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Unique Properties of Apicomplexan Mitochondria

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Abstract

Apicomplexan parasites constitute more than 6,000 species infecting a wide range of hosts. These include important pathogens such as those causing malaria and toxoplasmosis. Their evolutionary emergence coincided with the dawn of animals. Mitochondrial genomes of apicomplexan parasites have undergone dramatic reduction in their coding capacity, with genes for only three proteins and ribosomal RNA genes present in scrambled fragments originating from both strands. Different branches of the apicomplexans have undergone rearrangements of these genes, with *Toxoplasma* having massive variations in gene arrangements spread over multiple copies. The vast evolutionary distance between the parasite and the host mitochondria has been exploited for the development of antiparasitic drugs, especially those used to treat malaria, wherein inhibition of the parasite mitochondrial respiratory chain is selectively targeted with little toxicity to the host mitochondria. We describe additional unique characteristics of the parasite mitochondria that are being investigated and provide greater insights into these deep-branching eukaryotic pathogens.

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INTRODUCTION

The emergence of eukaryotes from an exclusively prokaryotic biosphere was a momentous event that charted a divergent course for life on Earth (82). This event coincided with the syntrophic adoption of mitochondria. The complexity of eukaryotes could not have existed and evolved but for the energy economy supported by mitochondrial physiology (64). Given the strong evidence for the monophyletic origin of mitochondria (37–39), it is probable that all extant mitochondria can trace their origin to that singular primordial event at the dawn of eukaryotes. Yet, evolution has resulted in a tremendous variety of mitochondria, their genomes, their regulation, and their functions, which are as diverse as the organisms they reside in, from *Paramecium* to the Pope. Each clade of eukaryotes appears to adjust its mitochondrial function to fit the niche in which it exists (39). Apicomplexan parasites demonstrate these evolutionary adjustments in an illuminating manner. These obligatory intracellular parasites consist of thousands of species, all making their living off a vast array of host animals (2, 3). Even corals, the deepest-branching animals, have intracellular apicomplexans, suggesting that the parasitic nature of apicomplexans is as ancient as the origin of Metazoa (61, 62).

The discovery of mitochondrial DNA (mtDNA) in malaria parasites as tandemly arrayed molecules with a unit length of 6 kb (51, 120, 125–127) led to the realization that a separate circular DNA of 35 kb in the parasite, then believed to be an mtDNA (34, 135), was in fact a remnant of a chloroplast genome residing in a separate organelle, now termed an apicoplast (for apicomplexan plastid). The apicoplast consists of a four-membrane structure and thus is proposed to have originated from a secondary endosymbiotic event common to all apicomplexans, in which an algal organism was engulfed by the progenitor of both the dinoflagellates and apicomplexans (56, 57). Most extant apicomplexans have three distinct genomes: the nuclear, the mitochondrial, and the apicoplast. However, the proposed secondary endosymbiotic event would have initially involved coexistence of five genomes—the progenitor's mitochondrial and nuclear DNAs plus the algal plastid, mitochondrial, and nuclear DNAs—before being reduced to three (**Figure 1**). The massive quantity of gene transfers and reductions that followed has resulted in apicomplexan genomes carrying a mixture of genes inherited from five distinct genomes (**Figure 1**). This complex provenance of apicomplexans, as well as their natural selection to fit a tremendous variety of parasitic niches, has resulted in many biological characteristics that are distinct from those of most model eukaryotes. In this review, we aim to describe such distinct aspects of the mitochondrion in apicomplexan parasites. Our goal is not to provide a comprehensive view of mitochondria in all apicomplexans but to describe their unique properties. We refer the reader to several excellent reviews that cover additional details of these organelles (11, 41–43, 79, 85, 124, 128).

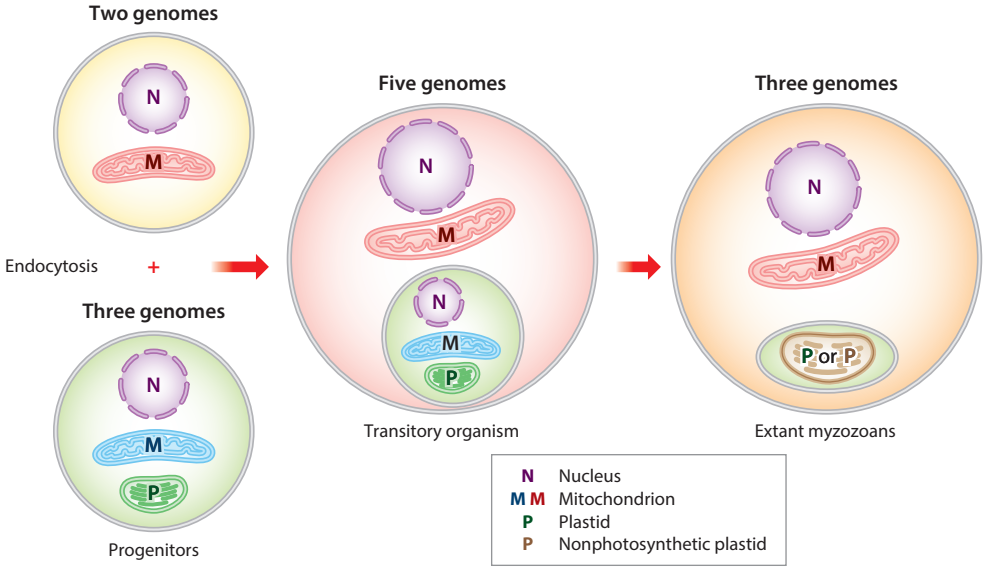

Figure 1

Schematic representation of the secondary endosymbiotic event involving the progenitor of myzozoans with two genomes and an alga with three genomes. Engulfment of the alga by the progenitor of Myzozoa resulted in a transitory organism with five genomes. Massive gene transfers and losses led to the elimination of the algal nucleus and mitochondrion, thus resulting in the three genomes seen in extant Myzozoa, which remain a conglomerate of five genomes.

DOWNSIZING AND DIVERSIFICATION

Apicomplexans belong to the superphylum Alveolata, which diverged approximately 850 million years ago into two main branches: ciliates and myzozoans. Whereas the progenitor of alveolates likely contained an mtDNA of around 50 kb coding for 40 or so genes, and most ciliates continue to have a similar-sized mtDNA, the myzozoans underwent a drastic reduction in their capacity and now code for only three proteins [cytochrome *c* oxidase subunits 1 and 3 (Cox1 and Cox3) and cytochrome *b* (Cyt*b*)] and fragmentary ribosomal RNA (rRNA) pieces (86, 128). The divergence of Myzozoa coincided with the secondary endosymbiotic acquisition of plastids (11, 49). Among the three main branches of Myzozoa, two (dinoflagellates and chromerids) consist of mostly free-living species and have plastids with photosynthetic capacity, whereas members of the third (apicomplexans) contain nonphotosynthetic plastids and parasitize animals.

The large reduction in the number of genes encoded by mtDNA in Myzozoa, therefore, is unlikely to be due to their dependence on an external source for their energy needs (23). One possibility is that the progenitor of the Myzozoa, after acquiring a plastid, became more dependent on the photosynthetic supply of energy and less on the mitochondrial contribution. All through the radiation of the metazoan clade, the reduction in the gene content of their mtDNAs also appears to be accompanied by large-scale rearrangements of genes as well as of the configurations of the genomes (**Figure 2**). Even among the apicomplexans, the synteny of the genes is highly divergent and the configuration of the genomes varies among different genera, and sometimes even within the same genus (**Figure 2**). Unlike the three common protein-coding genes, rRNA gene fragments are highly variable among different apicomplexans. The evolutionary forces that must have driven this high degree of divergence are not clear, but they suggest that events that occurred at branch

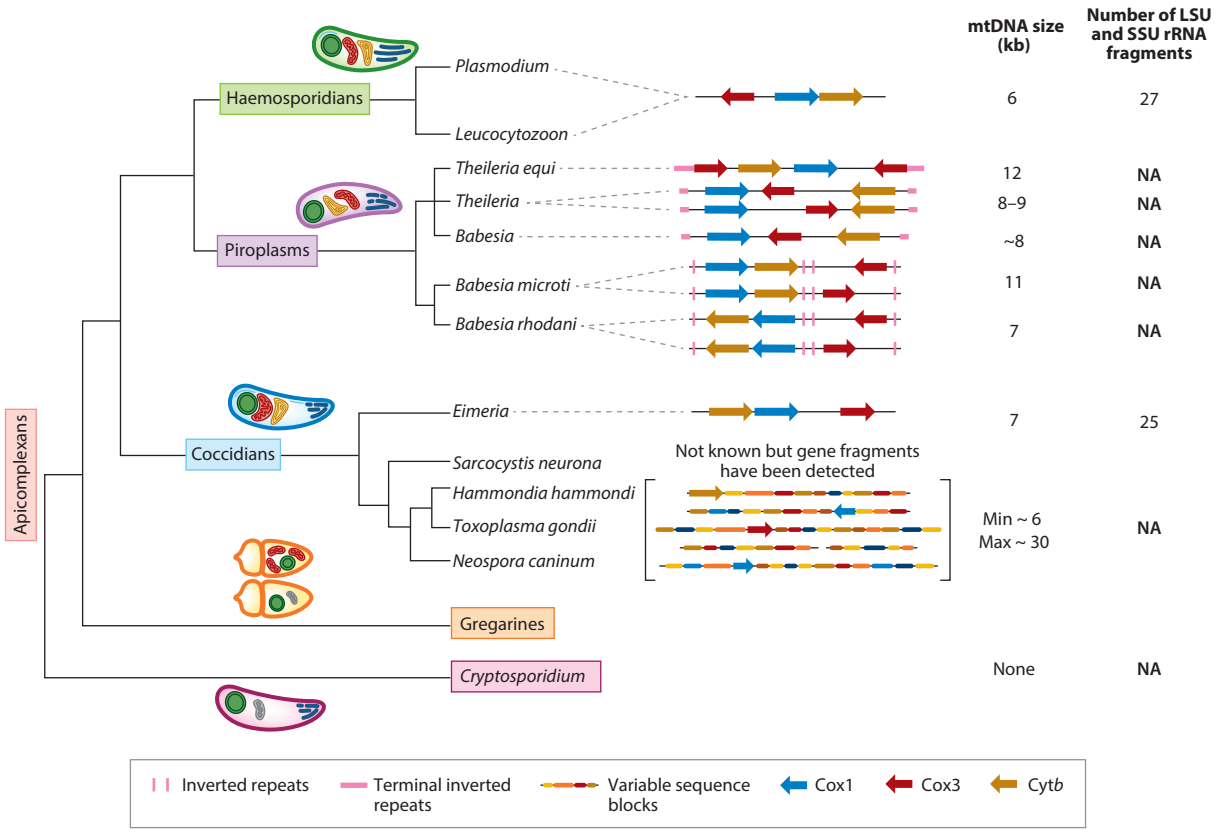

Figure 2

Phylogenetic tree illustrating the evolutionary relationships between the subclasses of apicomplexans (branch lengths are not drawn to scale). Arrows represent the synteny of protein-coding genes and their directionality in the apicomplexan mitochondrial genome of organisms in each subclass. The size of the mtDNA as well as the number of known fragmentary mitochondrial LSU and SSU rRNA transcripts in each subclass are indicated to the right of the tree. Abbreviations: LSU, large subunit; mtDNA, mitochondrial DNA; NA, not applicable; rRNA, ribosomal RNA; SSU, small subunit. Figure adapted with permission from References 11, 96, and 128.

points in the apicomplexan phylogenetic tree coincided with massive gene rearrangements within the mtDNA. Of interest is that the extent of divergence seen for the mtDNA is not observed in the apicoplast genomes of the extant apicomplexans (99, 129). This observation suggests differential evolutionary pressures on the two apicomplexan cytoplasmic genomes.

APICOMPLEXAN MITOCHONDRIAL GENOMES

All apicomplexan mtDNAs encode three proteins and rRNA fragments, but their sizes and gene arrangements vary greatly among different genera and, in some cases, within a genus (**Figure 2**). The mtDNAs range in size from 6 kb (e.g., *Plasmodium* spp.) to 12 kb (*Theileria equi*) (43). In general, apicomplexan mtDNAs are present as linear molecules, but in *Plasmodium* spp., they appear to form a few circular DNA molecules, which are likely to be molecules undergoing a rolling-circle mode of replication (105, 134). The tandem head-to-tail arrays with 6 kb unit length are the likely outcome of this mode of replication. Gene arrangements in *Plasmodium* spp. are highly conserved, with >90% sequence identity over an evolutionary distance among different species estimated to

span millions of years based on the divergence of nuclear DNA sequences (43). For instance, the overall G+C content of the nuclear DNA varies from ~21% in *Plasmodium falciparum* to ~34% in *Plasmodium vivax*, two malaria parasites infecting humans, but both their mtDNAs have ~31% G+C content (88). It is also remarkable that, while the codon usage frequency for the nuclear genes between these species is quite divergent, it remains identical for their mtDNA-encoded genes (88).

Compared with *Plasmodium* spp., *Theileria* and *Babesia* mtDNAs are divergent among different species. *T. equi* mtDNA is a 12 kb linear molecule with terminal repeats, while other *Theileria* spp. have an 8–9 kb mtDNA with genes arranged differently from those in the *T. equi* mtDNA. The size differences are also observed in *Babesia* species; *B. microti* has an 11 kb mtDNA compared with ~8 kb mtDNA in other species (43).

Toxoplasma gondii and related coccidial parasites, *Hammondia* and *Neospora*, contain the most diverse and dramatically rearranged mtDNA among all apicomplexans (10, 96). For many years, the authentic mtDNA sequence from *Toxoplasma* could not be established. One complicating reason was the presence of multiple sequences of what appeared to be portions of mtDNA interspersed throughout the nuclear genome of the parasite (101). The recent application of long-read DNA-sequencing technology has revealed a startling complexity in the mitochondrial genome of *T. gondii* (96). Multiple molecules ranging in size from 320 to 23,600 base pairs were detected. Further analysis of all the sequences revealed that these molecules were not linear concatemers of a unit molecule but did contain 26 distinct sequence blocks that were arranged in varying configurations. Overall, coding sequences for Cox1, Cox3, and Cyt*b* could be discerned but were arranged differently in different molecules (96). Fragments of rRNAs could also be detected, but attempts to assemble them into small subunit (SSU) and large subunit (LSU) rRNAs have not been carried out (96). It is a wonder that this complex arrangement of genes can result in the generation of mitochondrial complexes necessary for the survival of the organism. It is also interesting to note that the non-cyst-forming coccidial parasites of *Eimeria* species do not have this bizarre arrangement of their mtDNA but rather appear to consist of tandem arrays of 7 kb molecules (44, 71).

MITOCHONDRIAL rRNA

Since the emergence of mitochondria from their Alphaproteobacterial progenitor, mitochondrial rRNAs appear to have undergone dramatic changes (24, 29, 53). This is reflected in the highly divergent structures of mitoribosomes, particularly in the increase in the ratio of protein to RNA content, suggesting that the structural role of organization and scaffolding transferred from rRNAs to proteins in the mitoribosome (12, 106). Although the structure of the mitoribosome of an apicomplexan parasite has not been elucidated, analyses of mitoribosomes of unicellular organisms such as *Trypanosoma brucei*, *Tetrahymena thermophila*, and *Chlamydomonas reinhardtii* show a reduction in rRNA size and incorporation of new and enlarged mitoribosomal proteins. Mitoribosomal proteins form a shell that surrounds the rRNA core of the mitoribosome, stabilizing and orienting the critical regions of their fragmented rRNAs appropriately (106, 123, 132). With the much larger number of rRNA fragments encoded by apicomplexan mtDNA and the apparent absence of some of the conserved rRNA domains, the apicomplexan mitoribosomal structure, once determined, is likely to reveal many divergent and surprising features.

In contrast to most eukaryotes, in which mitochondrial rRNA subunits are encoded by continuous DNA sequences that are transcribed into long continuous polyribonucleotide chains, DNA sequences encoding mitochondrial rRNA in apicomplexans are fragmented and arranged in a scrambled manner originating from both strands of mtDNA (29, 125, 127). rRNA fragments have been identified in the mitochondria of all apicomplexans examined to date, including *Plasmodium*

spp. (28, 29, 125, 127), *Babesia* spp. (43, 45), *Theileria* spp. (45, 52), *Eimeria* spp. (44), and *T. gondii* (96). However, investigations of these mitochondrial rRNA fragments have been conducted mostly on *Plasmodium* species parasites (29). Just as the mtDNA of each apicomplexan varies in size and organization, these mitochondrial rRNA fragments also vary in size, arrangement, and length across various apicomplexans. *Eimeria* spp. and *Plasmodium* spp. have an mtDNA of ~6–7 kb in length. However, 14 LSU and 11 SSU rRNA fragments (69, 72) have been identified for most species of *Eimeria*, while 15 LSU and 12 SSU rRNA fragments have been identified for *P. falciparum* (29, 30). Furthermore, the DNA sequences encoding mitochondrial rRNA in *Plasmodium* spp. and *Theileria* spp. are arranged out of order and interspersed between protein-coding genes on either strand of the mtDNA (29, 30, 125, 127). The lengths of the mitochondrial rRNA fragments also differ vastly within each apicomplexan parasite (29, 72, 96).

Through both intra- and intermolecular complementary base pairing, rRNA fragments in apicomplexans can be predicted to form standard RNA secondary structures, such as hairpin loops, that are consistent with the structures formed by their continuous rRNA sequence counterparts (29, 50, 68, 125). Although these fragmented rRNAs are not linked covalently, their catalytic core is preserved, so they are likely to retain their function. Systematic transcript mapping of mitochondrial rRNA transcripts in *P. falciparum* demonstrated that some are conserved across apicomplexans and can be mapped to conserved sequences in the SSU and LSU rRNAs of *Escherichia coli* (29, 30). However, some portions of the rRNA in *E. coli* could not be identified among the pool of rRNA transcripts in *P. falciparum.* One possible reason is that these missing rRNA pieces may be imported from the cytoplasm, perhaps through the machinery required for transfer RNA import. It is also possible that they have been lost due to the evolutionary trend of reducing the size of mitoribosomal rRNA and transferring its function to mitoribosomal proteins (53, 132).

The process by which rRNA fragments are processed and assembled with ribosomal proteins into a functional mitoribosome remains to be elucidated. The mitoribosome of *P. falciparum* is associated with the inner mitochondrial membrane, and the essentiality of the mitoribosome in *P. falciparum* and *T. gondii* has been demonstrated (54, 70, 113). Based on amino acid sequence similarity to mitoribosomal proteins of other organisms, 43 proteins have been predicted to be *P. falciparum* mitoribosomal proteins, 6 of which are essential for mitochondrial functions (25, 54). Recently, two proteins with RNA-binding domains that are abundant in apicomplexans, *Pf*RAP01 and *Pf*RAP21, were identified and characterized as essential nuclear-encoded proteins that are targeted to the mitochondria, where they specifically bind mitochondrial rRNAs in *P. falciparum* (46, 47). *Pf*RAP21 is involved in the control of mitochondrial rRNA expression, while both RAP proteins play a role in RNA processing, translation, and expression of mitoribosomal subunits (46, 47).

It is interesting that mitochondrial rRNAs in *P. falciparum* and *Theileria parva* possess oligo(A) tails up to 21 nucleotides in length, which are added posttranscriptionally to their 3′ end (29, 36, 97). The length of the oligo(A) tails is transcript-specific, so their addition might serve a protective role in preventing exonucleolytic degradation of these rRNA fragments to allow for proper assembly, structure, and function of the mitoribosome. However, the roles of oligoadenylation of specific mitochondrial rRNA transcripts in apicomplexans remain uncertain. Several investigations have revealed that the genes encoding the 16S and 12S rRNA in humans encode small open reading frames (ORFs), which are translated into mitochondrial-derived peptides that are involved in important cellular processes (66, 67, 90, 139). These peptides, namely Humanin, small humanin-like peptides (SHLPs), and mitochondrial ORF of the 12 S rRNA-type c (MOTS-c), have significant physiological roles and demonstrate that mitochondrial rRNA could encode peptides with important functions. Although mitochondrial rRNA–encoded peptides have not been identified in apicomplexan parasites, a possible peptide-encoding function of small rRNA fragments could be

an important topic for exploration. In addition, researchers have observed several small mitochondrial RNA transcripts in *P. falciparum* with no homology to known rRNA sequences and no known function (29). It would be interesting to assess the potential presence of small ORFs within these RNA molecules that may have the capacity to encode peptides.

MITOCHONDRIAL PROTEOMES IN APICOMPLEXANS

Except for the three mitochondrial electron transport chain (mtETC) subunits encoded by the mtDNA, all other mitochondrial proteins are encoded by the nucleus and imported into the mitochondrion. Various approaches have been used to generate a predicted compilation of mitochondrial proteomes in *P. falciparum* and *T. gondii*. Bioinformatic (18) and manual (17) annotations initially generated a list of approximately 400 proteins likely to be imported into the *Plasmodium* mitochondrion. Since the mitochondrion contains multiple subcompartments with multiple import mechanisms, comprehensive lists are inevitably difficult to complete. Manual curation suggests a proteome size of approximately 500 proteins associated with the *P. falciparum* mitochondrion; the *T. gondii* proteome may be slightly larger, given its more complex metabolism. Experimental validations of the predicted mitochondrial proteome have been limited but are gradually accumulating. An approach consisting of mitochondrially targeted proximity biotinylation followed by enrichment and mass spectrometry was used to identify uncharacterized *P. falciparum* proteins putatively targeted to the mitochondrion (63). This approach generated a list of 122 putative mitochondrial proteins, including many that were unannotated and likely to be essential for parasite survival (63). However, biotinylated proteins identified in this study also included apparently cytoplasmic proteins, thereby pointing to the limitations of this approach.

A study using mitochondrially targeted proximity biotinylation approaches provided proteomic evidence suggesting a unique architecture of apicomplexan respiratory chain complexes in *T. gondii* (111). In addition to five canonical subunits (Cox1, Cox2, Cox3, Cox5b, and Cox6b) of the cytochrome *c* oxidase (Complex IV), this analysis identified 11 apicomplexan-specific Complex IV subunits (111). Complexome profiling, in which label-free quantitative mass spectrometry is paired with microscale fractionation of large complexes in blue native gels, has the power to identify novel protein subunits that comigrate with such complexes (107, 136). This approach has recently been applied to *P. falciparum* mitochondria–enriched samples, leading to the identification of novel components of mitochondrial respiratory chain complexes (27). Orthologs of all 11 previously reported Complex IV subunits were identified in the *P. falciparum* complexome profile, confirming the unique Complex IV architecture in these organisms (27). Five uncharacterized, mostly myzozoan-specific, putative subunits also comigrated with Complex IV, which were termed respiratory chain Complex 4–associated proteins 1–5 (C4AP1–5). In most eukaryotes, the Complex IV subunit Cox2 is encoded by the mtDNA. Uniquely, in apicomplexans and other myzozoans, the gene is split into two nuclear-encoded genes, *Cox2a* and *Cox2b* (131). Both of the proteins encoding these genes were detected in the *P. falciparum* complexome profile, confirming their mitochondrial targeting (27).

Canonically, at least four subunit proteins compose succinate dehydrogenase (Complex II): succinate dehydrogenase subunit A (SDHA), SDHB, SDHC, and SDHD. Only SDHA and SDHB have been experimentally validated in *Plasmodium* (121, 122), but candidates for SDHC (PF3D7_0611100) and SDHD (PF3D7_1010300) have been proposed (91). In the *Plasmodium* respiratory chain complexome profile, SDHA and SDHB were observed to comigrate together in an ~530 kDa complex, but the previously proposed SDHC and SDHD proteins did not comigrate with this complex (27). However, the authors of this study identified five putative subunits that all shared a common dominant band and assigned one as a candidate for *Pf*SDHC (PF3D7_1448900).

This assignment was based on the conserved DY motif found in SDHC in many species (93). The other putative Complex II subunits were termed respiratory chain Complex 2–associated proteins 1–4 (C2AP1–4). One of these proteins, PF3D7_0808450, is restricted only to myzozoans and was previously reported as a mitochondrion-localized protein essential for malaria transmission (59).

Complexome profiling has also been carried out on *T. gondii* mitochondria (78). In this study, 60 proteins were identified and assigned to Complexes II and IV and the F_1F_o-ATP synthase (Complex V). Recent investigations of F_1F_o-ATP synthase complexes in apicomplexans are discussed in detail below. Of these 60 respiratory chain complex proteins, 16 had not been previously identified. This study also determined the composition of *T. gondii* Complex III, a known drug target. In doing so, the authors identified two new homologous subunits and two parasite-specific subunits. All four of these proteins were found to be essential for Complex III stability and parasite viability. In addition, depletion of these four subunits led to collapse of the mitochondrial membrane potential, consistent with their assignment as Complex III subunits (78). Similar to *P. falciparum*, the *T. gondii* Complex II subunit SDHB migrates as an ~500 kDa complex (78), a much larger size than observed in yeast and mammalian Complex II (~130 kDa) (109, 110). This size was confirmed through endogenous tagging of *Tg*SDHB (TGGT1_215280) and subsequent blue native PAGE (polyacrylamide gel electrophoresis) analysis, making it a larger Complex II than reported in any other well-studied organisms outside of apicomplexans. Seven other proteins comigrated with *Tg*SDHB, all of which are annotated as hypothetical proteins that bear no obvious protein features indicating function, highlighting the unique nature of Complex II in apicomplexans.

Nucleus-encoded mitochondrial matrix proteins often have N-terminal targeting peptides that are cleaved during import by mitochondrial processing peptidases (MPPs) (33, 60). In yeast and mammals, MPPs are heterodimers of subunits α and β, which are located in the mitochondrial matrix (33). In addition to these matrix MPPs, the cytochrome bc_1 complex (Complex III) in eukaryotes contains Core1 and Core2 subunits with high amino acid sequence homology to MPP subunits α and β (137). In contrast, plant mitochondria do not have matrix-resident MPPs. Therefore, it has been suggested that Complex III has two functions in plants: ubiquinol oxidation and processing of mitochondrially targeted proteins (15, 16). The *P. falciparum* genome encodes annotated α and β MPP subunits that were clearly shown to be part of Complex III (27). This finding suggests a unique aspect of the malaria parasite mitochondrion in which MPPs are part of Complex III as in plants, thus playing a role in its structural assembly. In addition to comigration of all canonical components of Complex III, four other proteins comigrated with the complex in the *P. falciparum* mitochondrial complexome profile (27). Three of these putative subunits are represented almost exclusively in Apicomplexa, lacking any obvious sequence homology with characterized subunits in other phyla.

Another spatial proteomics approach, called hyperplexed localization of organelle proteins by isotope tagging (hyperLOPIT), has been used to determine the steady-state subcellular localization of thousands of *T. gondii* proteins (9). The hyperLOPIT technique capitalizes on specific abundance-distribution contours that organelles and other subcellular structures form after being subjected to density-gradient centrifugation (21, 95). This study identified 193 mitochondrial membrane-associated proteins and 274 soluble mitochondrial proteins, further enriching the proteomic landscape of *Toxoplasma* mitochondria (9).

MITOCHONDRIAL CONTRIBUTIONS TO METABOLISM

Since apicomplexans are phylogenetically distant from mammals and other model organisms, it is not surprising that their mitochondria exhibit significant differences from those of model organisms. They are also a large phylum containing diverse parasitic species that exist in numerous

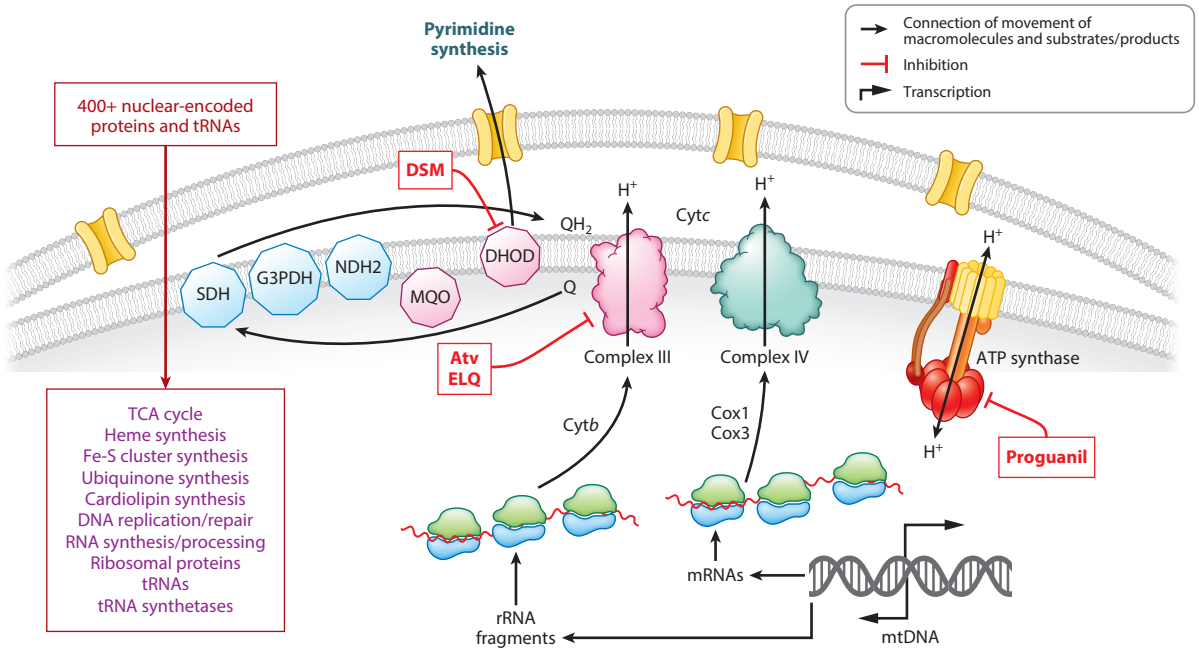

Figure 3

A schematic of mitochondrial functions in *Plasmodium falciparum* with associated drug targets. Dark red boxes depict the estimated 400-plus nuclear-encoded proteins targeted to the mitochondrion and their associated biological processes. Drugs that target respiratory chain proteins are shown in red. Reduced ubiquinone (QH_2) is generated by five dehydrogenases. Four of them (SDH, MQO, G3PDH, and NDH2) are not essential in blood stages, but DHOD is essential for its role in pyrimidine biosynthesis and is the target of DSM antimalarial compounds. QH_2 is oxidized by Complex III, a step that is inhibited by atovaquone, ELQs, and other inhibitors. Complex III transfers electrons to cytochrome *c*, which is then oxidized by Complex IV. Both Complexes III and IV pump H^+ across the inner membrane, generating protonmotive force. Only three components of these complexes are encoded by the mtDNA: Cyt*b*, Cox1, and Cox3. These are translated by unusual ribosomes assembled from fragmented rRNAs encoded by mtDNA and ribosomal proteins encoded on the nuclear genome and imported into the mitochondrion. ATP synthase does not seem to be a significant source of ATP in blood stages but can work in reverse by hydrolyzing ATP to pump H^+. This step is proposed to be targeted by proguanil, a component of the antimalarial drug Malarone. Abbreviations: Atv, atovaquone; Cyt*b*, cytochrome *b*; Cox1/3, cytochrome *c* oxidase 1/3; DHOD, dihydroorotate dehydrogenase; ELQs, endochin-like quinolones; G3PDH, glycerol-3-phosphate dehydrogenase; MQO, malate-quinone oxidoreductase; mtDNA, mitochondrial DNA; NDH2, type II NADH dehydrogenase; SDH, succinate dehydrogenase; TCA, tricarboxylic acid.

host species and environments. Therefore, there is a wide range of bioenergetic capabilities and metabolism associated with apicomplexan mitochondria and related organelles (86).

Expression of mitochondrial activities often varies significantly between parasite life cycle stages. Several metabolic pathways common in more familiar model organisms, such as β-oxidation of fatty acids and portions of amino acid and steroid biosynthesis, are not found in apicomplexan mitochondria. **Figure 3** schematically illustrates mitochondrial functions in *P. falciparum*, the parasite for which the most extensive information is available. Apicomplexan parasites of vertebrates, such as *Plasmodium* and *Toxoplasma*, have a nearly complete mtETC (lacking only Complex I) and an ATP synthase complex, providing robust oxidative phosphorylation, at least in some life stages. The metabolic partner of the oxidative phosphorylation ATP generation pathway is the oxidative tricarboxylic acid (TCA) cycle, which provides reducing equivalents to the mtETC.

In *Plasmodium* and *Toxoplasma*, a repurposed branched-chain α-keto acid dehydrogenase (BCKDH) complex in the mitochondrion, rather than a direct ortholog of the pyruvate

dehydrogenase found in model organisms, provides acetyl-CoA to the cycle (100), and a set of five ubiquinone-dependent dehydrogenases provide reducing equivalents to the mtETC. Among the dehydrogenases, only dihydroorotate dehydrogenase (DHODH) is essential in asexual blood stage *Plasmodium*, as a key enzyme in the pyrimidine biosynthesis pathway, and in *Toxoplasma*, as critical for virulence, even though the latter parasite has a salvage pathway for pyrimidines (31, 40, 102). In these parasites, 2-oxoglutarate (from glutamine), in addition to glycolytic pyruvate, is a major contributor to carbon flux through the TCA cycle (55, 80, 81). In *Toxoplasma*, the presence of a γ-aminobutyric acid shunt pathway allows for additional input to the TCA cycle of succinate derived from glutamine. Two of the TCA cycle enzymes are novel relative to those in more familiar organisms. Malate-quinone oxidoreductase is present in place of NADH-dependent malate dehydrogenase, which renders this step of the TCA cycle essentially irreversible. A class I fumarate hydratase containing an Fe-S cluster replaces the more familiar metal free class II fumarase, which renders the parasite enzyme sensitive to reactive oxygen species. The very low oxidation-reduction potential maintained in the parasite mitochondrion should, however, help protect Fe-S clusters in fumarate hydratase, aconitase, and other Fe-S-containing proteins (92).

Combined genetic and metabolomic experiments have found that acetyl-CoA from the *Plasmodium* mitochondrion is required for acetylation of histones and other cytoplasmic and nuclear proteins, which is provided by BCKDH, or by α-ketoglutarate dehydrogenase if BCKDH is ablated (22). *Plasmodium* spp., unlike animals and *Toxoplasma*, lack ATP-citrate lyase, which cleaves citrate exported from the mitochondrion to acetyl-CoA and oxaloacetate; for this reason, it is presently unclear how acetyl-CoA made in the *Plasmodium* mitochondrion reaches other compartments.

In the most virulent malaria parasite species, *P. falciparum*, expression of the enzymes of most mitochondrial pathways, including oxidative phosphorylation, is greatly reduced in the asexual blood stage, and the enzymes of the TCA cycle are dispensable (55). Nevertheless, the mtETC and ATP synthase complexes remain essential in this stage, as well as in the insect and liver stages. Expression of these complexes is greatly increased in the sexual blood stage gametocytes (27), although this stage is not fast growing and was initially thought to be less metabolically active. Stable isotope metabolomic experiments, however, suggest that gametocytes actually exhibit increased utilization of glucose with increased glycolytic flux directed through the TCA cycle (80). This increased expression of mitochondrial enzymes may occur in preparation for the increased oxidative phosphorylation activity needed in the following insect stages. In *Toxoplasma*, on the other hand, the fast-growing, invasive tachyzoite stage has relatively robust oxidative phosphorylation activity, but upon stress from the immune system or treatment with certain drugs, including inhibitors of the mtETC, *Toxoplasma* undergoes stage conversion to the slow-growing bradyzoite cyst form with decreased reliance on mitochondrial oxidative phosphorylation and TCA cycle activities (20, 103).

Many *Cryptosporidium* parasites of the mammalian lower intestine and gregarine parasites of invertebrates have far fewer mitochondria-related organelles, called mitosomes, that lack internal structure as well as a TCA cycle, mtETC complexes, and ATP synthase but do contain a small number of ubiquinone-dependent dehydrogenases, such as NADH dehydrogenase and malate-quinone oxidoreductase, and an ubiquinol-utilizing alternative oxidase (1, 73, 86, 138). It is not clear how such a reduced mitosome would energize its membrane to provide for protein import, which, at a minimum, is required to carry out the common essential function of all mitochondria and mitosomes (i.e., Fe-S cluster biogenesis). One possibility is a combination of external proton release concomitant with oxidation of ubiquinol and electrogenic exchange of ADP^{3-} for ATP^{4-} via the mitochondrial adenine nucleotide transporter.

MITOCHONDRIAL TARGETS FOR ANTIPARASITIC DRUGS

Early investigations using preparations enriched in mitochondria from malaria parasites showed that an antimalarial hydroxynaphthoquinone under development, subsequently named atovaquone, inhibited Complex III of the parasites (32). An examination of the sequence of *P. falciparum* Cyt*b*, the central subunit of Complex III, revealed subtle but significant differences in the ubiquinol oxidation (Q_o) and ubiquinone reduction (Q_i) sites of the parasite Cyt*b* compared with its host counterpart, which were proposed as the basis for selective toxicity of antimalarial drugs such as atovaquone (127). Atovaquone treatment also caused a collapse of the membrane potential across the inner membrane of parasite mitochondria (116). Subsequent studies revealed that mutations surrounding the Q_o site in the parasite Cyt*b* were responsible for resistance to atovaquone (58, 83, 87, 115). Structural studies using heterologous Complex III have supported the Q_o site as the area of interaction with atovaquone (13). Because Cyt*b* is largely conserved among mitochondriate apicomplexan parasites, atovaquone is also effective against parasites such as *Toxoplasma* and *Babesia* (4, 19).

While atovaquone is a potent drug, resistance to it arises rapidly when administered as a monotherapy (76). Therefore, a synergistic combination of atovaquone with a biguanide, proguanil, has been used for malaria prophylaxis and treatment (75, 77). The synergy between atovaquone and proguanil results from the ability of proguanil to inhibit a secondary pathway for generating membrane potential across the inner mitochondrial membrane in malaria parasites (117). Another series of compounds called endochin-like quinolones (ELQs) have been extensively investigated; one of them, ELQ-331, has been designated a clinical candidate (89, 98, 118). ELQ-331 is a prodrug that is converted to ELQ-300, which works at the Q_i site of the parasite Cyt*b* (89, 98).

Other ELQ compounds also inhibit the growth of *Toxoplasma* and *Babesia* (26, 65). Medicinal chemistry exploration of the ELQ compounds has revealed that subtle differences in their structure can result in compounds that target either the Q_i or Q_o site (118). A combination of ELQ-300 and atovaquone to treat rodent malaria was found to be highly effective without engendering the emergence of resistant parasites (119). Such a combination holds promise for a malaria treatment with a greatly reduced chance of succumbing to the development of drug resistance. Long-lasting implants of atovaquone or ELQ-331 in mice provided protection lasting months against experimental malaria (5, 114). Such a strategy has the potential for mass drug administration to control and eliminate malaria in endemic regions.

Plasmodium spp. are unable to salvage pyrimidines for their nucleic acid synthesis and must generate pyrimidines de novo (112). As mentioned above, an essential role of the parasite mtETC is to regenerate ubiquinone, which is used as the electron acceptor for mitochondrially localized DHODH, a critical enzyme in pyrimidine biosynthesis (102). Thus, DHODH is an attractive target for antimalarial drug discovery (8). Several compounds are potent inhibitors of parasite growth, and a few have undergone clinical trials (74, 104). As with atovaquone, resistance to DHODH inhibitors also arises rapidly (133) and thus will require partner drugs to mitigate this risk.

UNIQUE FEATURES OF APICOMPLEXAN ATP SYNTHASE

F-type ATP synthases are large rotary molecular machines that use electrochemical potential across a membrane to synthesize ATP, thus playing a central role in the energy economy of a wide range of organisms (14, 130). They are constituted from two subcomplexes: the soluble F_1 portion and the membrane-associated F_o portion. When the complete genome sequences of malaria parasites were assembled, genes encoding most of the F_1 subcomplex could be detected but, surprisingly, highly conserved and essential genes encoding the F_o subcomplex subunits, with the

exception of subunit c and OSCP, could not be detected through similarity searches (35). The absence of these conserved subunits was also noticed in other apicomplexan genomes (84). In *P. falciparum*, ATP synthase formed dimers, and genes encoding its subunit were refractory to disruption (7).

More recent research, however, revealed that the apicomplexan F_1F_o ATP synthase is highly divergent from its counterpart in other classes of organisms. Proteomic analyses of isolated ATP synthase from *T. gondii* carried out by two independent groups revealed the presence of multiple proteins in addition to the canonical F_1 subunits and subunit c (48, 108). Nearly all these newly observed proteins were conserved in other apicomplexan parasites as well as other myzozoans, except for *Cryptosporidium* and gregarine spp. that lack mtDNA (48, 108). Remarkably, these newly discovered ATP synthase subunits did not have apparent orthologous proteins in ciliates (6, 123). These studies indicate that the Myzozoa branch of the Alveolata clade possesses a central bioenergetic complex that had a divergent evolutionary provenance. These Myzozoa-specific subunits of the ATP synthase are divergent even from their closest sister clade of ciliates. These subunits might have originated from the ATP synthase that was part of the mitochondrion or the plastid belonging to the alga that was engulfed by the progenitor of myzozoans at the transitory stage (**Figure 1**). We acknowledge that there is no phylogenetic evidence supporting this proposition, but that could be due to an absence of sequence information from the algal progenitor. Chimeric origins of critical pathways are known to exist among unicellular parasites.

The unusual nature of the ATP synthase of these organisms became more apparent with the determination of a high-resolution cryogenic electron microscopic (cryo-EM) structure of the *T. gondii* complex (**Figure 4**) (94). The complex was observed both as dimers and as hexamers (trimers of dimers). There were 32 different proteins in each monomer, 15 of which could be discerned as structural equivalents of canonical subunits observed in ATP synthases of model organisms. Remarkably, 17 subunits were Apicomplexa-specific (identified as ATPTG1–17, for ATP synthase subunit from *T. gondii*). While 14 such ATPTG were observed in previous proteomic studies, 3 were identified directly from the cryo-EM structure (ATPTG1, ATPTG7, and ATPTG16). A gentler extraction revealed that the dimers come together to form trimers, thus constituting hexamers, which are more akin to their native configuration in the parasite mitochondria. In the *T. gondii* ATP synthase dimer, the angle between the monomers is a low 19°—very different from the 100° angle seen in yeast and mammalian complexes. Because dimerization of ATP synthase is responsible for inducing curvature in the inner mitochondrial membrane, this narrow angle in the *T. gondii* dimer would induce reduced curvature compared with the yeast and mammalian membranes.

Cryogenic electron tomography of mitochondrial membranes showed bulbous vesicles that were decorated with icosahedral arrangements of ATP synthase hexamers to form pentagonal pyramids localized to the regions of curvatures in the membranes, indicating that hexamer packing is likely the primary determinant of cristae morphology in *T. gondii*.

Overall, this research (94) constitutes the first structural elucidation of any mitochondrial complex from an apicomplexan parasite and reveals in remarkable detail the divergence and intricacies of a critical molecular machine. Further studies using cryo-EM and cryogenic electron tomography to investigate mitochondrial complexes and their arrangements in situ are likely to reveal further amazing details.

UNRESOLVED ASPECTS OF APICOMPLEXAN MITOCHONDRIA

The unusual features of apicomplexan mitochondria summarized above raise many additional questions. These involve the machinery used by the parasites to manage the separate genetic

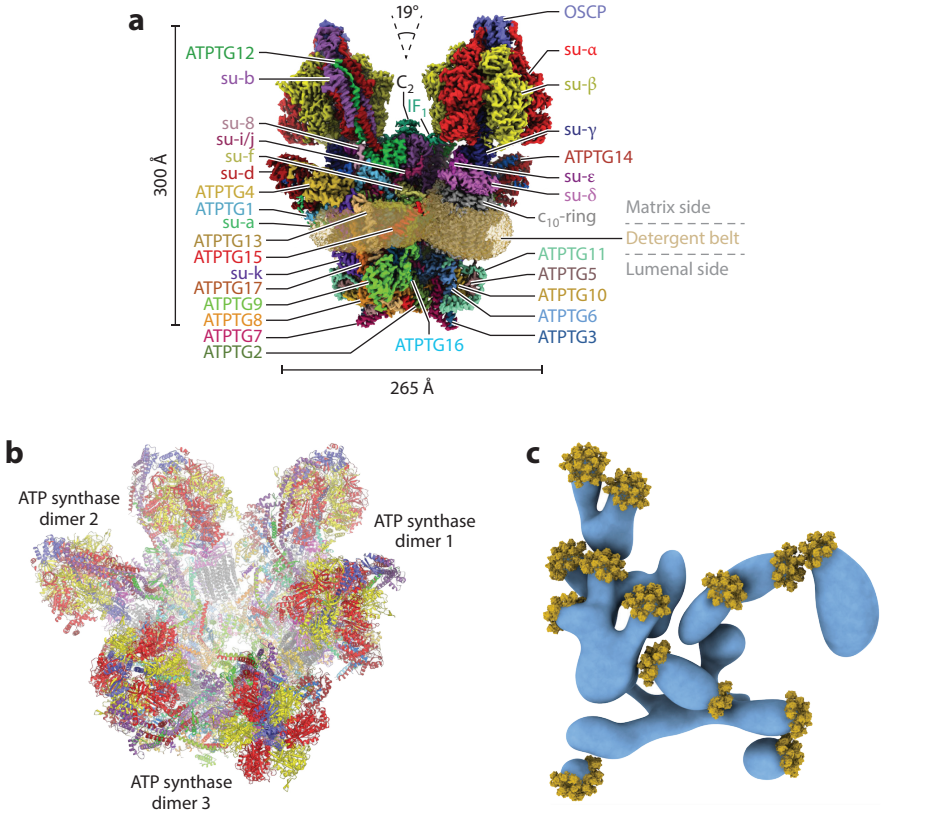

Figure 4

Cryogenic electron microscopic structure of the ATP synthase complex from *Toxoplasma gondii*. (*a*) A dimer of *T. gondii* ATP synthase. There are 32 subunit proteins in each monomer; 15 of them have structural equivalents in other ATP synthases and are identified with the prefix su-, and the other 17 (ATPTG1–ATPTG17) are unique to *T. gondii*. The monomers form a 19° angle relative to one another in the dimer, compared with the 100° angle seen in yeast and mammalian ATP synthase. (*b*) In the native state, *T. gondii* ATP synthases form hexamers (trimers of dimers). (*c*) The hexamers associate at the bulbous mitochondrial inner membranes in pentameric pyramids that impart the curvature to the cristae. Figure adapted with permission from Reference 94.

systems relegated to the mitochondria. The remarkable conservation of the mtDNA in *Plasmodium* spp. suggests a robust system of DNA replication, repair, and recombination. While some of the components of this system have been identified, many remain to be elucidated. For instance, drug resistance mutations that arise in the mtDNAs of both *Plasmodium* and *Toxoplasma* quicky achieve fixation in the multiple copies of the genome such that wild-type sequences are no longer detected; such mutations are also very stable, with no reversion to the wild type. These findings suggest a "copy-correction" machinery that remains unexplored.

Plasmodium mtDNA molecules in linear arrays with intermediates suggesting strand invasions were observed in an early study (105). This finding suggests extensive gene conversion events among the multicopy mtDNA molecules, requiring a variety of enzymes that would mediate this process. At this point, none of these mediators have been identified or investigated. Transcription regulation and processing of RNA encoded by mtDNA have also been underexplored. Approximately 40 distinct RNA molecules are generated from the 6 kb mtDNA of *Plasmodium* species.

Enzymes involved in precise processing of these molecules are not known. The finding that a large number of proteins with RNA-binding motifs (RAP proteins) amplified in Apicomplexa appear to be localized to the parasite mitochondrion suggests their potential role in posttranscriptional processing of mitochondrial RNA molecules. The most intriguing aspect of mitochondrial functions in apicomplexans is the manner in which the three mtETC proteins encoded by the mtDNA are translated. The structure of the mitochondrial ribosomes remains unresolved, as does the process by which such ribosomes are assembled from multiple rRNA fragments and a large number of mitoribosomal proteins imported from the cytoplasm. With the availability of powerful new technologies, we anticipate that many of these questions will be addressed in the coming years.

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