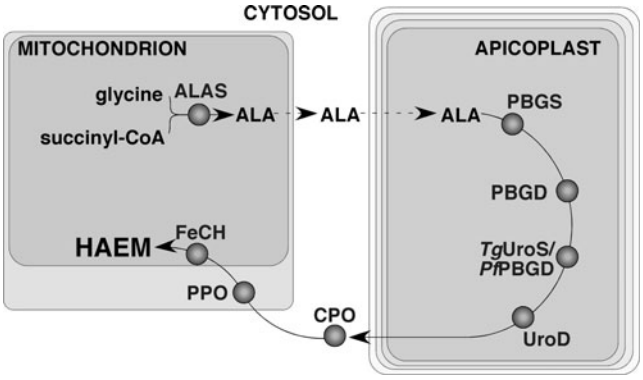

FIG. 7. Heme biosynthesis in *Toxoplasma* and *Plasmodium*. Heme biosynthesis commences with the ALAS-catalyzed formation of ALA in the mitochondrion of *Toxoplasma* and *Plasmodium*. ALA is then transported from the mitochondrion into the stroma of the apicoplast. Here, the next four reactions of heme synthesis take place, leading to the formation of coproporphyrinogen III. Coproporphyrinogen III must then be exported from the apicoplast into the cytosol. Here, the enzyme CPO oxidizes coproporphyrinogen III to form protoporphyrinogen IX. Like in animal and fungal cells, the last two enzymes of heme synthesis localize to the mitochondria.

plastidic pathways, which we previously termed a "hybrid" heme pathway (160).

The subsequent reactions up to the creation of CPIII—catalyzed by the enzymes PBGD, UroS, and UroD—also occur in the apicoplast in both *P. falciparum* and *T. gondii* [Fig. 7; (97, 98, 129, 175); A.T.K. and G.v.D., unpublished]. Initially, we were unable to identify homologues of UroS in the *P. falciparum* genome (160). One study found that *P. falciparum* PBGD has both PBGD and UroS activity, suggesting it may take the place the UroS enzyme (98). More recently, a possible UroS homologue has been identified in the *P. falciparum* genome, although the localization and activity of this enzyme are unknown (70).

The next step of the pathway is catalyzed by CPO, which localizes to the cytosol in both *P. falciparum* and *T. gondii* [Fig. 7; (100, 175)]. This reflects the localization of CPO in fungi, and phylogenetic analyses indicate that CPO in Apicomplexa is more closely related to CPO from fungi and animals than from plants and other photosynthetic organisms (15, 70). The final two enzymes of heme biosynthesis in Apicomplexa (PPO and FeCH) localize to the mitochondrion [Fig. 7; (99, 101, 160, 175)], indicating that the mitochondrion is the ultimate site of heme synthesis. A curious feature of PPO is that it does not utilize oxygen as an electron acceptor (compare Fig. 3), and instead may reduce coenzyme Q in the mitochondrial ETC (99).

The evolution of heme synthesis in apicomplexan parasites

The localization of enzymes for heme synthesis in Apicomplexa presents an unusual hybrid of the pathways found in photosynthetic and nonphotosynthetic organisms. To understand how such a biochemical mosaic could evolve, Koreny *et al.* examined heme biosynthesis in *Chromera velia* (70). *C. velia* is a single-celled, photosynthetic alga and is probably the sister group of the Apicomplexa (94). The plastid of *C. velia* and the apicoplast of Apicomplexa were derived from the same endosymbiotic event, likely from a red algal symbiont incorporated into the common ancestor of Apicomplexa and a related group of algae called dinoflagellates (58). Koreny *et al.* identified homologues for seven of the eight enzymes required for heme synthesis in *Chromera* (they did not identify PPO). They identified an ALAS homologue (*Cv*ALAS) and performed radiolabeling experiments with glycine and glutamate to demonstrate that only glycine could label into chlorophyll. This makes *C. velia* the only known photosynthetic eukaryote to synthesize chlorophyll using glycine as a direct metabolic precursor. *Cv*ALAS contains a putative N-terminal mitochondria-targeting peptide, so likely localizes to the mitochondrion as in other eukaryotes (Fig. 8C). However, all the other putative *C. velia* heme synthesis enzymes contain N-terminal leaders that resemble those found in apicoplast-targeting leaders of Apicomplexa, suggesting that the remainder of the *C. velia* heme (and chlorophyll) synthesis pathway occurs in the plastid (Fig. 8C).

To elucidate the evolutionary source of each enzyme, Koreny *et al.* performed extensive phylogenetic analyses, asking whether the *C. velia* and apicomplexan enzymes originate from the red alga that gave rise to the apicoplast, or from the proto-apicomplexan host cell. As expected (given that red algae lack an ALAS gene), *C. velia* and apicomplexan ALAS is derived from the host cell. Some of the apicoplast/plastid-localized enzymes—such as PBGS, PBGD, and UroS—likely derive from the red algal symbiont. Interestingly, Koreny *et al.* found that there were two or three paralogues of UroD, CPO, and FeCH in *C. velia*, all of which contained plastid-targeting leader sequences. In the case of UroD and CPO, one paralogue is clearly descended from the algal symbiont, whereas the paralogue that is also found in Apicomplexa is likely derived from the host cell (Fig. 8C). The situation is slightly more complex for FeCH, where one paralogue is clearly derived from the symbiont whereas the paralogue that is also found in Apicomplexa may have derived from a proteobacterium through lateral gene transfer either before or after symbiont acquisition. Curiously, apicomplexan PPO, which localizes to the mitochondrion of both *P. falciparum* and *T. gondii*, appears to derive from the red algal symbiont (Fig. 8D).

So how did the hybrid heme pathway of Apicomplexa and *Chromera* evolve? When the red algal symbiont was first acquired, the two tetrapyrrole pathways likely co-existed, as still occurs in *Euglena* (69). One pathway likely supplied heme to the mitochondrion, and the other supplied chlorophyll and heme to the plastid (Fig. 8A). The first step in the evolution toward the hybrid heme pathway of Apicomplexa was the loss of the plastid-based ALA synthesis pathway, with mitochondrially localized ALAS taking over supplying ALA to both the mitochondrion/cytosol and plastid tetrapyrrole synthesis pathways (Fig. 8B). The evolutionary rationale for losing the plastid ALA synthesis enzymes (GluTR and GSA-AT) is not clear. When the symbiosis that led to the apicoplast first evolved, the ALAS gene would have been encoded in the host cell nucleus, whereas GluTR and GSA-AT would have been encoded in the nucleus of the red algal symbiont. ALA synthesis is the major rate-limiting (and therefore regulatory) step of tetrapyrrole biosynthesis in both animals and plants (see section on Regulating Heme Metabolism). One intriguing possibility is that making the symbiont dependent on host-derived ALA enabled the host cell to control photosynthesis in its recently enslaved symbiont. A simpler explanation is that redundancy in ALA synthesis pathways was rationalized without reduction in evolutionary fitness. Regardless of why it occurred, the loss of GluTR and GSA-AT requires the evolution of a mechanism to transport ALA from the mitochondrion to the apicoplast. PBGS (which uses ALA as a substrate) probably resides in the stroma (lumen) of the apicoplast, meaning that ALA must be exported across the two membranes of the mitochondrion and then imported across the four membranes bounding the apicoplast. A mechanism was already in place to export ALA from the mitochondrion, so this was no obstacle to the loss of GluTR and GSA-AT (Fig. 8B). But the conjoining of these pathways would have required novel apicoplast-localized transporters capable of importing ALA (160). The exchange of ALA between the mitochondrion and apicoplast may be facilitated by the close association of the apicoplast and mitochondrion in *P. falciparum* (52, 159), although such an intimate relationship between the organelles does not always occur in *T. gondii* (G.v.D., unpublished observations).

The fact that all remaining *C. velia* heme synthesis enzymes localize to the plastid suggests that the next step in the evolution of the hybrid heme pathway may have been the wholesale loss of the remaining cytosolic and mitochondrially localized enzymes (Fig. 8C). Interestingly, rather than being lost from the genome, some of these enzymes (UroD and

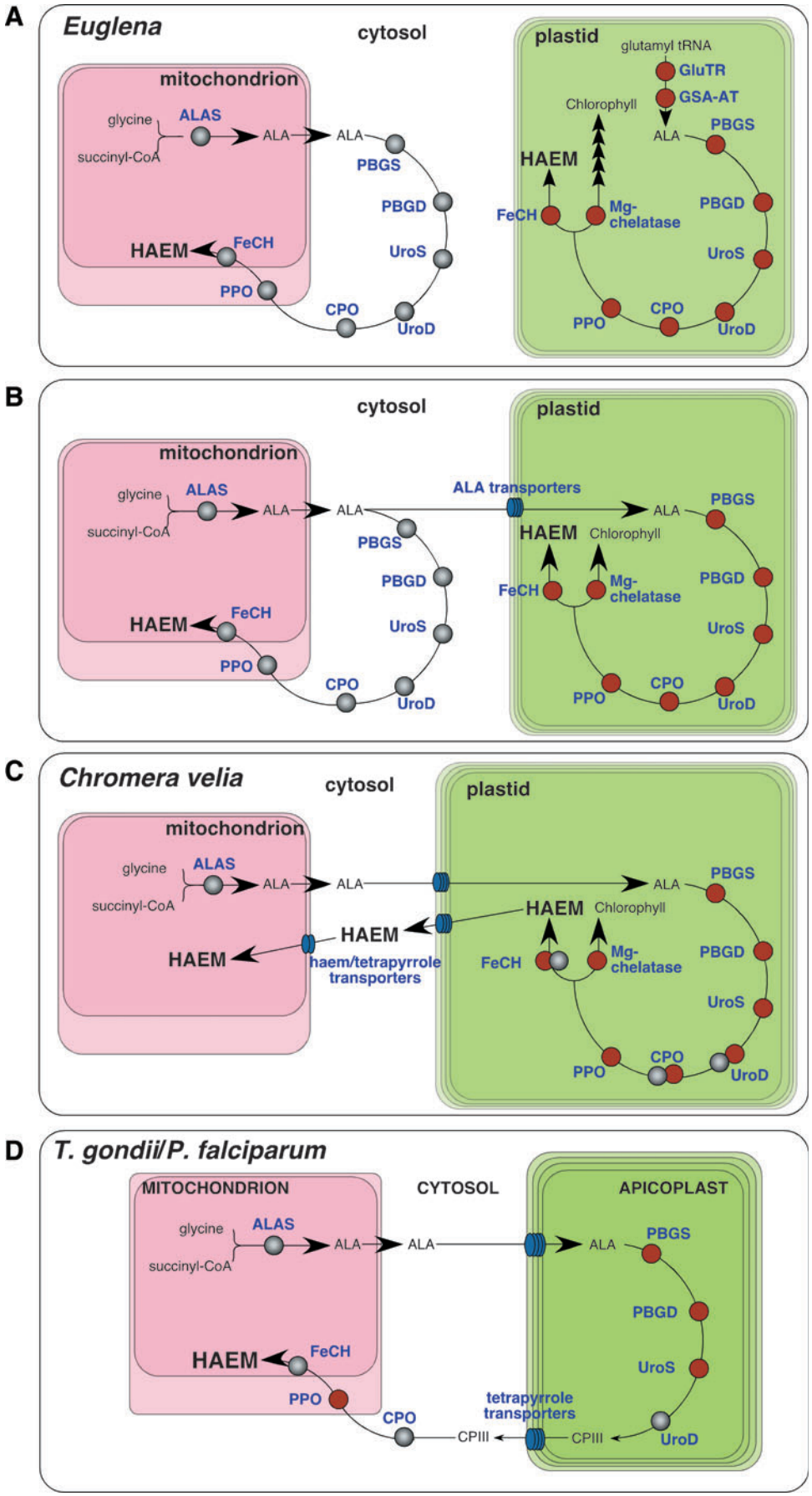

FIG. 8. A proposed scheme for the evolution of the heme biosynthesis pathway of Apicomplexa. (A) When the ancestor of Apicomplexa first evolved a plastid, both the mitochondrial/cytosol and plastid pathways of tetrapyrrole synthesis co-existed, a situation found today in *Euglena*. **(B)** The evolution of transporters that allowed ALA import into the plastid (*blue*) facilitated the loss of the plastid ALA synthesis enzymes, with ALA now provided solely by the mitochondrion. **(C)** Evolving transporters that export heme from the plastid (*blue*) facilitated the loss of the cytosolic and terminal mitochondrial steps of the pathway. This is seen in *Chromera velia* today, where all the steps beyond ALA synthesis likely occur in the plastid. Most of the plastid enzymes in *Chromera* were derived from the plastid-bearing symbiont (*orange*). Curiously, *C. velia* houses several enzymes that were likely acquired from the mitochondrial/cytosol pathway of the host cell (*gray*). **(D)** The loss of photosynthesis in Apicomplexa meant that tetrapyrroles were no longer required in the plastid. The terminal steps of heme biosynthesis then shifted back to the cytosol and mitochondrion, reflecting the pathway that exists in *P. falciparum* and *T. gondiii* today. The plastid (*orange*) and cytosolic (*gray*) ancestry of heme synthesis enzymes provides clues to the apparent shift in the localization of these enzymes during apicomplexan evolution (see text for details). To see this illustration in color the reader is referred to the Web version of this article at www.liebertonline.com/ars.

CPO) were retargeted to the plastid where they apparently coexist with the plastid-derived version, although the rationale for maintaining multiple paralogues of a particular enzyme is not clear (Fig. 8C). A likely reason for the loss of the remaining cytosolic and mitochondrial steps of the pathway was that, like in plants, the plastid of the photosynthetic apicomplexan ancestor had the greatest need for tetrapyrroles. Evolving a mechanism to export already abundant heme from the plastid and import heme into the mitochondrion (Fig. 8C) would have made the cytosolic/mitochondrial heme pathway redundant.

The catalyst for the final stage in the evolution of the hybrid heme pathway was the loss of photosynthesis. With tetrapyrroles no longer required in the apicoplast, the mitochondrion became the major site of heme usage. Likely, the final three enzymes in the pathway were retargeted to the cytosol and mitochondrion to enable heme synthesis in the mitochondrion. In this scenario, CPIII represents the final product of the apicoplast steps of heme synthesis (Fig. 8D). A means of exporting CPIII from the apicoplast must have evolved to enable the final stages of heme pathway evolution. Given its structural similarity to heme, it is possible that the transporters that evolved to export heme from the apicoplast in the previous step could also transport CPIII.

An alternative scenario for the final stage of heme pathway evolution posits that the *C. velia* pathway represents a derived rather than an intermediate stage. In this scenario, the stage represented in Figure 8B featuring separate cytosolic/mitochondrion and plastid-localized pathways was maintained until after the divergence of *Chromera* from the apicomplexan lineage. At some point in the apicomplexan lineage, the first three cytosolic enzymes of the heme pathway became redundant and were lost, possibly as a result of the evolution of plastid transporters that could export CPIII from the organelle. The subsequent loss of photosynthesis in Apicomplexa meant that the final enzymes in the plastid pathway were no longer required, leading directly to the pathway seen in Figure 8D (160). It is unclear how long both pathways for tetrapyrrole synthesis co-existed in the apicomplexan/chromerid lineage. Intriguingly, Perkinsids, a nonphotosynthetic sister group to Apicomplexa and chromerids that nevertheless retain a plastid, may have lost the plastid pathway for tetrapyrrole synthesis (36, 70). Little is known about the localization of tetrapyrrole synthesis pathways in dinoflagellates, a photosynthetic sister group of Apicomplexa and chromerids, but some tetrapyrrole synthesis enzymes in these algae retain plastid signatures (70). Establishing the route of tetrapyrrole biosynthesis in dinoflagellates, perkinsids, and other early diverging sister groups of Apicomplexa likely holds the keys to unraveling the complex evolutionary steps that led to the "hybrid" pathway observed today.

Heme scavenging in apicomplexan parasites

Surprisingly, there are numerous Apicomplexa that lack the genes required for heme biosynthesis. These include *Theileria*, *Babesia*, and *Cryptosporidium*, all of which contain enzymes that require heme [Table 1; (79)]. These organisms must therefore have an alternative source for heme. Organisms lacking heme synthesis pathways are not uncommon, and include numerous bacteria, trypanosomatid parasites, and the nematode worm *C. elegans* (5, 68, 121). These organisms obtain their heme through numerous scavenging mechanisms. Gram-negative bacteria contain receptors on their outer membrane that bind heme or heme-containing proteins and traffic them into the periplasmic space (5, 152). Heme is then transported across the inner membrane, often *via* an ABC transporter. Heme acquisition in *C. elegans* involves the HRG proteins mentioned earlier (119).

Trypanosomatids are single-celled eukaryotic parasites that include the agents of African sleeping sickness (*Trypanosoma brucei*), Chagas disease (*Trypanosoma cruzi*), and leishmaniasis (*Leishmania* species). Much like *Plasmodium*, trypanosomatids have both insect and mammalian life stages. The mechanisms for heme acquisition in trypanosomatids are not well understood. *In vitro* growth of trypanosomatids requires the addition of heme to the growth medium (19), suggesting that these parasites can scavenge heme from their host organisms. Uptake of fluorescent heme analogues in *T. cruzi* is reduced in the presence of ABC transporter inhibitors, suggesting a role for these transporters in heme scavenging (77). ABC transporters may also have a role in heme scavenging in *Leishmania*, with mutants in one ABC transporter inhibiting the scavenging of heme bound to hemoglobin (16). Bloodstream forms of *T. brucei* express a receptor protein on their plasma membrane that binds to hemoglobin-bound heme in the form of a haptoglobin/hemoglobin complex, and mediates the internalization of this complex by endocytosis (162). Notably, this receptor may also be the entry point into the parasite of trypanosome lytic factor (TLF), a high-density lipoprotein that contains haptoglobin-related protein bound to hemoglobin. TLF serves as a critical innate immune response that targets certain strains of *T. brucei*.

So how might *Babesia*, *Theileria*, and *Cryptosporidium* acquire heme from their host? Stages of the *Babesia* and *Theileria* lifecycles occur within heme-rich erythrocytes. Unlike *Plasmodium*, *Babesia* and *Theileria* do not endocytose vast quantities of hemoglobin from erythrocytes, so another mechanism of heme internalization might exist. Another life stage of *Theileria* occurs within the cytosol of leukocytes, where the parasite may have access to heme synthesized by the host cell, although it would presumably require plasma membrane transporters to do so. *Cryptosporidium* lives in an unusual extracytoplasmic compartment of gut epithelial cells. With very limited *de novo* metabolic capabilities, *Cryptosporidium* is adept at scavenging molecules such as sugars and nucleotides from its host (1). It is possible that *Cryptosporidium* can also scavenge heme, although with only one apparent heme-requiring protein (Table 1), the parasite's need for heme might be minimal. Clearly, there is still much to learn about how Apicomplexa lacking a heme synthesis pathway acquire their heme.

Alternative mechanisms of heme acquisition in apicomplexan parasites

Inhibitors of cytochrome *b* such as atovaquone stop growth of erythrocytic and liver stages of *Plasmodium* as well as tachyzoite and tissue cyst stages of *T. gondii* (6, 103). This indicates that heme is required for parasite survival at various stages of the lifecycle in both *P. falciparum* and *T. gondii*. But is parasite heme synthesis the main supplier of heme during these stages? Surprisingly, in blood stages of *P. falciparum*, the answer appears to be no. The apicoplast of *P. falciparum*

houses enzymes involved in several biosynthetic pathways beyond heme synthesis, including isoprenoid and fatty acid synthesis (120). Isoprenoid biosynthesis results in the formation of the molecule isopentenyl pyrophosphate (IPP) that is probably exported from the apicoplast and becomes an important component of several parasite molecules, including coenzyme Q, heme *a*, and dolichols (102). Derivatives of IPP also function in the prenylation of proteins such as Rabs to enable their association with cellular membranes. Fosmidomycin, a drug that targets IPP synthesis, kills blood-stage *P. falciparum* (59), suggesting that apicoplast IPP biosynthesis is essential for parasite survival in blood stages. In a recent study, Yeh and DeRisi supplemented the *in vitro* growth medium of blood-stage *P. falciparum* with exogenous IPP (176). They found that parasites were no longer sensitive to fosmidomycin, indicating that the addition of exogenous IPP overcame the requirement for isoprenoid synthesis in the apicoplast. Further, they found that treating parasites with inhibitors of apicoplast housekeeping processes results in the complete loss of apicoplasts from parasites, but that parasites lacking apicoplasts survived in the presence of IPP.

These experiments indicate that IPP synthesis is the only essential function of apicoplast metabolism during the erythrocytic cycle of *P. falciparum*. Genetic ablation of apicoplast fatty acid synthesis genes revealed that, indeed, this pathway is not essential in blood stages (164, 178). No gene knockout studies have yet been reported for heme synthesis genes, but, considering that apicoplast minus parasites are viable so long as they have a source of IPP, heme synthesis enzymes are very likely also not essential. So what is the alternative source for heme in blood stages of *P. falciparum*?

There is some evidence that the heme synthesis pathway of *Plasmodium* is not only built of parasite-encoded enzymes but also contains at least part of a parallel pathway constructed with the enzymes imported from the host erythrocytes (109). This so-called extrinsic heme synthesis pathway invokes the import of several heme biosynthesis proteins of the erythrocyte host cell into the parasite (11, 12). These enzymes then function in parasite heme synthesis. The best characterized of these imported enzymes is host cell PBGS, but also include FeCH, CPO, and possibly others (11, 12). Host cell ALAS is apparently not imported into the parasite. It remains unclear exactly how host cell enzymes are imported into the parasites, or how these enzymes are integrated into the heme synthesis pathway (*e.g.*, is host cell FeCH imported into the mitochondrion, and if so, how?). There has been no direct test of the extrinsic pathway hypothesis for over a decade. One obvious way of testing the hypothesis is to generate genetic knockouts of parasite heme pathway enzymes, such as PBGS and FeCH, and determine whether mutants can still generate heme from precursors such as glycine or ALA. If so, then the extrinsic pathway likely has a role in parasite heme synthesis. If not, then likely the extrinsic pathway has no role.

If imported host heme synthesis enzymes do not have a role in parasite heme synthesis in *Plasmodium* blood stages, and the parasite heme synthesis pathway is not essential for parasite growth, where do these parasites obtain their heme? One possibility is that, like in *Theileria*, *Babesia*, and possibly *Cryptosporidium*, *Plasmodium* blood stages are able to scavenge heme from their hosts. There are three possible routes of heme uptake in *Plasmodium*. The vast majority of heme released from hemoglobin in the parasite digestive vacuole is polymerized as hemozoin (31). Nevertheless, the levels of heme taken into the digestive vacuole should be more than sufficient for parasite heme requirements. If parasites scavenge only minor proportion of heme released from hemoglobin digestion, then this may be enough to meet their requirements. Free heme also occurs in erythrocytes in concentrations of $\sim 1\,\mu M$ (84). Heme could therefore be scavenged directly from the host cell by importing free heme through membrane receptors and transporters. A final route of entry could be heme derived directly from serum in the short time period between parasite egress from one host cell and the invasion of another. Intriguingly, a fluorescent analogue of heme (zinc-protoporphyrin IX) can be rapidly transported into the parasite cytosol in intraerythrocytic parasites (128), supporting the model of direct heme scavenging from the erythrocyte.

How might *Plasmodium* parasites scavenge heme from their hosts? Scavenging will likely involve membrane transporters. One requirement of these transporters is that they localize to the membrane where scavenging occurs, likely the parasite plasma membrane or digestive vacuole membrane. ABC transporters, which have a role in heme and tetrapyrrole import in a range of bacteria, mammals, and trypanosomatids (5, 16, 72, 73, 77), are strong candidates. The *P. falciparum* genome encodes $\sim$16 ABC-domain-containing proteins. Little is known about the native functions of these proteins. Curiously, one ABC transporter (*Pf*MDR1) localizes to the digestive vacuole membrane and has a role in mediating parasite resistance to certain drugs (23). A second ABC transporter (*Pf*MDR2) localizes, at least partly, to the plasma membrane, although this protein has been implicated in efflux of heavy metals from the parasite (125, 126). Another potential mechanism for scavenging heme is through exporting hemophore proteins that bind to free heme, as occurs in some bacteria (5). Receptors on the parasite surface may then bind to the hemophore and mediate heme transport into the parasite. No hemophores or hemophore receptors have been described in *Plasmodium*. Intriguingly, the heme-binding protein HRP2 is secreted into the host cell cytosol (2, 53) where it could conceivably function as a hemophore.

Determining the role of heme synthesis and scavenging in Apicomplexa that live inside nucleated host cells, such as *T. gondii* and liver-stage *Plasmodium* parasites, will also be particularly interesting. These parasites live inside host cells that have an active heme synthesis pathway, and parasites may be able to scavenge heme from these host cells. By analogy, intracellular stages of the *T. cruzi* and *Leishmania* lifecycles as well as some intracellular bacteria [such as the aphid symbiont *Buchnera*; (112)] live in nucleated cells and lack a complete heme synthesis pathway. These organisms likely scavenge heme from their hosts. *Mycobacterium tuberculosis* is a bacterium that lives inside macrophages. *M. tuberculosis* has its own heme synthesis pathway, but can also scavenge heme from its host using a hemophore-based mechanism (155). It is certainly possible that Apicomplexa that live inside nucleated cells can similarly scavenge heme. Genetic knockout and biochemical labeling experiments of parasites at these life stages will directly address these possibilities.

Regulating Heme Metabolism

The cytotoxic effects of free heme mean that organisms with an active heme synthesis pathway must tightly regulate this

pathway to obtain a balance between generating enough required heme without generating harmful levels. Regulation of heme biosynthesis is well characterized in both animals and plants. In both cases, a major regulatory step of the pathway is the synthesis of ALA (89, 150). Regulation of mammalian ALAS is complex and occurs at multiple levels, likely reflecting the importance of this step. Mammals express two isoforms of ALAS—one is expressed exclusively in erythroid cells, where there is a large need of heme, and a second is expressed in all other cells. Both ALAS isoforms are regulated at a transcriptional level. In addition, mRNA of the erythroid form of ALAS contains an iron responsive element, a stem-loop structure that is bound by iron regulatory protein (IRP) in iron limiting conditions (90). When bound, IRP inhibits ALAS translation, meaning that heme synthesis is down-regulated in iron-poor conditions. Another unique ALAS regulation mechanism occurs during protein import into the mitochondrion. The N-terminal targeting leader of ALAS contains multiple cysteine-proline (CP) motifs that bind heme (78). When heme is bound, the targeting peptide of ALAS adopts a secondary structure that prevents its import into the mitochondrion. Thus, high levels of heme inhibit the initiation of further heme synthesis by keeping the enzyme away from its substrates. In mammalian cells, mRNA and protein turnover of ALAS is very rapid. Depending on organism and cell type, mRNA and protein half-life can both be as little as 1 h (60). Such a fast turnover enables heme synthesis to adjust rapidly in response to other regulatory mechanisms. Further, increased cellular heme levels can markedly decrease the half-life of both ALAS mRNA transcripts and protein (30, 151).

In plants, ALA synthesis is primarily regulated through GluTR activity. Since plants must control both heme and chlorophyll syntheses, GluTR activity can be regulated directly by heme, as well as by other mechanisms that apparently respond to chlorophyll precursor levels (149, 150). Although ALA production is a critical step in tetrapyrrole regulation in both plants and animals, it is important to note that there are several other points of regulation in the synthesis pathway. In addition to regulating tetrapyrrole synthesis, plants must also respond to the metabolic state of their plastids. Tetrapyrroles exported from plastids are critical retrograde signaling molecules in plants, where they regulate the expression of numerous genes in the plant nucleus (144). The exact identity of the retrograde signaling molecule(s) involved has been controversial, with Mg-protoporphyrin and heme both proposed as candidates (96, 144, 174).

Little is known about regulation of heme synthesis in Apicomplexa. These parasites often have complex lifecycles and requirements for heme probably vary substantially between different stages. This is particularly true for parasites such as *Plasmodium* that have life stages in both heme-rich and heme-poor niches. A study of ALAS and PBGS enzyme activity in blood stages of the simian malaria parasite *Plasmodium knowlesi* found that heme could partly inhibit activity of both enzymes (142), suggesting a possible direct means of regulating heme synthesis. Curiously, the mitochondrial targeting peptide of ALAS in both *T. gondii* and *P. falciparum* contains CP motifs that may bind heme (70). Indeed, *in vitro* import of *P. falciparum* ALAS into heterologous mitochondria can be inhibited by the addition of heme (163), suggesting that this unusual mechanism of ALAS regulation is conserved across a broad swath of eukaryotes. Nothing is known about the possible role of heme as a signaling molecule in Apicomplexa. It is perhaps surprising that *Plasmodium* blood-stage parasites still actively synthesize heme when there is an apparent alternative means of heme acquisition that should be more than sufficient to meet the requirements of the parasite (147, 176). It will be intriguing to determine whether there is cross-talk between heme synthesis, heme scavenging, and heme detoxification pathways in blood-stage *Plasmodium* parasites, all of which must be carefully regulated to ensure a sufficient supply of heme while at the same time negating potential toxic effects of this molecule.

Conclusions

Heme metabolism in apicomplexan parasites is complex, varied, and, in many aspects, understudied. Parasites utilize different mechanisms to acquire heme, both within and between different life stages. Further, there is surprising diversity among Apicomplexa in their means of acquiring heme. Drugs that target heme proteins such as cytochrome *b*, and drugs that target heme detoxification, are potent inhibitors of growth in numerous parasite species, indicating that heme metabolism is critical for parasite survival. Yet there are many aspects of heme detoxification, heme synthesis, heme scavenging, and heme usage in these parasites that are totally unknown. A better understanding of these processes, therefore, offers the potential to develop much needed therapeutic interventions against some of the most potent parasites on earth.

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Abbreviations Used

$\Delta\Psi$ = membrane potential
ABC transporter = ATP-binding cassette transporter
ALA = δ-aminolevulinic acid
ALAS = δ-aminolevulinic acid synthase
ATP = adenosine triphosphate
CCHL = cytochrome c/c_1 heme lyase
CP = cysteine-proline
CPIII = coproporphyrinogen III
CPO = coproporphyrinogen III oxidase
C-X-X-C-H motif = cysteine-X-X-cysteine-histidine amino acid motif
DHOD = dihydroorotate dehydrogenase
ETC = electron transport chain
FAD = flavin adenine dinucleotide
FeCH = ferrochelatase
GluTR = glutamyl-tRNA reductase
GSA-AT = glutamate semialdehyde aminotransferase
HCP1 = heme carrier protein 1
HDP = heme detoxification protein
HO = heme oxygenase
HRG = heme responsive gene
HRP = histidine-rich protein
IPP = isopentenyl pyrophosphate
IRP = iron regulatory protein
mRNA = messenger ribonucleic acid
NADH = nicotinamine adenine dinucleotide
NADPH = nicotinamine adenine dinucleotide phosphate
PBGD = porphobilinogen deaminase
PBGS = porphobilinogen synthase
PPO = protoporphyrinogen IX oxidase
TLF = trypanosome lytic factor
TNF-α = tumor necrosis factor-α
UroD = uroporphyrinogen III decarboxylase
UroS = uroporphyrinogen III synthase