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Review article

Putative metabolic roles of the mitochondria in asexual blood stages and gametocytes of *Plasmodium falciparum*

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Abstract

Upon infection into human red cell, *Plasmodium falciparum* differentiates into asexual and sexual (gametocyte) stages. The mitochondrion is a tubular-cristate organelle, functionally and structurally different between the two stages. Genes and proteins involving metabolic and functional roles, protein targeting and import to this organelle, are comprehensively reviewed. The genes and proteins of the electron transport system are identified, partially characterized in human and rodent malaria parasites consisting of a single subunit of NADH dehydrogenase, two subunits of succinate dehydrogenase, cytochrome C reductase and cytochrome Coxidase. One of the primary functional roles of the mitochondrion in the parasite is the coordination of pyrimidine biosynthesis, the electron transport system and oxygen utilization through dihydroorotate dehydrogenase. All enzymes of tricarboxylic acid cycle, pyruvate dehydrogenase complex and some enzymes of ATP synthase, are identified and partially characterized using the completed *P. falciparum* genome. Some metabolic and functional roles of the organelle include oxidative phosphorylation, ubiquinone and heme biosynthesis, antioxidant defense and redox balance. Recent physiological studies involve membrane potential maintenance, cellular signaling and cation homeostasis. The organelle is a target for antimalarial drug, i.e. atovaquone. Based on the lines of evidence, we hypothesize that the parasite exhibits metabolic adaptation of the underdeveloped mitochondrial organelle to life in the mosquito vector and the human host.

Keywords: malaria; mitochondrion; metabolism; electron transport system; oxygen utilization; pyrimidine biosynthesis; tricarboxylic acid cycle; heme biosynthesis; ubiquinone biosynthesis; protein synthesis; antimalarial drug target

INTRODUCTION

Malaria remains one of the most important diseases of the world, causing annual infections to at least 515 million people from the developing countries and 1.5-2.7 million deaths mainly in sub-Saharan Africa[1-3]. Of the four *Plasmodium* species of blood-borne apicomplexan parasite, *P. falciparum* is responsible for the most severe form of human malaria. Disease symptoms include fever, chills, prostration, anemia, delirium, metabolic acidosis, cerebral malaria, multi-organ system failure, coma and death[2,4]. To combat the disease, there is an urgent need to develop new drugs due to the increasing prevalence of drug resistant parasites to currently use antimalarials, chloroquine and sulfadoxine-pyrimethamine, which consequently accounts for increase morbidity and mortality[5,6].

Upon blood stage infection, *P. falciparum* grows and differentiates into various asexual stages, i.e., ring, trophozoite and schizont, as well as into infectious sexual stages (gametocytes) which are taken up during the mosquito blood meal. In the *Anopheles* mosquito, sporozoite-stage parasites develop after fertilization of male and female gametes

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role in the transfer of electrons between the mtETS complexes and acts as a proton carrier that supports ATP synthesis [80]. Thus the inhibition of the electron transfer at this site represents an important target for chemotherapeutic attack.

One class of compounds extensively investigated over fifty years for antimalarial activity is the quinone analog, hydroxynaphthoquinone. One of the compounds, atovaquone which is 2-(trans-4-(4′-chlorophenyl) cyclohexyl) -3-hydroxy-1, 4-naphthoquinone, is currently used as a potent antimalarial [113]. Atovaquone affects multiple sites in which Q_{8-9} plays a significant catalytic role in the target enzyme, e. g. complex Ⅲ[111], and DHODase [39]. The malarial parasites synthesize their own ubiquinones and cannot salvage this from the host. Furthermore, all genes necessary for a complete ubiquinone biosynthetic pathway, starting from chorismate to ubiquinone, are also identified. We have found that atovaquone at micromolar levels strongly inhibits ubiquinone biosynthesis in *P. falciparum*, similar to its effect on *Pneumocystis carinii* [114].

Many derivatives of 5-hydroxy-2-methyl-1, 4-naphthoquinone have been synthesized and tested for their antimalarial activities on the in vitro growth of *P. falciparum* [115]. These include fifteen quinoline quinones[40]. 5-Hydroxy-2-methyl-1, 4-naphthoquinone exhibits a strong effect on the malarial mtETS complex I, II and mitochondrial oxygen consumption [74, 76]. Thus, quinoline quinones may represent a new class of compounds with potent antimalarial activity.

Proteomics

There are 246 (~4.6% of total parasite proteins) proteins targeted to the mitochondrion after translation using the TargetP and MitoProtII methods as originally identified [34, 61]. Using recently developed PlasMit program for prediction of mitochondrial transit peptides, Bender *et al* [62] have predicted 381 (~7.1%) mitochondrial proteins, based on 5334 annotated genes in the *P. falciparum* genome, close to the numbers of the mitochondrial proteins in other organisms. However, the human mitochondrial proteomics indicate about 1000 proteins involved for bioenergetic function, carbohydrate and lipid metabolisms, and regulatory functions in apoptosis and cell death, and homeostasis [116]. Most of the parasite mitochondrial protein sequences identified to date are hypothetical proteins. Nevertheless, homology searching using bioinformatics approaches have identified some components of metabolic pathways, transporter and import proteins (Table 1).

Import mechanism

Basic knowledge on mitochondrial import mechanism has been limited[117]. A long N-terminal sequence having a typical feature of a mitochondrial targeting signal has been identified in the de novo pyrimidine synthetic DHODase gene homologue for the first time in *P. falciparum* [118]. The DHODase is then verified as the mitochondrial protein by using a polyclonal antibody raised against the purified protein from *P. falciparum* and immunogold-labeled electron microscopy [26].

Recently, Wilson's group has used the GFP chimeric protein targeted to the mitochondrion and identified the import signal within a region of 68 amino acids in the mitochondrial protein HSP-60 (Table 1) which may play a role as a molecular chaperone[93-96]. Most recently, the transferring protein orthologue (translocase of outer membrane) has also been identified in *P. falciparum*[119]. The proteins contain N-terminal sequences with homologies to mitochondrial transfer peptides which use to enter the organelle, similar to higher eukaryotes[62]. Sienkiewicz *et al*[102], in contrast, recently demonstrates that the mitochondrial iron superoxide dismutase (FeSOD) has a 70-residue long N-terminal extension that shows a typical bipartite apicoplast organelle targeting sequence[120], but the FeSOD protein targets the GFP fusion into the parasite's mitochondrion. The verification of the proposed mitochondrial proteins will, therefore, be necessary.

Comparing mitochondrial and apicoplast organelles

A non-photosynthetic chloroplast, or apicoplast, has been demonstrated in malaria parasites since eleven years ago[121]. The apicoplast, as well as the mitochondrion, is considered to be a target for drug development [122]. The apicoplast retains a circular

Table 1 *P. falciparum* mitochondrial proteins and their biochemical functions

No	Name	Function
1	Coq4	CoQ biosynthesis
2	Lon protease homologue	Chaperone/protease
3	Prohibitin/BAP37 (PHB2 homologue)	Respiratory chain assembly
4	Valyl-tRNA synthetase	Translation
5	Elongation factor g (EF-G)	Translation
6	50s RPL24	Translation
7	50s RPL17	Translation
8	DNA-directed RNA polymerase	Transcription
9	EF-Tu	Translation
10	50S RPL2	Translation
11	Citrate synthase	TCA cycle
12	Isocitrate dehydrogenase (NADP-dependent)	TCA cycle
13	Succinyl-CoA sythetase beta subunit	TCA cycle
14	Succinate dehydrogenase Fe-S subunit	TCA cycle/complex II
15	Fumerate hydratase class I	TCA cycle
16	NADH dehydrogenase	Complex I
17	Rieske Fe-S protein 3	Complex III
18	Cytochrome c1	Complex III
19	ATP synthase beta subunit	Complex V
20	ATP synthase gamma subunit	Complex V
21	ATP synthase alpha subunit	Complex V
22	ATP synthase delta subunit	Complex V
23	Dihydroxy hexaprenylbenzoate methyltransferase	CoQ biosynthesis
24	Geranyl diphosphate synthase/prenyl transferase (Coq1)	CoQ biosynthesis
25	Coq2	CoQ biosynthesis
26	Coq5 (CoQ synthesis methyltransferase)	CoQ biosynthesis
27	Coq8 (ubiquinol-cytochrome c reductase assembly protein, ABC1)	CoQ biosynthesis
28	Branched-chain alpha keto-acid DH E1 alpha subunit	Amino acid degradation
29	Branched-chain alpha keto-acid DH E1 beta subunit	Amino acid degradation
30	Mitochondrial serine hydroxymethyltransferase	One carbon metabolism
31	Rhodanese	Amino acid degradation/ Cyanide detoxification
32	Mitochondrial intermediate processing peptidase (MPP)	Import
33	MPP alpha subunit	Import
34	MPP beta subunit	Import/complex III
35	HSP-60/CPN-60	Import
36	Mitochondrial phosphate carrier	Transport
37	Delta aminolevulinate synthase	Heme synthesis
38	HSP-70/DnaK	Protein folding
39	Dihydroorotate dehydrogenase	Pyrimidine biosynthesis
40	GrpE	Protein folding

Table 2 Structure, genomics and proteomics of mitochondrial and apicoplast organelles in the asexual stage of *P. falciparum*

Property	Mitochondrion	Apicoplast
Size (in trophozoite stage)	1.0x0.2 μm	1.6x0.3 μm
Membrane layer	2	3-4
Number per cell	single	single
DNA:		
-size	6 kb	35 kb
-shape	linear	circular
-copy number	>20	>15
-% A + T content	69%	86%
- gene encoding protein	3	23
tRNA	import	import
Protein identified´	381	551
Replication:		
-mechanism	phage-like	bi-directional theta, rolling circle
-replicating enzyme	unknown	multienzyme complex
Transcription/Translation	bacterial-type, 70S ribosome	bacterial-type, 70S ribosome

Representing ~7.1% and ~10% of the total proteins encoded by the parasite genome.

Table 3 Functional/metabolic roles between mitochondrial and apicoplast organelles in the asexual bloog stage *P. falciparum*

Property	Mitochondrion	Apicoplast
Electron transport system	present	absent
Oxygen consumption	present	absent
Oxidative phosporylation/ ATP synthesis	absent	absent
Pyrimidine synthesis	involved	not involved
Tricarboxylic acid cycle	present	absent
Ubiquinone biosynthesis	present	absent
Heme biosynthesis	involved	involved
Fatty acid biosynthesis´	absent	present
Isoprenoid biosynthesis	absent	present
Redox and antioxidant system	present	possibly present
Cellular signaling/homeostasis	present	unknown

The operating fatty acid biosynthesis requires the utilization of NADH as an electron donor and the production of fatty acid as an electron acceptor, however, it is presently unknown for an antioxidant defense system in the apicoplast.

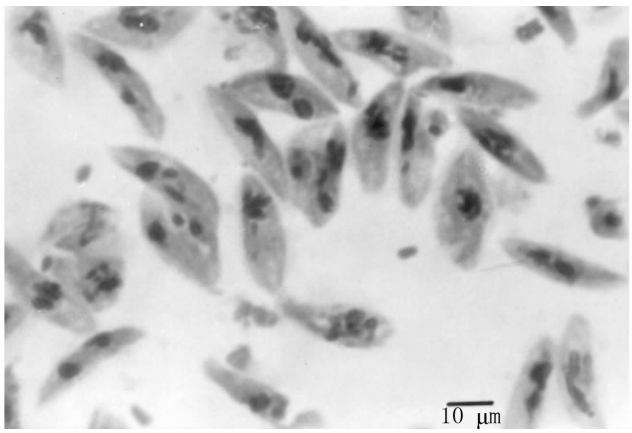

Figure 1 Maturing sexual satge of *Plasmodium falciparum* from an *in vitro* culture. (Giemsa)

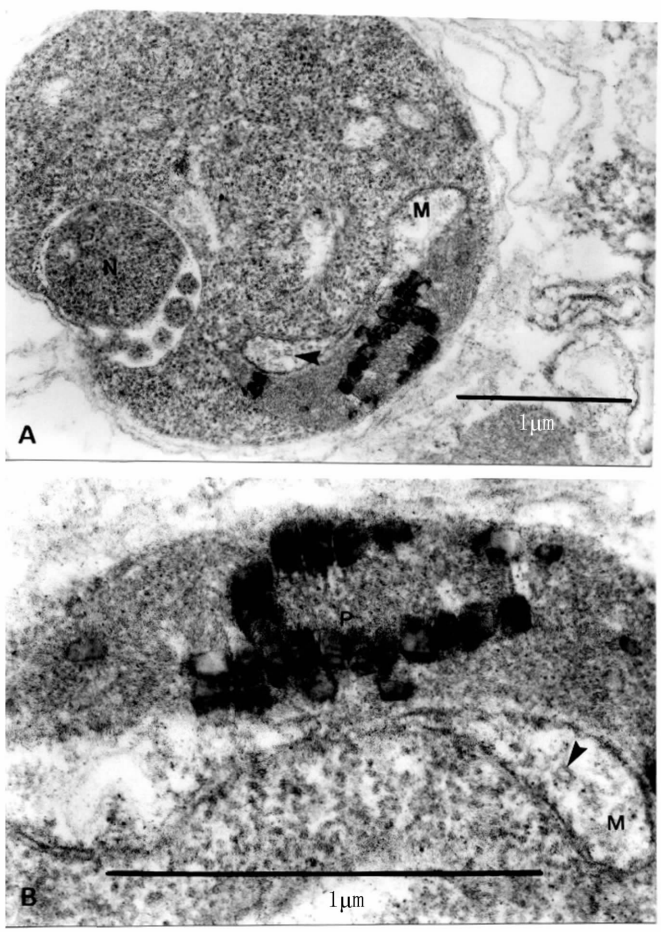

Figure 2 Asexual stage of *Plasmodium falciparum* (trophozoite stage) polymerized from catabolized heme in a food vacuole (TEM) . A: Single mitochondrion with a clearly double membrane organelle, and an elongated form, preparing for binary fission; B: Single tubular cristate structure(arrowhead) in the organelle at higher magnification, classified as type I mitochondrion . N: Nucleus; M: Mitochondria; P: Crystalline hemozoin pigment.

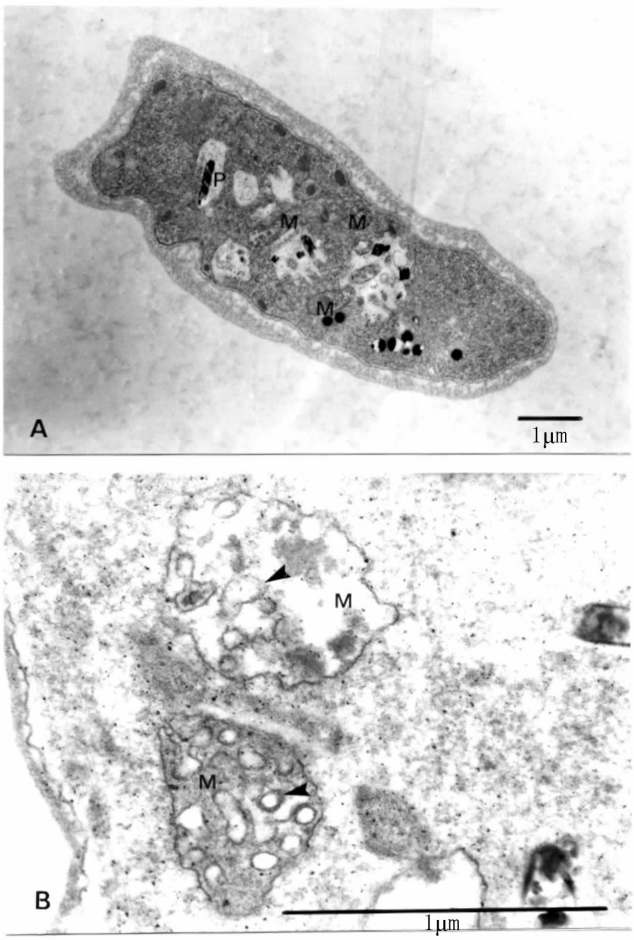

Figure 3 Sexual stage of *Plasmodium falciparum* (gametocyte stage IV) (TEM). A: Many mitochondria in a single parasite B: Two organelles containing several cristae (arrowheads) clssified as type II mitochondrion. M: Mitochondria; P: Pigment.

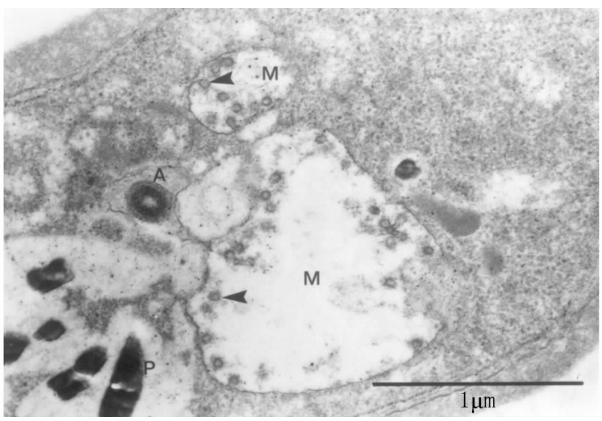

Figure 4 Type III mitochondria in the maturing sexual stage of *Plasmodium falciparum* (TEM). Electron-dense and finely compact of the tubular cristae (arrowheads) are typically found with this type of the mitochondrion. An apicoplast, associated to one of the mitochondria, was observed with a multi-membraneous organelle, containing electron-dense matrix and absence of internal cristae. M: Mitochondria; P: Pigment; A: Apicoplast. It is noted that the mitochondrial localization is closely associated to the food vacuole, a place where hemoglobin is degraded to free amino acids and hemozoin pigment is formed.

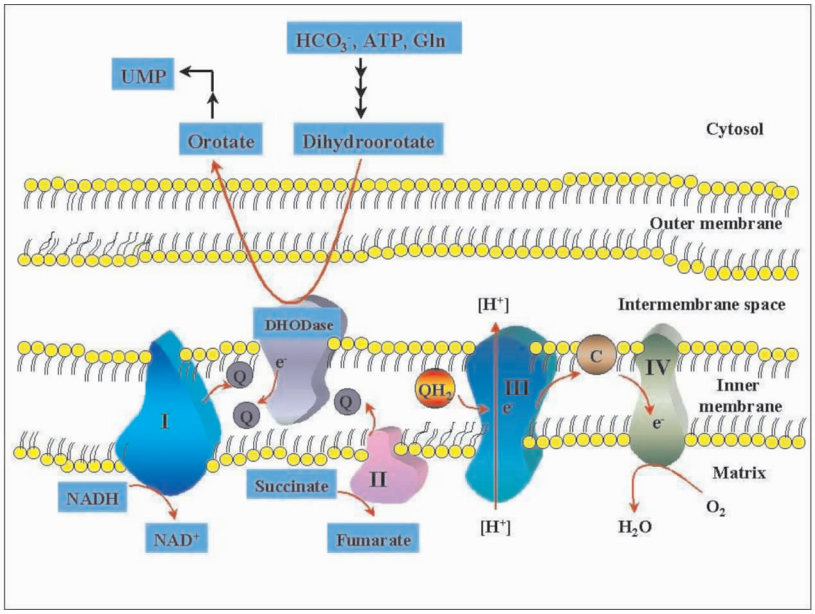

Figure 5 A diagram of the proposed arrangement of the mitochondrial electron transport system and its link with pyrimidine biosynthesis *via* dihydroorotate dehydrogenase in the asexual and sexual blood stages of *Plasmodium falciparum*. Dihydroorotate dehydrogenase (DHODase), an inner membrane protein of the pyrimidine pathway, generates electrons to the mitochondrial electron transporting complexes, containing NADH – ubiquinol reductase (a single component of complex I, NADH dehydrogenase), succinate – ubiquinone oxidoreductase (succunate dehydrogenase, complex II), cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV) which is the final electron acceptor. Q, oxidized ubiquinone; QH_2, reduced ubiquinone; C, cytochrome C.

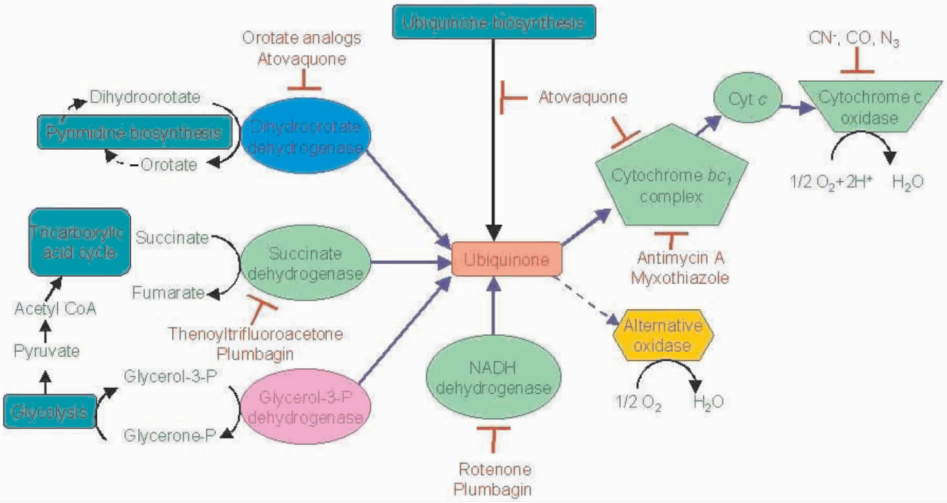

Figure 6 A diagram of proposed mitochondrial metabolic networks of *Plasmodium falciparum*. The mitochondrial electron transport complexes contain a single polypeptide NADH dehydrogenase, two – subunits succinate dehydrogenase, cytochrome c reductase (cytochrome bc_1) and cytochrome c oxidase. Alternative oxidase as a cyanide (CN^-) insensitive – branched pathway (shown in a broken line) and glycerol – 3 – phosphate (glycerol – 3 – P) dehydrogenase are also included in the respiratory chain. A part of the pyrimidine biosynthesis is shown and linked to coenzyme ubiquinone. The ubiquinone plays a central role for electron and proton transferring coenzyme. Cytochrome c (Cyt c, a component of cytochrome bc_1 complex) is also shown serving an electron transferring protein. Glycolysis, possibly links to the TCA cycle, is operating in the cytosol. The tree – bars indicate known active sites of inhibitors and the antimalarial atvaquone, plumbagin. The diagram is adapted from web site: http://sites.huji.ac.il/malaria.

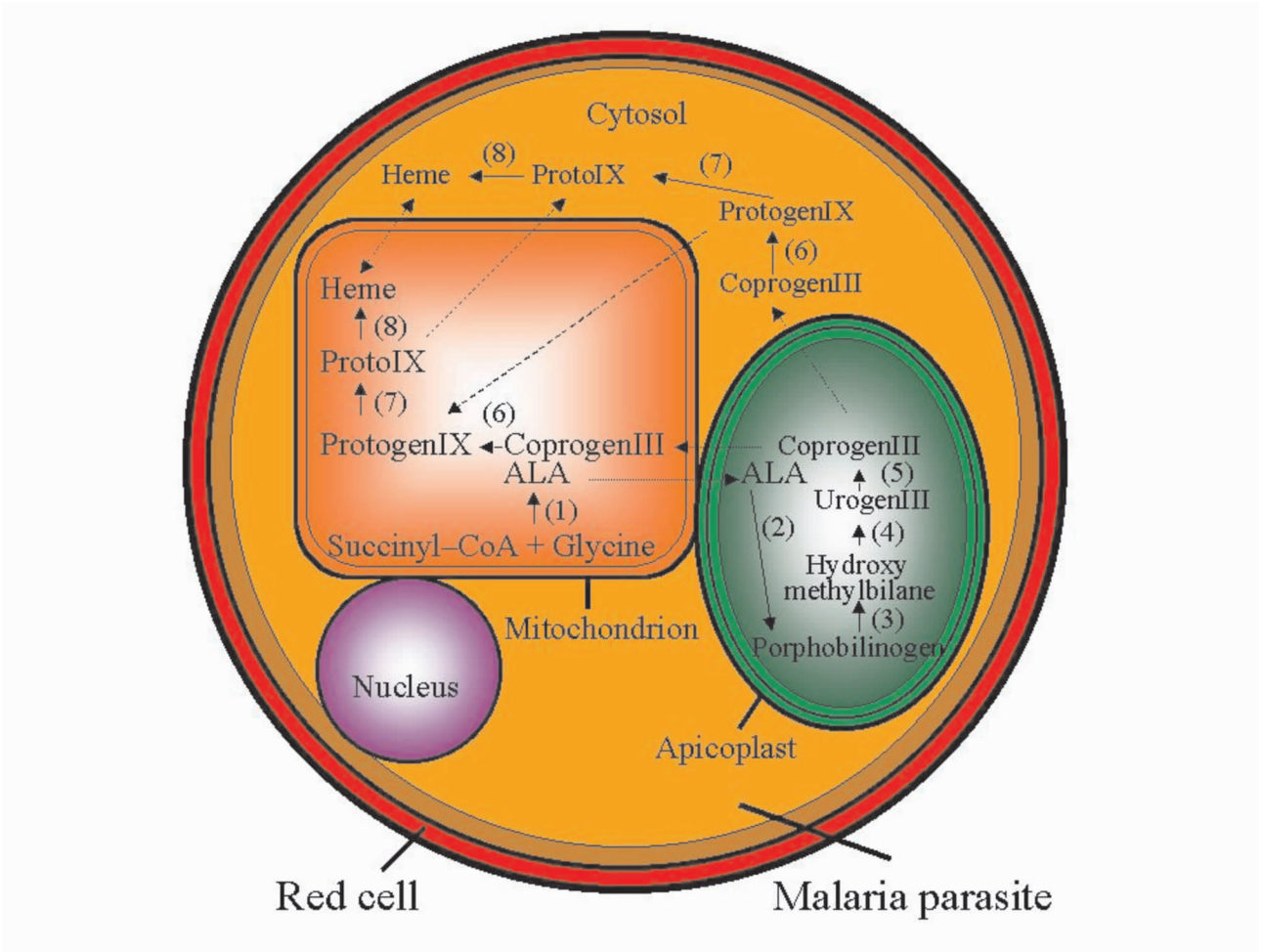

Figure 7 A schematic representation of *de novo* heme biosynthetic pathway of *Plasmodium falciparum*. The parasite's heme pathway operates in three possible compartmentations: mitochondrion, apicoplast and cytosol. Solid and broken indicate eight enzymatic Reactions (1→8) and proposed exchange of metabolites among three subcellular compartments, respectively. δ-aminolevulenic acid synthase catalyzes the condensation of succinyl – CoA and glycine (Reaction 1) to produce δ-aminolevulenic acid (ALA) in the mitochondrion, porphobilinogen synthase and hydroxylmethylbilane synthase catalyze ALA to prophobilinogen (Reaction 2) and then to hydroxymethylbilane (Reaction 3) in the apicoplast. The fourth and the fifth enzymes, uroporphyrinogenIII (UrogenIII) synthase (Reaction 4) catalyzing the production of UrogenIII, and UrogenIII decarboxylase (Reaction 5) converting UrogenIII to coproporphyrinogenIII (CoprogenIII), occur also in the apicoplast. The last three enzymes of the pathway, CoprogenIII oxidase (Reaction 6) catalyzing CoprogenIII to protoporphyrinogenIX (Protogen IX), ProtogenIX oxidase (Reaction 7) converting ProtogenIX to protoporphyrin IX (ProtoIX), and ferrochelatase (Reaction 8) producing heme from ProtoXI, are possibly operating in either cytosol or mitochondrion. The hypothetical pathway for the functional heme biosynthesis in the parasite is adapted from Sato *et al* [94].

35 kb of its original algal plastid genome and synthesizes 23 proteins[123]. Apicoplast DNA is inherited only through the female gamete [55], and the organelle DNA replication occurs by a unique enzyme complex synthesized by an open reading frame encoding contiguous DNA polymerase, DNA primer and DNA helicase components [124]. More than 500 proteins predicted to function in the apicoplast have been identified using the bioinformatic approaches and experimental evidences, leading to rapid advancement in metabolic maps and functional determinations [125]. We have summarized the present knowledge on properties, stuctures, genomics, transcriptomics, proteomics, and metabolic/functional roles in the mitochondrion and apicoplast of *P. falciparum* (Table 2, 3).

Aided by the recent progress of the malarial genome database, we now have a better understanding of several metabolic networks confined with the apicoplast. The primary ones are type II fatty acid biosynthesis (acetyl-CoA ---> fatty acids) and isoprenoid biosynthesis (non-mevalonate pathway). The type II *de novo* fatty acid synthetic pathway is catalyzed by separate enzymes as demonstrated in bacteria and plants, unlike the type I fatty acid pathway in mammals which were shown to be multifunctional enzymes [125]. Detailed characterization of lipoylation pathways involving pyruvate dehydrogenase complex indicates that the apicoplast can function in converting pyruvate to acetyl-CoA for use in fatty acid biosynthesis which it is absent in the mitochondrion [126, 127], but the other lipoylation pathway involving α-keto acid dehydrogenase complex is located in the parasite´s mitochondrion[106]. This is an unusual property of the mitochondrion, named as the strange organelle [128]. The organelle cannot produce acetyl-CoA, but takes it up from the apicoplast for the TCA cycle to generate NADH and other metabolites. In the isoprenoid biosynthesis using isopentenyl diphosphate as the precursor, the apicoplast operates the non-mevalonate pathway using the enzymes 1-deoxy-D-xylulose-5-phosphate (DOXP) synthase and DOXP reductoisomerase, similar to those of bacteria and algae [123].

It has been proposed earlier that the malaria parasite imports a nearly complete set of host-cell heme biosynthetic enzymes to use for its own machinery apparatus to produce heme [129]. Later, all eight enzymes required for the heme *de novo* pathway have been identified in the nuclear genome of the parasite [61]. The malaria parasite, unlikes any other organisms, has the ability to synthesize heme *de novo* by sharing the pathway within the boundaries of the apicoplast and the mitochondrial organelle (Figure 7) [94, 125, 130]. The localization of the first three enzymes, δ-aminolevulenic acid synthase (ALAS), porphobilinogen synthase (PBGS) and hydroxylmethylbilane synthase (HMBS) operating in the pathway has been verified using a GFP reporter in live transfected *P. falciparum* [94]. ALAS is targeted to the mitochondrion, but PBGS and HMBS are targeted to the apicoplast. The fourth and the fifth enzymes, uroporphyrinogen III synthase (UROS, Reaction 4) and uroporphyrinogen III decarboxylase (UROD, Reaction 5), have apparent apicoplast targeting sequences at their N-terminus. The last three enzymes of the pathway, coproporphyrinogen III oxidase (CPO, Reaction 6), protoporphyrinogen IX (PPO, Reaction 7) and ferrochelatase (FC, Reaction 8), lack the bipartite sub-structures at their N-terminus [94]. Hence enzymes UROS and UORD are predicted to be apicoplastic proteins, and enzymes CPO, PPO and FC are either cytosolic or mitochondrial proteins. This suggests a mechanistic model for multiple intracellular localization of the parasite organelle proteins, especially in the *de novo* heme biosynthesis. Some human mitochondrial proteins have also multiple subcellular compartments, their detailed targeting mechanisms recently reviewed[131]. Compartmentation of all heme enzymes in the parasite remains to be verified. It has been hypothesized that an exchange of metabolites in the pathway to produce heme between the two organelles ensues, including organelle attachment[94, 125]. The proposed contact/attachment of both organelles is evident by visualizing their close apposition on subcellular fractionations and electron micrographs using either *P. falciparum* (Figure 4) or other malaria parasites [30, 132].

CONCLUSIONS AND FUTURE PROSPECTS

Based on the morphological, biochemical and genetic findings in the asexual and sexual stages of the human malaria parasite *P. falciparum*, mitochondrial heterogeneity may have functional significance for growth and development and completion of life cycle. The mitochondrial structures and functions may

reflect an evolution of *Plasmodium* spp. in which they are living in relatively low oxygen environments of the human host to maintain their redox balance, and also an organelle metabolic adaptation to life in the mosquito vector. It is necessary to study the biochemistry and physiology of the mitochondrion in more detail, for instance, membrane potential, differences in the mechanism of energy metabolism, functionality of the tricarboxylic acid cycle, oxidative phosphorylation and ATP synthesis, functional properties of the electron transport complexes, roles of ubiquinone and heme biosynthesis, and oxygen tension on the survival of the parasites circulating in the human blood[74, 107, 133].

Regulation of the tricarboxylic acid cycle, the electron transport system and the oxidative phosphorylation for energy metabolism needs to be considered [77-79, 134]. Understanding of the parasite´s organelle biogenesis is still requiring, including the involvement of cellular signaling essential for the process [71]. In addition, detailed internal organization of the organelle related to its metabolic adaptation and heterogeneity should be further elucidated using novel electron microscopic tomography [45, 135].

Special thanks to the malarial genome database [61], about 250-380 proteins are predicted to target to the mitochondrion post-translationally. These include some enzymes of the pyruvate dehydrogenase complex, the complete tricarboxylic acid cycle enzymes, many electron transport complexes and ATP synthase[46-47, 51, 61-63, 128]. Functional analyses remain to be elucidated using techniques such as gene knock-out, RNA interference, microarray and metabolomics [32, 35, 42]. The mitochondrion is a chemotherapeutic target for antimalarial drug development, for example, the enzyme dihydroorotate dehydrogenase [136]. In our post-genomics era, proteomics should be performed with mitochondria from all stages of the human parasite, in the presence or absence of any novel compounds affecting biogenesis and functions of the organelle. Identification of genes/proteins responsible for the mitochondrial heterogeneity throughout the life cycle of the parasite is, likewise, necessary.

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