Mitochondrial Evolution and Functions in Malaria Parasites

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Key Words

Plasmodium, tricarboxylic acid cycle, mitochondrial electron transport chain, alveolates, dinoflagellates, ATP synthase

Abstract

Mitochondria in malaria parasites have some unusual evolutionary and functional features. The drastic reduction in the size of their mitochondrial genome, encoding just three proteins, appears to have originated at the point of divergence of dinoflagellates and apicomplexan parasites from ciliates and may have accompanied the acquisition of plastids by the former. Unusual translational machinery as revealed by the highly fragmented mitochondrial ribosomal RNA genes also appears to have originated at this deflection point. Some of the biochemical properties of malarial mitochondria also appear to be unconventional. Although tricarboxylic acid cycle enzymes are encoded by the genome, they do not appear to be involved in the full oxidation of glucose to fuel mitochondrial ATP synthesis in the blood stages of malaria parasites. A critical role of the mitochondrial electron transport chain appears to be to serve pyrimidine biosynthesis. In spite of their minimal nature, *Plasmodium* mitochondria are attractive targets for antimalarial drugs.

Contents

INTRODUCTION

The realization that mitochondria play a critical role in life and death decisions by metazoan cells has enhanced the significance of these organelles, already cherished as the brokers of cellular energy economics, to an even greater level. The title of the recent book *Power, Sex, Suicide: Mitochondria and the Meaning of Life* (45) is not too far off the mark. Yet, our understanding of mitochondria continues to be somewhat parochial, mainly limited to metazoan and fungal systems. The vast ecological niches occupied by unicellular eukaryotes demand myriad physiological adjustments, many of which are likely driven by the evolution of their mitochondria. Even a limited survey of mitochondria from a few protists provides a glimpse of an enormous diversity of their forms and functions.

Malaria: disease caused by eukaryotic unicellular parasites of the genus *Plasmodium*

mtDNA: mitochondrial DNA

Alveolata: a high-level eukaryotic taxon consisting of unicellular microorganisms having cortical alveoli, flattened vesicles packed into a continuous layer supporting the plasma membrane

Here, we aim to review some salient features of mitochondria possessed by an important group of human pathogens, malaria parasites. The discovery that the mitochondrial genome of malaria parasites was an unusually small molecule arranged in tandem arrays (88–90) led to a reevaluation of a circular DNA molecule then considered to be the mitochondrial genome (103). Recognition of the latter molecule as a relic plastid genome has fundamentally altered our view of these important pathogens. Both the mitochondrion and the plastid are highly divergent from their conventionally studied counterparts and provide opportunities for antiparasitic drug development. We do not attempt an exhaustive review of malarial mitochondria; the reader may wish to consult many recent reviews for additional details (40, 54, 75, 87, 91, 93).

MITOCHONDRIAL DNA IN ALVEOLATES: DRAMATIC CHANGES WITHIN AN EVOLUTIONARY CLADE

The origins of extant mitochondria can be traced to symbiotic arrangements involving Alphaproteobacteria that gave rise to eukaryotic cells. Thus, the mitochondrial DNAs (mtDNAs) of all eukaryotes are evolutionary descendants of an alphaproteobacterial genome. In spite of this common ancestry, mtDNAs in different evolutionary lineages display enormous diversity in their gene content and organization (33). Organisms within the top-level clade Alveolata provide some of the most striking examples of such mtDNA diversity shaped by evolutionary forces. A common feature of most alveolate mitochondria is the presence of tubular cristae. However, as shown in **Figure 1**, the three major groups of alveolates—ciliates, dinoflagellates, and apicomplexans—possess mitochondrial genomes that are dramatically divergent in their organization and gene contents. Ciliates have the largest mtDNA among the three, with a ~47-kb telomere-flanked linear mtDNA encoding 45 open reading frames (ORFs) in the

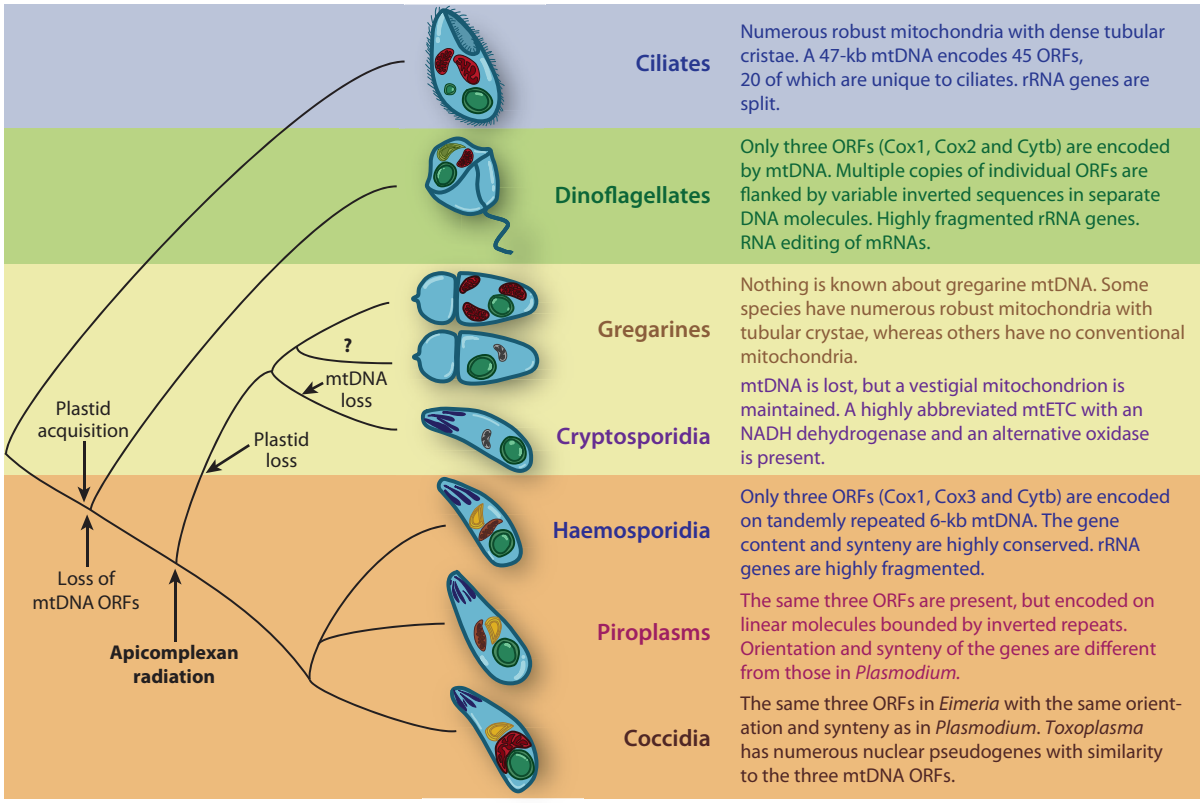

Figure 1

Mitochondrial DNA in alveolates. The phylogenetic relationship among members of the clade Alveolata is depicted (branch lengths are not to scale). Major events involving plastid and mitochondria are indicated at different branches. The column on the right provides salient descriptions of mitochondrial DNA in each of the representative organisms.

case of *Tetrahymena* spp. (8, 11, 59). Remarkably, 20 of these ORFs encode proteins with no obvious homologues in any other organisms; these encoded proteins appear to be limited to the ciliate branch (59). The strangeness of this becomes apparent when one considers that the jacobid protozoan *Reclinomonas americana*, the record holder of the largest number of mtDNA-encoded ORFs, has 97 ORFs, of which 96 have assigned functions (46). Proteomic analysis has shown that at least 13 of the unassigned mitochondrial ORFs in *Tetrahymena thermophila* are detectably expressed as proteins (77). It is unclear what evolutionary force drove such divergence of gene content in ciliate mtDNA. An analysis of mtDNA sequences encoding unassigned ORFs in four *Tetrahymena* species suggests an enhanced rate of divergence indicated by the presence of mutational hot spots that seem to accelerate acquisition of nonsynonymous substitutions (59). Nevertheless, much remains unknown about the mitochondrial physiology in ciliates, with possible implications for their sister clades, the dinoflagellates and apicomplexans.

Dinoflagellates have drastically reduced gene content within their mtDNA, encoding just three ORFs, but have highly complex gene organization (60, 61). The three proteins—cytochrome *c* oxidase subunits I and III (Cox1 and Cox3) and cytochrome *b* (Cytb)—are encoded on multiple DNA molecules from which one or two of the ORFs may originate. These genes are flanked by a varying number of

Apicomplexa: a phylum of parasitic eukaryotic microorganisms characterized by the presence of a unique apical complex of microtubules

Cox1: cytochrome *c* oxidase subunit 1

Cox3: cytochrome *c* oxidase subunit 3

Cytb: cytochrome *b*

inverted repeat sequences; in some instances such repeats make up 85% of the mtDNA. Multiple copies of the ORFs are detected, with their flanking repeats often different from one another. These different flanking sequence arrangements are likely to have been generated through recombination events. It is proposed that the mitochondrial genome of dinoflagellates consists of several nonidentical molecules of about 30 kb, but it is not clear whether these molecules are circular or linear (61). In addition to the unusual organization of mtDNA, dinoflagellates also seem to have acquired RNA editing, with a high prevalence of A to G (or A to I) changes; G to C changes are also observed, which is unique to dinoflagellate mitochondrial genes. There do not seem to be any encoded tRNAs, but fragments of rRNA genes with homology to highly conserved regions can be detected.

Apicomplexan mtDNAs also have highly reduced gene content compared with ciliates and remarkably encode the same three ORFs (Cox1, Cox3, and Cytb) as the dinoflagellate mtDNA. This would suggest that the dramatic gene reduction in mtDNA preceded the divergence of apicomplexans from the dinoflagellates. Organization of these genes, however, is different from the dinoflagellate mtDNA. The first apicomplexan mtDNA to be recognized was from a rodent malaria parasite, *Plasmodium yoelii*, as head-to-tail tandemly repeated arrays with a unit length of about 6 kb (88, 90). Prior to this recognition, apicomplexan mtDNA was believed to be a 35-kb circular DNA molecule, which in fact was a relict chloroplast genome, the result of a secondary endosymbiotic event (101, 102). Cox1 and Cytb are encoded by one strand of the mtDNA, whereas Cox3 is encoded by the other strand. In addition, multiple regions on either strand, varying in length from 20 to 200 nucleotides, appear to encode fragments of large and small subunits of rRNA (23, 81). No tRNA genes can be detected. In sharp contrast to the dinoflagellate mtDNA, this arrangement of protein- and rRNA-coding genes is highly conserved among all *Plasmodium* spp. Indeed, the sequence conservation within the mtDNAs from evolutionarily distant *Plasmodium* spp. is much higher than that of the nuclear genes; codon usage differences seen among the nuclear genes of these species (reflected in the overall GC content of their genomes) are not observed for the mitochondrially encoded genes (57). This would suggest divergent evolutionary forces affecting nuclear and mitochondrial genes in malaria parasites.

As one examines mtDNA in other apicomplexans, the divergent evolutionary forces working on the nuclear and mitochondrial genomes become more apparent. The piroplasm parasites *Theileria* and *Babesia* have a different gene arrangement of their mtDNA compared with *Plasmodium*: The 7-kb mtDNA is linear and bounded by inverted repeat sequences; Cytb and Cox3 are encoded on one strand, whereas Cox1 is encoded on the other; the order in which rRNA gene fragments are arranged is also different from that in *Plasmodium* (36). On the basis of unpublished sequences available from the Sanger Institute (A. Pain & M. Berriman, personal communication), an evolutionarily more distant coccidian parasite, *Eimeria*, appears to have tandemly arrayed mtDNA with gene arrangements identical to *Plasmodium*. Another coccidian parasite, *Toxoplasma*, has a large number of nuclear pseudogenes that appear to have a mitochondrial origin (65); because of this the sequence arrangement and gene content of *Toxoplasma* mtDNA have not been unequivocally determined. Recent evidence suggests that an important human pathogen, *Cryptosporidium*, belongs to a deep-branching group of apicomplexans called gregarines (47, 48). Although little is known about mtDNA in gregarines, electron microscopic observations indicate that whereas some of the gregarine species have robust mitochondria with dense tubular cristae, others are devoid of discernible mitochondria (B.S. Leander, personal communication). *Cryptosporidium* spp. fall in the latter category: Genomic sequencing revealed the absence of mtDNA, as well as genes encoding any of the proteins of the mitochondrial electron

transport chain (mtETC) (1, 108). However, a vestigial mitochondrion, called a mitoplast, is present in *Cryptosporidium*, as has been the case for many other so-called amitochondriate organisms such as *Giardia* and *Entamoeba* (37, 50, 69).

The gains, losses, and rearrangements of mitochondrial genomes in alveolates seem to point to a remarkable plasticity of mitochondrial functions necessary to accommodate the disparate environmental niches in which the varied members of this protozoan group have to survive. For instance, plastid acquisition by dinoflagellates through secondary endosymbiosis may have permitted a reduced role for mitochondria, resulting in the loss of the majority of mitochondrial genes possessed by the common ancestor of ciliates and dinoflagellates. Similarly, parasitic adaptation by the apicomplexans may have permitted the loss of photosynthetic genes within the plastid. These physiological adjustments provide opportunities for understanding some of the unique aspects of early eukaryotic radiation, as well as for developing means for the control of pathogenic species belonging to this clade.

HINTS OF UNUSUAL TRANSLATIONAL MACHINERY

The three proteins encoded by the mtDNA are likely synthesized by an unconventional translational apparatus. At least 15 different RNA fragments encoded by either mtDNA strand evidently associate with each other in *trans* and combine with nuclearly encoded ribosomal proteins to form mitochondrial ribosomes (mitoribosomes) in malaria parasites (22, 23, 56). These rRNA fragments associate with each other through conserved stem-loop structures and represent major portions of catalytic regions such as the peptidyl transferase domain of a ribosome, although certain other critical regions of rRNA do not appear to be represented within these fragments. It is unclear whether and how these apparent deficiencies are compensated for in the mitoribosomes. Sequence analysis of the genome identifies a number of ribosomal proteins encoded in the nucleus with potential mitochondrial localization signals (R. Perrault & A.B. Vaidya, unpublished data). Some of these putative mitoribosomal proteins are significantly larger than their orthologues in other systems, suggesting the possibility that extra domains of the mitoribosomal proteins may compensate for the missing rRNA domains. Although direct evidence for protein synthesis in *Plasmodium* mitochondria is lacking at this point, there is some evidence that the rRNA fragments are present in large Mg^{2+}-requiring complexes that resemble ribosomes (56).

The mtDNA does not encode any tRNAs. This suggests the importation of a full set of tRNAs from the cytosol, requiring a machinery devoted to this purpose. Such import systems exist in other organisms (71), and tRNA import into the *Toxoplasma* mitochondrion has been reported (21). There has also been some speculation that tRNAs encoded by the plastid genome may be imported by the mitochondrion in apicomplexan parasites. Each tRNA requires its cognate tRNA synthetase that charges it with the appropriate amino acid. Examination of genomic sequences suggests the lack of a complete set of tRNA synthetases required to serve the three compartments in which protein synthesis occurs in malaria parasites. One possibility is dual or triple targeting of the encoded enzymes through use of alternate exons that encode appropriate targeting signals for the plastid and mitochondrion; however, there is no evidence for this yet.

Of the three ORFs, only Cytb has an AUG triplet at the 5′ end that could serve as the initiation codon. Alternative initiation codons have been demonstrated in other mitochondrial systems, but in the absence of authentic amino acid sequences of the encoded proteins, the mechanism of translation initiation for these genes remains unclear. A peptidyl deformylase is encoded in the parasite genome but is likely targeted to the plastid (6, 44).

The unusual nature of the translational machinery could be an attractive target for antimalarial compounds. Indeed, several antibiotics

Mitochondrial electron transport chain (mtETC): a series of enzyme complexes in the inner membrane of the mitochondrion that transfer reducing equivalents (electrons) through a series of redox cofactors, ultimately reducing molecular oxygen

that inhibit bacterial protein synthesis have antiparasitic activity. Although these antibiotics are believed to target plastid protein synthesis (15), further investigations into mitochondrial protein synthesis may be useful in the search for novel antimalarial compounds.

Tricarboxylic acid (TCA) cycle: a cyclic metabolic pathway that oxidizes the acetyl group of acetyl-CoA to CO_2 through a series of enzymatic steps that generate NADH, ubiquinol, and GTP or ATP

TRICARBOXYLIC ACID CYCLE ENZYMES: SERVING FUNCTIONS OTHER THAN GLUCOSE OXIDATION

Conventionally, the mitochondrion is viewed as the source of cellular energy in the form of ATP. Mitochondrial ATP synthesis is closely linked to oxidative degradation of substrates that power the mtETC, resulting in the generation across the mitochondrial inner membrane of a proton gradient, which then drives the forward rotary movement of the ATP synthase. A key metabolite initiating this complex set of events, acetyl-CoA feeds a two-carbon unit to the tricarboxylic acid (TCA) cycle (**Figure 2**). Oxidative reactions catalyzed by enzymes within the TCA cycle generate electrons in the form of NADH and ubiquinol, which go on to power the mtETC. In the absence of acetyl-CoA, the TCA cycle cannot function as a cycle. Mitochondrial acetyl-CoA is generated in three ways: conversion of pyruvate (usually generated through glycolysis) by a multiprotein pyruvate dehydrogenase complex; degradation of fatty acids through β-oxidation; and catabolism of branched-chain amino acids leucine and isoleucine via transaminase, dehydrogenase, hydratase, and lyase reactions. Of these, the most common pathway is through glycolysis-generated pyruvate.

The role of the TCA cycle and its contribution to the bioenergetics of erythrocytic stages of malaria parasites has been debated for a number of years (76). It was known in the 1960s that ^{14}C-labeled glucose fed to malaria parasites was not converted to any significant amount of $^{14}CO_2$ (4, 9), suggesting a lack of entry by pyruvate-derived acetyl-CoA into the TCA cycle. Indeed, glucose is converted almost entirely into lactate by malaria parasites (76). The recent observation that pyruvate dehydrogenase is exclusively located in the apicoplast (26) provides further evidence that argues against the full oxidation of glucose as a driving force for the putative mitochondrial TCA cycle. The second way to generate mitochondrial acetyl-CoA is through β-oxidation of fatty acids. However, sequence analysis of the available malaria parasite genomes fails to identify genes encoding enzymes needed for this pathway. There is also no biochemical evidence to suggest fatty acid degradation by malaria parasites, thus making this pathway to acetyl-CoA unlikely for malaria parasites. The third pathway involves degradation of branched-chain amino acids. In the first reaction in this pathway, a branched-chain amino acid transaminase transfers the amino group to α-ketoglutarate to produce glutamate and a corresponding branched-chain α-keto acid. Several subsequent steps involving branched-chain keto acid dehydrogenase (BCKDH) complex, a large complex resembling pyruvate dehydrogenase, as well as other enzymes, lead to the formation of acetyl-CoA. Whereas the *Toxoplasma* genome encodes all the enzymes involved in branched-chain amino acid degradation (75), malaria parasites possess none of these except BCKDH. One recent suggestion has been that BCKDH could instead act on pyruvate, albeit inefficiently, to produce acetyl-CoA (75). However, it is not clear how well BCKDH can compete with lactate dehydrogenase for utilization of pyruvate.

The evidence to date, then, suggests that the TCA cycle in erythrocytic stages of malaria parasite mitochondria is unlikely to perform as a cycle. However, all the enzymes that participate in the TCA cycle are encoded in the genome and, except for pyruvate dehydrogenase, are known or predicted to localize to the mitochondrion by means of mitochondrial targeting signals. Citrate synthase is localized to the mitochondrion and is expressed at relative high levels, as judged from transcriptome analysis. However, in the absence of

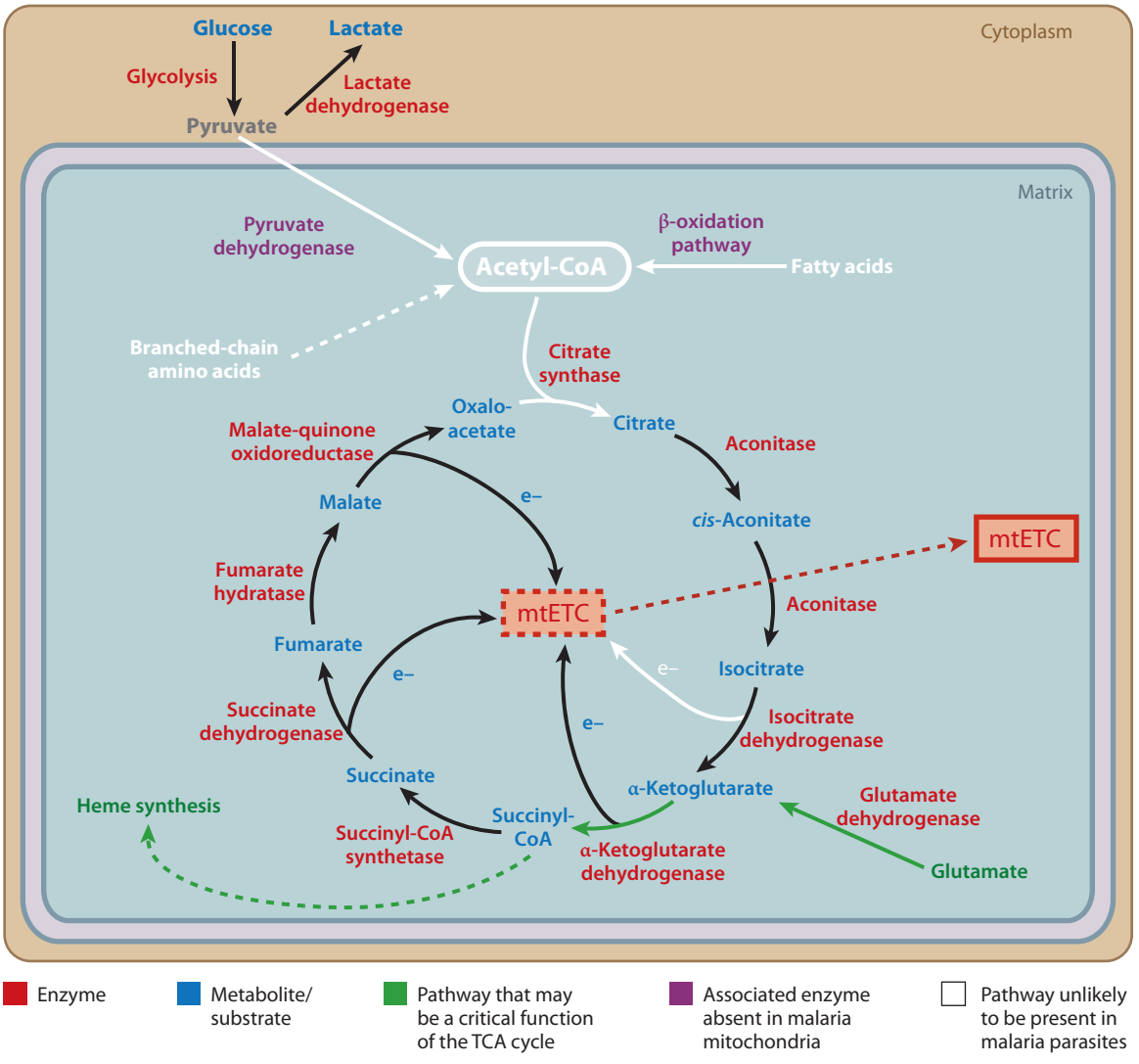

Figure 2

Tricarboxylic acid (TCA) cycle in malaria parasites. Enzymes and substrates of the classical TCA cycle are shown. Questions regarding the source of acetyl-CoA are depicted in white. Because pyruvate dehydrogenase is localized to the apicoplast, pyruvate generated through glycolysis is unlikely to be the source of acetyl-CoA, nor are pathways involving fatty acid β-oxidation or catabolism of branched-chain amino acids, as discussed in the text. The NADP-dependent isocitrate dehydrogenase is unlikely to provide electrons to the mitochondrial electron transport chain (mtETC), as depicted by the white arrow. A main entry point within the cycle is likely to be α-ketoglutarate generated from glutamate and utilized for the production of succinyl-CoA to be used in heme biosynthesis, as shown by green arrows.

mitochondrial acetyl-CoA, its function is uncertain at this point. Aconitase appears to serve dual functions, both as a mitochondrial enzyme and as a cytosolic iron response element binding protein to regulate mRNAs involved in iron homeostasis (35). The next enzyme in the cycle, isocitrate dehydrogenase, is an $NADP^+$-dependent rather than an NAD^+-dependent enzyme (70, 96). Its expression increases under oxidative stress, and thus it has been proposed

Protonmotive force (Δp or $\Delta\mu_H$): potential energy stored in a transmembrane proton gradient, consisting of a chemical component and an electrostatic component

Q: ubiquinone or Coenzyme Q

NDH: NADH dehydrogenase

to be important in generating NADPH, which is necessary for redox balancing within the mitochondrial matrix (106). NADPH may also serve biosynthetic processes relegated to the mitochondrion, but is unlikely to provide electrons to the mtETC (**Figure 2**).

The major entry point for the TCA "cycle" in malaria parasites appears to be α-ketoglutarate produced from glutamate through the likely reaction of glutamate dehydrogenase. Glutamate could be derived through a variety of pathways including hemoglobin degradation as well as amino acid uptake. A recent metabolomic study provides strong support for this view (K.L. Olszewski & M. Llinas, personal communication). A critical role of the TCA cycle enzyme α-ketoglutarate dehydrogenase appears to be the production of succinyl-CoA (**Figure 2**), a substrate for 5-aminolevulinate synthase, which condenses it with glycine to produce 5-aminolevulinate in the rate-limiting first step of heme biosynthesis. At this point, this metabolite production is the principal known function of the TCA cycle enzymes in the blood stages of malaria parasites. Recently, Daily et al. (17) have reported that *Plasmodium falciparum* parasites isolated fresh from a subgroup of patients (which consist of only ring stages of parasites and possibly early gametocyte stages) have increased levels of mRNAs that encode TCA cycle enzymes. They suggest that this change in a subpopulation of parasites may be a response to nutrient stress in a manner similar to that found in *Saccharomyces*. In the absence of any biochemical evidence for such a response, this suggestion remains provocative. Indeed, the recent report of Lemieux et al. (49) indicates that such apparent variation in the expression patterns of parasite samples isolated from patients may arise from differences in developmental synchrony and the proportion of early gametocytes in the samples. When these factors were taken into account, these authors found little variation in the transcriptional patterns of numerous patient parasite samples and in vitro parasite cultures.

MITOCHONDRIAL ELECTRON TRANSPORT CHAIN: A TARGET OF ANTIMALARIAL DRUGS

In most eukaryotic cells, the mtETC is required for generation of the protonmotive force (Δp or $\Delta\mu_H$), which is central to oxidative energy metabolism (reviewed in Reference 72). However, in organisms or under conditions favoring a glycolytic metabolism, which includes the blood stages of malaria parasites and some other parasite species, the role of the mtETC is often reduced, and mitochondria may even become consumers rather than producers of energy (14, 58, 84, 94, 97). In higher eukaryotes the mtETC is generally composed of four integral membrane enzyme complexes in the mitochondrial inner membrane: NADH:ubiquinone oxidoreductase (Complex I), succinate:ubiquinone oxidoreductase (Complex II), ubiquinol:cytochrome *c* oxidoreductase (Complex III, or cytochrome bc_1), and cytochrome *c* oxidase (Complex IV), with ubiquinone (Coenzyme Q, or Q) and cytochrome *c* functioning as electron carriers between the complexes and Complexes I, III, and IV functioning as sites that generate protonmotive force. *Plasmodium* spp. have lost the large multisubunit Complex I of the mitochondrial inner membrane but have retained electron transfer Complexes II through IV (**Figure 3*a***). They do possess a single-subunit, non-proton-pumping NADH dehydrogenase (NDH) that reduces ubiquinone. The apparent subunit composition of the mtETC complexes (54) deduced from the genome data (31) suggests that mtETC complexes of the malaria parasite have a much simpler subunit composition than their counterparts in mammals and yeast. Complex III in *Plasmodium* has 7 identifiable subunits instead of 10 as seen in yeast, and Complex IV has only 6 recognizable subunits instead of the 12 seen in yeast. However, it is possible that in these deep-branching organisms, the mitochondrial complexes may possess additional highly divergent subunits that may not yield easily to bioinformatic identification. Subunit II of Complex IV is

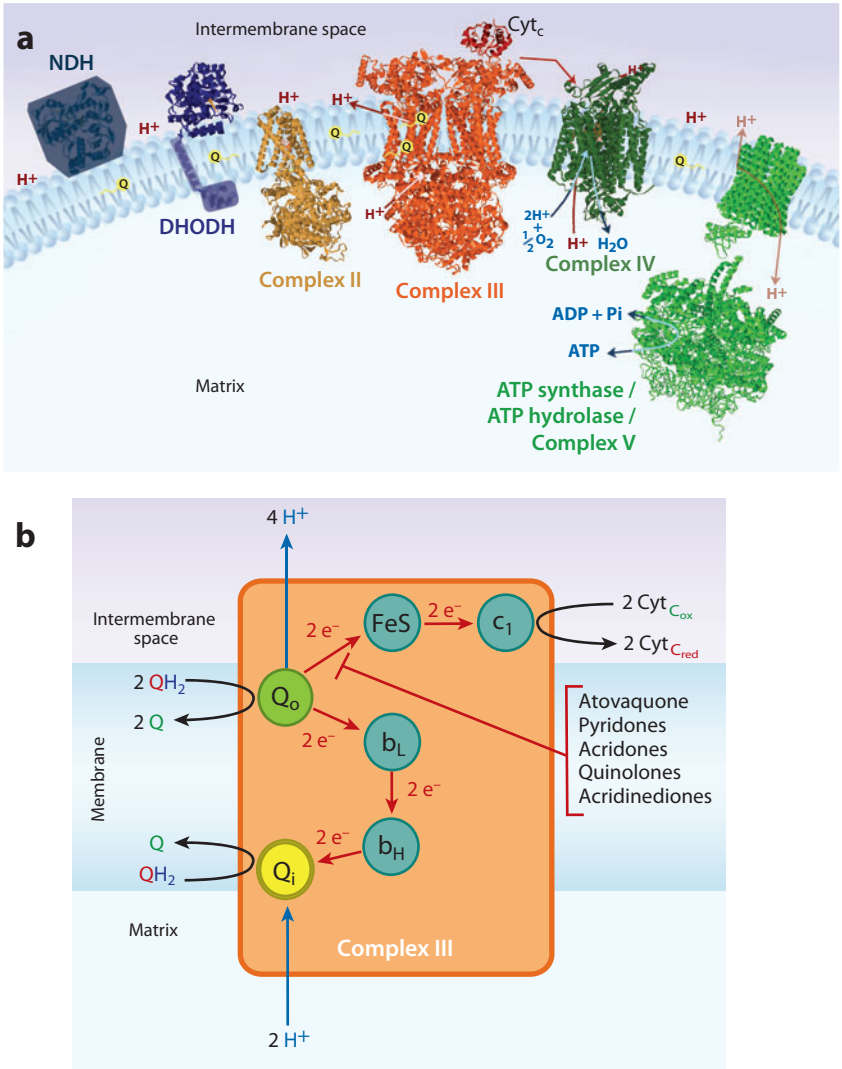

Figure 3

Mitochondrial electron transport chain (mtETC) of malaria parasites and Complex III reactions targeted by drug candidates. (*a*) The enzyme complexes of the mtETC are embedded in the inner membrane of the mitochondrion. The reactions at Complex III and Complex IV result in translocation of protons from the matrix to the intermembrane space. Also shown are additional dehydrogenases that provide electrons to the mtETC via ubiquinone (yellow structures labeled "Q"; malate:ubiquinone oxidoreductase and glycerol-3-phosphate dehydrogenase are not shown). Complex V is shown in two sections. The enzymes are drawn as ribbon diagrams of the structures of orthologues available in the Protein Data Bank. (*b*) The schematic depicts Complex III, with the substrates ubiquinol (QH_2) and ubiquinone (Q) interacting with their reaction sites [ubiquinol oxidation site (Q_o) and ubiquinone reduction site (Q_i), respectively]; the electron and proton transfers of the ubiquinol:cytochrome *c* oxidoreductase reaction, according to the Q-cycle mechanism; and the five classes of drug or drug candidates that block the reaction at the Q_o site. For a full explanation of the current modification of the Q-cycle as applied to the *bc*-type complexes see Reference 13.

Q_o: ubiquinol oxidation site in Complex III

split, and the genes for the two parts are not found in the mitochondrial DNA but have migrated to two different chromosomes. Verification of the in silico compositions by biochemical studies is still largely lacking owing to the difficulty of purifying a meaningful amount of the complexes (55, 87), which is exacerbated by the apparent low levels and/or activities of the respiratory enzymes in the blood stages of the parasites (27, 41, 42, 66). The low activities of the mtETC complexes are consistent with the largely glycolytic carbon and energy metabolism of the blood stage parasites (95).

Despite the relatively low activity of the mtETC in malaria parasites, it still appears to be the primary source of the mitochondrial proton electrochemical gradient (66, 79). Furthermore, the sensitivity of parasites to inhibitors of the mtETC indicates that it is indispensible to the parasites. Hydroxynaphthoquinone inhibitors of Complex III are lethal to apicomplexan parasites including *Plasmodium* spp. (28, 29), which lead to the development of the antimalarial drug atovaquone. Atovaquone targets the ubiquinol oxidation site (Q_o) of cytochrome *b* with high selectivity (29). Unfortunately, high-level resistance to atovaquone occurred at a relatively high frequency and correlated with mutations at position 268 (Tyr) of cytochrome *b*. This position lies near a highly conserved motif and participates in forming the Q_o site. Similar resistance mutations at this site and other nearby positions in cytochrome *b* also arose in *P. yoelii* raised in mice treated with suboptimal levels of atovaquone (78). A cause and effect relationship between a mutation introduced at Tyr-268 and the resulting two-orders-of-magnitude reduction in the susceptibility of cytochrome bc_1 to inhibition by atovaquone was demonstrated using a bacterial system (53). Experiments with this system also provided additional information on the drug's mode of action (53).

To obviate the resistance problem, atovaquone is sold commercially as Malarone™, a synergistic combination of atovaquone and proguanil. In the presence of proguanil, the effective dose of atovaquone is significantly reduced; however, once a malaria parasite acquires the resistance mutation in cytochrome *b*, the synergistic effect of proguanil is lost. The precise molecular mechanism by which it potentiates the action of atovaquone (and other inhibitors of cytochrome bc_1) has yet to be elucidated. We demonstrated that proguanil lowers the concentration of atovaquone required to significantly reduce the mitochondrial membrane potential in *P. yoelii* by six- to eightfold (79, 80), while qualitative measurements of *P. falciparum*–infected erythrocytes indicated that the degree of membrane potential reduction is increased when atovaquone is combined with proguanil (66).

Atovaquone is an effective drug, but is expensive and subject to relatively facile development of resistance. Drug resistance is a major problem for malaria treatment and prevention, and the search for new, effective, and inexpensive drugs is a continuing and urgent need. Even though Complex III of the mtETC is essential to both the human host and the malaria parasite, it continues to be a promising target for the development of antimalarial drugs. Extensive structural and genetic evidence suggests that the Q_o site of Complex III is large and capable of accommodating two ubiquinol molecules. Various subclasses of Complex III inhibitors bind to nonidentical but overlapping regions of the Q_o site. In agreement with this view of the Q_o site, several classes of chemicals provide promising leads with antimalarial activities. 4-Pyridone analogs of the clopidol class that selectively inhibit *Plasmodium* Complex III are at an advanced stage of development (107, 110). Winter et al. have synthesized (104) haloalkoxyacridone derivatives that appear to target the bc_1 complex and inhibit human malaria parasite growth with inhibitory concentrations in the picomolar range. Quinolones related to the early antimalarial candidate endochin that also seem to target Complex III while exhibiting minimal atovaquone cross-resistance have been developed (105). The dihydroacridinedione WR249685 is a selective inhibitor of the cytochrome bc_1 complex (3). Thus, at least five

different structural entities have the potential to be developed as antimalarials by having the capacity to selectively inhibit *Plasmodium* Complex III (**Figure 3*b***).

PYRIMIDINE BIOSYNTHESIS: A CRITICAL MITOCHONDRIAL FUNCTION

In addition to NADH dehydrogenase, at least four known or predicted oxidoreductases may donate reducing equivalents to the ETC via ubiquinone (54, 93), including the essential enzyme dihydroorotate dehydrogenase (DHODH), which catalyzes the conversion of dihydroorotate to orotate, the sole redox reaction in the de novo pyrimidine biosynthesis pathway. *P. falciparum*, like many parasites, has a streamlined metabolism, and many biosynthetic pathways are absent (32), with essential metabolites salvaged from the host (31). In the case of pyrimidines, however, it is the salvage pathway that is missing, rendering their biosynthesis indispensible for nucleotide metabolism (34). This has naturally led to consideration of the enzymes of the pyrimidine pathway as possible targets for drug development, even though the steps of the pathway are the same as in the host; evolutionary distance likely has resulted in significant differences between the host and parasite enzymes that can be exploited, as in the case of Complex III. Recent results have shown that the parasite DHODH, in particular, is indeed a promising target (2, 67, 68). Phillips et al. (68) reported a substituted triazolopyrimidine compound that is highly species selective and exhibited an IC_{50} below 100 nM against *P. falciparum*. The triazolopyrimidines could be prepared by a relatively simple and inexpensive synthetic route, an important consideration for antimalarial drugs. Patel et al. (67) identified compounds from several chemical classes that demonstrated submicromolar potency against cultured *P. falciparum* parasites with low toxicity for mammalian cells and that appeared to represent more than one mode of action.

There are two classes of DHODH. One class, found in animals as well as apicomplexan parasites, utilizes ubiquinone as cofactor (called type 2) and is found in the inner mitochondrial membrane. Members of the type 1 class, present in *Saccharomyces* and certain protozoa, require soluble electron acceptors, such as fumarate or NAD^+, and are not associated with mitochondria. Our group has capitalized on this difference to explore the intersection of the pyrimidine biosynthetic pathway and the ETC with startling results. Painter et al. (66) expressed the fumarate-dependent DHODH from yeast in *P. falciparum* D10 parasites. The transgenic parasites, cultivated in vitro, are resistant to all complex III inhibitors tested, including atovaquone. Thus, the mtETC can become dispensable in blood stages of the human malaria parasite upon acquisition of a means to synthesize pyrimidines independently of ubiquinone regeneration by the mtETC. These results have been essentially reproduced by other groups. This observation appears to have far-reaching implications in that other functions dependent on a functional mtETC, including the ubiquinone-dependent oxidation of other substrates by mitochondrial dehydrogenases and oxidative phosphorylation by ATP synthase, if present, must also be dispensable under pyrimidine bypass conditions (66, 92, but see Reference 24 for an opposing view).

We were surprised to find that resistance to mtETC inhibitors in these transgenic parasites was completely reversed when treated in combination with proguanil. This finding appears to indicate that proguanil interferes with a function that becomes essential only when the mtETC is blocked. Observations with the lipophilic cationic fluorescent probe Mitotracker™ Red suggest that this effect is related to maintenance of the mitochondrial membrane potential, which is likely to be essential for protein import and the operation of electrogenic carriers. In the presence of atovaquone or another ETC inhibitor alone, a substantial potential is maintained, but when combined with proguanil the potential is fully dissipated (66).

We have proposed a hypothetical model (figure S4 in Reference 66) based upon the

DHODH: dihydroorotate dehydrogenase

known mechanism of mitochondrial membrane potential generation in mammalian ρ°, yeast ρ⁻/ρ°, and dyskinetoplastic trypanosome cells, all of which lack an active mtETC (10, 19, 74). In these cells, the continuous import of ATP (produced by glycolysis) into the mitochondrion through the electrogenic adenine nucleotide carrier (ADP/ATP carrier) establishes a transmembrane potential due to the exchange of ATP^{4-} for ADP^{3-}, producing a net charge difference (negative inside). The mitochondrial phosphate carrier, which is electroneutral, exports a phosphate ion ($H_2PO_4^-$) in exchange for an hydroxide ion, maintaining chemical balance. To maintain the ATP/ADP flux, the F_1 ATPase hydrolyzes imported ATP^{4-}, yielding ADP^{3-} and phosphate. The model then hypothesizes that proguanil inhibits one or more of the components of this alternative pathway for potential generation. Thus, in the presence of proguanil alone, the mtETC will generate a mitochondrial membrane potential; in the presence of atovaquone or other mtETC inhibitors alone, the alternative electrogenic system can maintain a membrane potential, but in the simultaneous presence of both proguanil and a mtETC inhibitor, a membrane potential cannot be maintained. This is a testable hypothesis consistent with the presently available information, but it is by no means the only mechanism that can be envisioned by which the parasites might maintain a (proguanil-sensitive) mitochondrial membrane potential in the absence of electron transport. Further experimental characterization is desirable and could conceivably reveal a potential new therapeutic target.

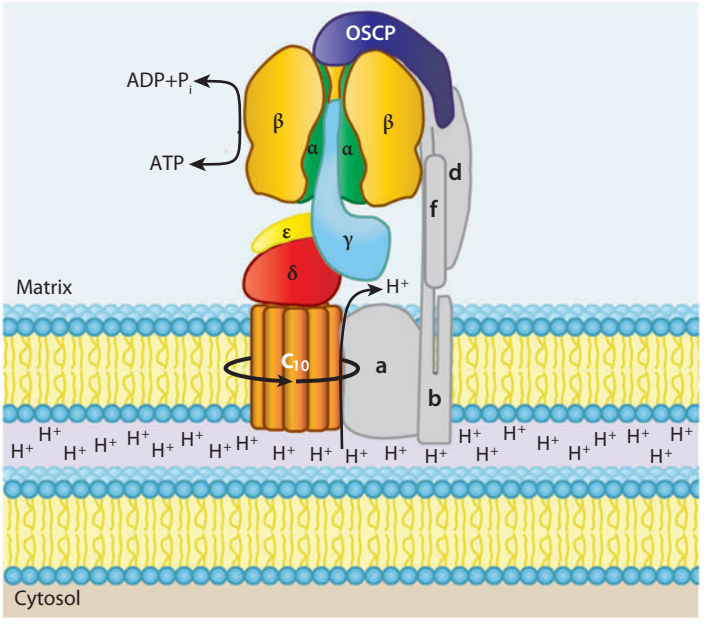

Figure 4

Schematic representation of the ATP synthase/hydrolase, or Complex V. Complex V is embedded in the inner mitochondrial membrane and couples transmembrane proton transfer to ATP synthesis or hydrolysis via a rotary mechanism. c_{10}, δ, ε, and γ form the rotor, while the rest of the subunits comprise the stator. Subunits are shown in approximately the correct proportions and location as found in the mammalian enzyme (99). A portion of the F_1 head domain has been sliced away to show the penetration of the γ subunit (the remaining α subunit is hidden behind the visible head subunits). Additional minor subunits, some of which are involved in dimerization, are not shown. Putative orthologues of the colored subunits are present in *Plasmodium*, while orthologues of the gray membrane and peripheral stalk subunits (a, b, f, and d) are unknown (see text).

COMPLEX V: FOR WHAT PURPOSE?

Complex V, or F_1F_o-ATP synthase, is an elegant rotary machine that couples the synthesis or hydrolysis of ATP to the transmembrane protonmotive force (5, 12, 98). It consist of an extrinsic catalytic domain that interconverts ATP and ADP plus inorganic phosphate, traditionally called F_1 (with an $\alpha_3\beta_3\gamma\delta\varepsilon$ subunit structure), and a membrane domain, known as F_o, that forms a conditional proton channel, requiring rotation of the central ring of c subunits to complete the transport of protons across the mitochondrial inner membrane (**Figure 4**). The rotating channel is formed by the interface of the integral membrane a subunit with the c ring. A peripheral stalk formed by the b, OSCP, and other species-specific subunits connects the a subunit to the α and β catalytic subunits, thus holding the stator of the motor immobile against the central rotor composed of the c ring and the γ, δ, and ε subunits (**Figure 4**) (100).

The genomic sequence data indicates the likely presence of all the core subunits of F_1 (α, β, γ, δ, and ε) and some of the F_o/stator subunits

(c and OSCP) in *P. falciparum* (54). Conspicuously absent are the a and b subunits, which are essential to the function of known F-type ATP synthases. As more apicomplexan genomic sequences were reported, it became apparent that recognizable a and b subunits are probably absent from the entire phylum, which caused us to question whether the whole apicomplexan clade had lost the capacity for coupled ATP synthesis and hydrolysis (55). However, the recently reported genome sequence of *T. thermophila* (8, 20) in the sister phylum Ciliophora points to another possibility. F_o a and b subunits also appear to be absent from this organism, yet *Tetrahymena*, which form large cells with numerous mitochondria that can be isolated with relative ease, contain significant ATP synthase activity (16, 62), albeit insensitive to many of the classical inhibitors, including oligomycin. This suggests that ciliates, and perhaps also apicomplexan species, employ highly divergent or novel subunits to fulfill the functions of the classical a and b subunits. Uncovering the details of the interactions of novel subunits to form the proton channel and the stator would certainly be of great biochemical interest, and the differing inhibitor spectrum suggests that a pharmaceutical opportunity might also result from such studies.

In the many eukaryotic species that rely on oxidative phosphorylation, ATP synthesis is the final energy-conserving step, but it is still not certain that this process is utilized in *P. falciparum* parasites. As discussed above, oxidative phosphorylation does not appear to function as a source of cellular ATP in the blood stages of *P. falciparum*. Alternatively, the parasites could use the coupled hydrolysis of ATP to maintain the proton electrochemical gradient, as in the bloodstream form of *Trypanosoma brucei* (7, 63, 74). But this seems unlikely because the ETC appears to be the primary source of the proton gradient (vide supra). Of course it is possible that whereas this function has been largely abrogated in cultured asexual *P. falciparum* parasites, which are maintained in the continuous presence of glucose, oxidative phosphorylation may be important in other stages of the life cycle. Although this suggestion is largely unexplored to date owing to the difficulty in working with these nonculturable stages, it has been supported by some RNA expression and proteomic studies that indicate an apparent increase in the levels of a number of mitochondrion-associated proteins, especially in the sexual gametocyte stage (25, 38, 43, 64, 111).

Uyemura et al. (85, 86) have reported that digitonin-permeabilized rodent malaria parasites, *P. berghei* and *P. yoelii*, show evidence of oxidative phosphorylation as judged by the indirect measure of respiratory control. They also reported that this was subject to oligomycin inhibition. These findings are in contrast to observations that *P. yoelii* mitochondria do not demonstrate respiratory control and that oligomycin does not appear to inhibit mitochondrial ATP hydrolase activity (30; M.W. Mather & A.B. Vaidya, unpublished observation). The possibility of mouse mitochondrial contamination was not entirely ruled out in these reports. Therefore, the question of oxidative phosphorylation in rodent malaria parasites remains unsettled.

METABOLITE SYNTHESIS IN MITOCHONDRIA

Besides its key function in supporting pyrimidine biosynthesis in the cytoplasm, the mitochondrion in malaria parasites apparently performs or contributes to important biosynthetic pathways in its own right. These may include biogenesis of iron-sulfur clusters and hemes and biosynthesis of ubiquinone, all of which are required for the function of the mtETC, as well as other processes.

Iron-sulfur cluster biosynthesis is an ancient and essential metabolic pathway present in nearly all mitochondria and mitochondrion-like organelles (39, 109). Although the mitochondrion is the site of primary biosynthesis in eukaryotes, additional components are present in the cytosol to provide for insertion into cytosolic and nuclear-targeted iron-sulfur proteins (51, 52). A large number of enzymes, chaperones, and transporters are required for this

process, and we found 18 putative proteins in *P. falciparum* (54) that may participate. One conspicuous difference in the pathway compared with that found in mammalian mitochondria and most other organisms is the absence of the mitochondrial iron carrier frataxin.

Despite the presence of residual heme from the digestion of hemoglobin in the food vacuole, malaria parasites appear to require de novo heme biosynthesis (82). In an unusual split pathway, it appears that the first and last enzymes of the pathway, 5-aminolevulinic acid synthase and ferrochelatase, are located in the mitochondrion, where the final product will be utilized, but the next three enzymes are targeted to the apicoplast (73, 93). The location of the remaining intermediate steps is still unclear. Aminolevulinic acid synthase catalyzes the condensation of glycine with succinyl-CoA to form 5-aminolevulinic acid, hence it may have remained a mitochondrial enzyme despite the location of subsequent steps in the apicoplast to access succinyl-CoA, the product of the TCA cycle enzyme α-ketoglutarate dehydrogenase.

Plasmodium parasites are also dependent on de novo synthesis of ubiquinone, but the product has a slightly shorter isoprenoid tail than host ubiquinone (Q_{8-9} versus Q_{10}) (18). The final steps of ubiquinone biosynthesis, beginning with the formation of polyprenyl diphosphate, are normally completed in the mitochondrion, although the enzymes of the pathway are not well characterized even in model organisms. Genome data suggest that orthologs of six of seven enzymes present in yeast are also found in *Plasmodium* (54). *P. falciparum* octaprenyl diphosphate synthase (Coq1p orthologue) has been recombinantly expressed and is inhibited by the terpene nerolidol (83).

SUMMARY POINTS

1. Mitochondrial DNAs in organisms within the taxon Alveolata, to which malaria parasites belong, have undergone dramatic changes in size, organization, and encoded functions. These changes reflect evolutionary adjustments necessary for the physiological demands of different environmental niches in which these organisms exist.
2. Malaria parasites have the smallest mitochondrial genome, encoding just three proteins and highly fragmented ribosomal RNA. An unconventional translational machinery is likely present within the malarial mitochondria.
3. The TCA cycle does not seem to operate as a cycle in malaria parasites. A principal function is to provide precursors for heme biosynthesis.
4. The mtETC is abbreviated in malaria parasites. It exhibits a relatively low level of activity but is nevertheless essential. Complex III of the chain is the target of the antimalarial drug atovaquone. This enzyme's active sites are divergent relative to their counterparts in the human complex. At least five different chemical classes are being explored as potential antimalarials that target Complex III.
5. A critical role of mtETC in the blood stages of *P. falciparum* is to serve the mitochondrially located DHODH within the pyrimidine biosynthesis pathway. Transgenic parasites expressing cytosolic yeast DHODH become independent of mtETC.
6. Genes for critical subunits of mitochondrial ATP synthase subunits cannot be detected in any of the apicomplexan parasites or in ciliates. This suggests that ATP synthase in Alveolata has an unconventional nature.
7. The minimal mitochondrion in malaria parasites is still important for their survival. In addition to the pyrimidine biosynthesis, several other metabolites are likely to be synthesized in the mitochondrion.

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103. A review of mitochondrial and plastid genomes of apicomplexan parasites.

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Contents

Index

Errata

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