Metabolic maps and functions of the *Plasmodium* mitochondrion

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Abstract

The mitochondrion of *Plasmodium* species is a validated drug target. However, very little is known about the functions of this organelle. In this review, we utilize data available from the *Plasmodium falciparum* genome sequencing project to piece together putative metabolic pathways that occur in the parasite, comparing this with the existing biochemical and cell biological knowledge. The *Plasmodium* mitochondrion contains both conserved and unusual features, including an active electron transport chain and many of the necessary enzymes for coenzyme Q and iron-sulphur cluster biosynthesis. It also plays an important role in pyrimidine metabolism. The mitochondrion participates in an unusual hybrid haem biosynthesis pathway, with enzymes localizing in both the mitochondrion and plastid organelles. The function of the tricarboxylic acid cycle in the mitochondrion is unclear. We discuss directions for future research into this fascinating, yet enigmatic, organelle.

Introduction: the malaria jigsaw puzzle

Plasmodium species are the causative agents of malaria. They divide their life cycle between an *Anopheles* mosquito vector and a vertebrate host, which, depending on the species, ranges from primates and rodents to birds and reptiles. In humans, four species of *Plasmodium* cause malaria, with the disease manifesting during what is known as the asexual or intra-erythrocytic cycle of the parasite. During this cycle, *Plasmodium* parasites invade erythrocytes, consume the host cell haemoglobin, before undergoing a complex division process known as schizogony (Aikawa *et al*., 1966; Aikawa, 1971; Francis *et al*., 1997; Cowman & Crabb, 2002). Schizogony culminates in the formation of multiple daughter parasites known as merozoites, which burst from the erythrocyte and re-invade uninfected erythrocytes (Cowman *et al*., 2002; Wickham *et al*., 2003). The four species of human malaria differ in the periodicity of this cycle, as well as in the outcomes of the disease. *Plasmodium falciparum* is the species responsible for most of the approximately 1 million deaths caused by malaria every year (Greenwood *et al*., 2005). The species targets a protein to the erythrocyte cell surface that is capable of sequestering infected erythrocytes to blood vessels, and it is thought that this process is the cause of cerebral malaria, a major cause of malaria-related deaths (Crabb *et al*., 1997; Miller *et al*., 2002; Wassmer *et al*., 2003).

Despite years of effort, the development of an effective vaccine against malaria remains an unrealized goal. To date, antimalarial drugs remain the major way to treat the disease. Well-established drugs, such as sulphadoxine/pyrimethamine, chloroquine derivatives and artemisinin, are the most common treatments for malaria throughout the world. However, there is widespread resistance to many of these drugs, resulting in an urgent need to identify and develop alternatives (Greenwood *et al*., 2005). Identifying novel drugs requires a sound biological understanding of the parasite at both a cellular and a biochemical level. Given the medical and economic importance of the disease, it is perhaps surprising that the biological basis of major antimalarials such as chloroquine and artemisinin are still not fully understood (Eckstein-Ludwig *et al*., 2003; Bray *et al*., 2005; Li *et al*., 2005). This lack of understanding reflects a broader gap in our scientific knowledge

about *Plasmodium* parasites that is only slowly being bridged.

A major step forward in reaching a more comprehensive understanding of *Plasmodium* biology was the publication, in 2002, of the entire genome of *P. falciparum* (Gardner *et al.*, 2002). This provided a list of the 5000 or so genes that encode all the necessary biological functions of the parasite. However, much like opening a box containing 5000 jigsaw pieces, the information from the malaria genome provides, at best, a scrambled image of how the parasite functions. The challenge for malaria researchers in the post-genomic era is to piece together the genomic jigsaw and obtain a meaningful picture of the malaria parasite.

Malaria's enigmatic organelle

The mitochondrial organelle was probably acquired at the dawn of eukaryotic evolution, through an endosymbiotic process whereby an α-proteobacterium became incorporated into a proto-eukaryote (Martin & Muller, 1998; Moreira & Lopez-Garcia, 1998). The textbook function of mitochondria is in the oxidation of substrates in the tricarboxylic acid or citric acid cycle. Electrons from this process are fed into an electron transport chain at the inner mitochondrial membrane, which ultimately reduces oxygen to water. The energy released in this transport of electrons is used to generate a proton gradient across the inner membrane, which is harnessed by ATP synthase in the generation of ATP, the so-called energy currency of cells. This process of ATP generation through electron transport is known as oxidative phosphorylation.

It is becoming increasingly clear that mitochondrial functions are considerably more diverse than textbooks would suggest. In recent years, there has been extraordinary focus on the role of mitochondria in programmed cell death, or apoptosis (Desagher & Martinou, 2000). Mitochondria of mammalian cells also function in β-oxidation of fatty acids, whereby fatty acids are broken down to substrates that feed into the tricarboxylic acid cycle. Mitochondria are also involved in several biosynthetic processes, creating compounds necessary to support the electron transport chain. These include the biosynthesis of coenzyme Q (ubiquinone), an important electron carrier in the electron transport chain, haem, a prosthetic group in cytochromes, several of which are crucial in the electron transport chain, and iron-sulphur clusters, which also function as prosthetic groups in several proteins of the electron transport chain.

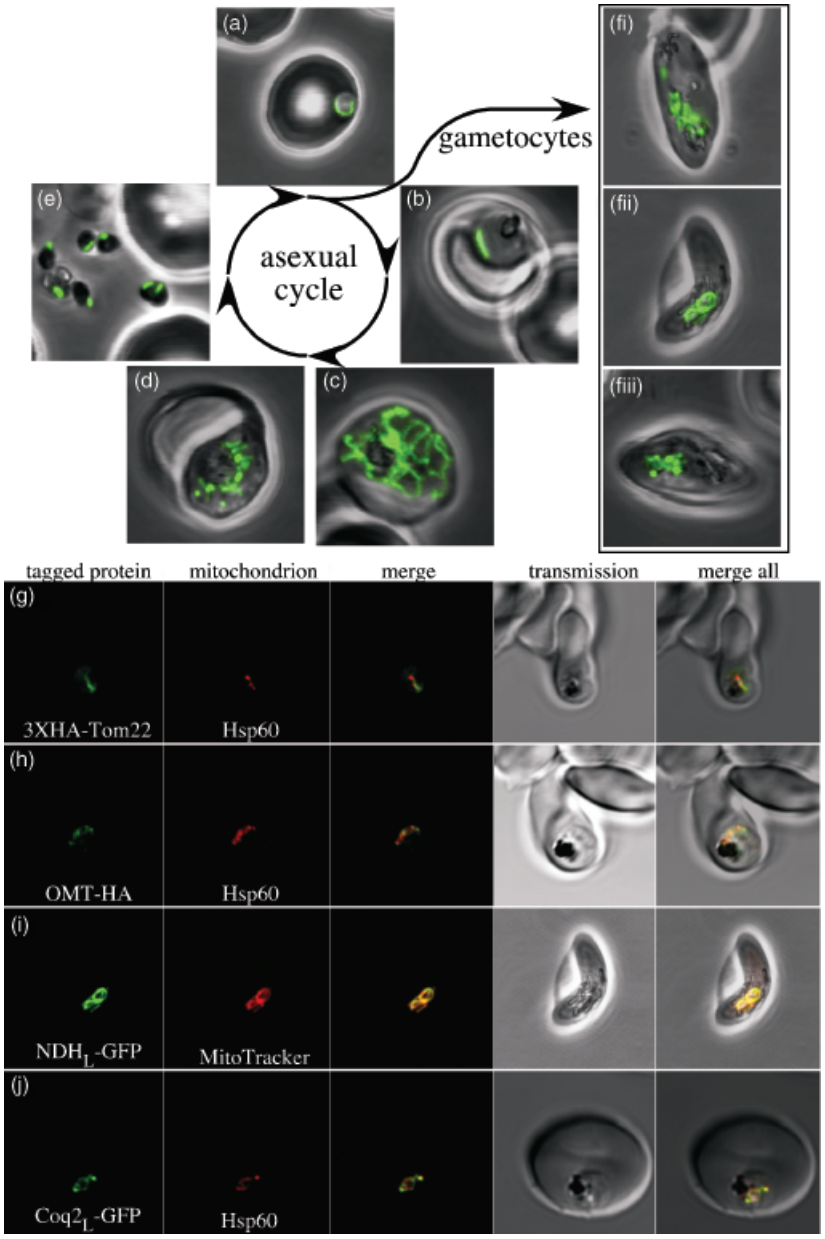
Despite well-characterized roles in other organisms, the functions of the mitochondrion of *Plasmodium* have remained somewhat enigmatic. Early biochemical work indicated that during asexual stages, *Plasmodium* converts the vast majority of glucose obtained from the host blood serum via glycolysis to lactate (Bryant *et al.*, 1964; Scheibel & Pflaum, 1970). These studies found that when fed radioactive glucose, *Plasmodium* converted most of this to lactate, with minimal breakdown to tricarboxylic acid cycle intermediates (Bryant *et al.*, 1964; Scheibel & Pflaum, 1970), suggesting the tricarboxylic acid cycle does not function during asexual stages. With a major purpose of the mitochondrion seemingly absent, many interesting questions arise regarding the function of the *Plasmodium* mitochondrion. In this post-genomic era for malaria research, the potential exists to obtain many new insights into these questions as well as re-evaluating the existing research. In this paper we attempt to gather together the genomic jigsaw pieces that make up the mitochondrial organelle of malaria parasites, and provide a scaffold around which future research can complete the picture of how this organelle functions. We have generated a putative metabolic map of the mitochondrion, based on gene homologies, and we attempt to synthesize our putative metabolic pathways with what is already known about this organelle, and generate hypotheses about what the *Plasmodium* mitochondrion is really doing. A list of putative mitochondrial genes generated by our analysis is presented as Supplementary Table 1. In this review, we will not cover proteins involved in the replication, transcription and translation of the mitochondrial genome. Identifying many of these 'housekeeping' proteins is a difficult task, made more so by the presence of a second endosymbiotic organelle, the apicoplast, in these parasites. The housekeeping machinery of the mitochondrion and apicoplast are very similar, and indeed some proteins may be shared between these two organelles, or with the nuclear housekeeping machinery. Identifying housekeeping proteins, such as tRNA synthetases and ribosomal proteins, will require a rigorous experimental-based approach, and will, no doubt, yield many interesting insights.

Structure of the *Plasmodium* mitochondrion

The evolutionary history of mitochondria dictates that these organelles are essentially bacterial cells living inside a eukaryote. Much like the alpha-proteobacteria from which they are derived, mitochondria are surrounded by two membranes. The innermost of these membranes typically forms invaginations known as cristae. Electron microscopy dating from the late 1960s clearly identified double membrane-bound mitochondrial organelles in *Plasmodium* parasites (Aikawa, 1966; Hepler *et al.*, 1966; Rudzinska, 1969). Rudzinska noted the presence of cristae in bird malaria species, but found that asexual stages of mammalian malaria species such as *P. falciparum* lacked obvious cristae (Rudzinska, 1969). Located in tight association with the mitochondrion is a multi-membrane bound organelle, which

early electron microscopists dubbed the spherical body (Aikawa, 1966; Hepler *et al.*, 1966). These researchers speculated that the spherical body functioned as a metabolic store for the mitochondrion, either as an energy reservoir (Aikawa, 1966) or a lipid store (Hepler *et al.*, 1966). We now know this spherical body to be the plastid (apicoplast) organelle of *Plasmodium* (Kilejian, 1991; McFadden *et al.*, 1996; Kohler *et al.*, 1997), and that the association between these organelles exist throughout the asexual life cycle (Hopkins *et al.*, 1999; van Dooren *et al.*, 2005).

During the asexual life cycle, the mitochondrion exists as a single organelle (Divo *et al.*, 1985; Slomianny & Prensier, 1986), which is generally elongated or branched (van Dooren *et al.*, 2005) (Fig. 1a–e). The bacterial history of the mitochondrion means that it must properly divide and segregate into daughter cells, a daunting prospect in asexual stage *Plasmodium* cells, which undergo a complicated form of cell division known as schizogony. Schizogony begins with multiple rounds of nuclear division that are not coupled to cytokinesis. This produces a multinucleated schizont cell, containing upwards of 30 nuclei. Schizonts then undergo cytokinesis, with each nucleus properly segregating into a daughter merozoite cell. Observations of the mitochondrion during schizogony indicate that it becomes highly branched, before dividing into multiple organelles very late in schizogony (van Dooren *et al.*, 2005) (Fig. 1c–e).



Divided mitochondria associate with divided apicoplasts, and as an organellar pair they segregate into daughter merozoites (van Dooren *et al*., 2005). Electron microscope images suggest that the organellar pair moves into daughter merozoite buds posterior or lateral to the nucleus (Aikawa, 1966; Hepler *et al*., 1966). The molecular mechanisms by which *P. falciparum* mitochondria divide are unclear. No homologue of the bacterial division protein FtsZ is present in the *P. falciparum* genome, suggesting that mitochondria do not undergo the binary fission seen in some mitochondria (Beech *et al*., 2000). Mitochondrial division is well studied in yeast and mammalian cells. Here, dynamin-like proteins are thought to play an important role. The *P. falciparum* genome contains two homologues of dynamin-related proteins (PlasmoDB identification numbers PF10_0368 and PF11_0465), although the role of these in mitochondrial fission has not been determined. *Plasmodium falciparum* also contains a homologue of Fis1 (MAL13P1.139), an outer membrane protein crucial to mitochondrial fission in both yeast and mammalian cells (Mozdy *et al*., 2000; GvD and GIM, unpublished), suggesting that mitochondrial fission is similar to that in other organisms. Although *P. falciparum* mitochondria undergo fusion processes (van Dooren *et al*., 2005), no homologues of mitochondrial fusion proteins identified in yeast, such as Mgm1, Fzo1 and Ugo1, are apparent in the genome.

Plasmodium parasites have a complex life cycle that consists of other proliferative stages in the liver and the mosquito mid-gut. Nothing is known about organellar division and segregation in these cells, and very little is known about the general appearance of mitochondria in any of the other life stages. As will become apparent during this paper, the lack of knowledge about the structure of mitochondria mirrors the paucity of general knowledge about them during these life stages. During each round of asexual division, a small number of merozoites differentiate into gametocyte cells. When ingested by the mosquito, gametocytes differentiate into gametes and sex occurs. Gametocytes often contain multiple rounded mitochondria (Krungkrai *et al*., 2000; Fig. 1, F.i), although their mitochondria may also be branched (Fig. 1, F.ii) or clustered (Fig. 1, F.iii). Gametocyte mitochondria retain the close association with the apicoplast seen in asexual stages (GvD and GIM, unpublished), and it is thought that the gametocyte mitochondria in species such as *P. falciparum* develop distinct tubular cristae (Krungkrai *et al*., 2000). Electron microscope work suggests that oocysts, the proliferative stage of *Plasmodium* that occurs in the mosquito midgut, also contain multiple mitochondria (Aikawa, 1971).

Destination mitochondrion

Plasmodium mitochondria contain a small 6-kb genome that encodes three protein-encoding genes (namely cytochrome *b*, cytochrome oxidase I and cytochrome oxidase III) and fragmented rRNA genes (Aldritt *et al*., 1989; Vaidya *et al*., 1989; Feagin, 1992; Feagin *et al*., 1997). As such, it one of the smallest mitochondrial genomes yet sequenced, although the mitochondria-like organelles of some organisms have probably lost their entire genome. The mitochondrial genome of *P. falciparum* lacks many genes typically found on the genome of other organisms. These include tRNA genes, subunits of NADH dehydrogenase, and the gene encoding the cytochrome oxidase II (CoxII) protein. By analogy with the related parasite *Toxoplasma gondii*, *P.*

Fig. 1. (a–f) Mitochondrial structure during the intraerythrocytic stages of *Plasmodium falciparum*. Cells were transfected with the N-terminal leader sequences of citrate synthase (a–e, f.i) and f.iii); Tonkin *et al*., 2004; van Dooren *et al*., 2005) or NAD(P)H dehydrogenase (f.ii); see also **i** below) fused to green fluorescent protein. a–e) Development of the mitochondrion during the asexual cycle. In ring (a) and trophozoite (b) stages, the organelle is typically small and tubular in appearance. By mid schizogony, the organelle is highly branched in its appearance (c). The mitochondrion divides late in schizogony (d) and a single mitochondrion segregates into each daughter merozoite (e). *Plasmodium falciparum* cells leave the asexual cycle by differentiating into gametocytes, which are ingested by mosquitoes, where they further differentiate into gametes. The mitochondrial organelle of gametocytes can adopt several morphologies. It may comprise several mitochondria, clearly separate from one another (F.i). It may be a single, branched organelle (f.ii), or may consist of a cluster of mitochondrial lobes towards the centre of the cell (f.iii). The relationships between these different morphologies are not understood. The morphology of the mitochondrial organelle in other stages of the life cycle is also largely unknown. g) *Pf*Tom22 localizes to the mitochondrion of *P. falciparum*. *Pf* Tom22 was tagged with a 3X-HA epitope tag at its N-terminus. An immunofluorescence assay was performed as previously described (Tonkin *et al*., 2004), with 3XHA-*Pf*Tom22 labelled in green and the mitochondrial marker Hsp60 labelled in red. The merged image (central panel) shows apparent overlap between the two proteins, indicating that *Pf*Tom22 is a mitochondrial protein. h) The oxoglutarate/malate transporter homologue of *P. falciparum* (*Pf*OMT) localizes to the mitochondrion of *P. falciparum*. *Pf*OMT was tagged with a 1XHA tag at its C-terminus (green) and co-labelled with anti-Hsp60 antibody (red). The merged image shows apparent overlap between the two proteins, indicating that *Pf*OMT is a mitochondrial protein. **i**) The single-subunit NAD(P)H dehydrogenase homologue of *P. falciparum* (*Pf*NDH) localizes to the mitochondrion. The N-terminal extension of *Pf*NDH was fused to green fluorescent protein (GFP, green), with live parasites co-labelled with MitoTracker Red (red), a mitochondrial marker. In this gametocyte image, there is clear overlap, indicating that *Pf*NDH localizes to the mitochondrion. In asexual stages, when protein expression levels are probably higher, a considerable portion of *Pf*NDH_L-GFP also localizes to the cytosol, suggesting that import of this fusion protein is somewhat inefficient. j) The coenzyme Q biosynthesis protein Coq2 homologue of *P. falciparum* (*Pf*Coq2) localizes to the mitochondrion. The N-terminal portion of *Pf*Coq2 was fused to green fluorescent protein and labelled with anti-green fluorescent protein antibody (green). These fixed parasites were co-labelled with anti-Hsp60 antibody (red), with the apparent co-localization indicating that *Pf*Coq2 is a mitochondrial protein.

falciparum probably imports tRNA molecules from the nucleus and possibly the apicoplast (Esseiva *et al.*, 2004; Barbrook *et al.*, 2006). The lack of NADH dehydrogenase subunits results from the loss of this complex in *P. falciparum* parasites (see later), and an unusual splitting of the gene encoding the typically hydrophobic CoxII probably enables this protein to be imported from the nucleus (Funes *et al.*, 2002; Waller *et al.*, 2003).

During and after the process of endosymbiotic acquisition, the mitochondrion gave up most of its genes to the host cell nucleus (McFadden, 1999; Gabaldon & Huynen, 2004). The products of these prokaryote-derived genes, as well as mitochondrially targeted gene products of eukaryotic origin, must be trafficked back to the mitochondrion. The publication of the *P. falciparum* nuclear genome enabled us to begin looking for proteins that had a putative role in the *P. falciparum* mitochondrion through homology searching. We obtained the sequences of proteins with a known mitochondrial, or mitochondrial-related, function from well-studied organisms such as *Saccharomyces cerevisiae*, humans and *Arabidopsis* and performed BLASTP searches of the *P. falciparum* genome (www.plasmodb.org). We accepted a protein as probably having a mitochondrion function if it clearly grouped with other mitochondrial proteins in a phylogenetic analysis, or if the best BLAST hit outside the Apicomplexan phylum to which *Plasmodium* belongs was a likely mitochondrial protein.

Proteins can be targeted to the mitochondrion by several different pathways. The most common of these is via an N-terminal leader peptide, or presequence (Pfanner & Geissler, 2001). By fusing the presequence of mitochondrial proteins to a green fluorescent reporter protein, it has been shown that such presequences can be sufficient to target proteins to the mitochondrion (Fig. 1) (Sato *et al.*, 2003; Tonkin *et al.*, 2004). We compiled a list of proteins that very likely contained such a presequence, and used this as a positive data set in the generation of a computer algorithm called PlasMit, based on an artificial neural network, to identify other presequence-containing proteins (Bender *et al.*, 2003). We used this algorithm to further trim our list of putative mitochondrial proteins.

There are several other mechanisms by which proteins can target to the mitochondrion of other organisms. These include transmembrane domains, such as tail-anchor sequences, which typically target proteins to the outer mitochondrial membrane, and ill-defined internal signals that can target proteins to the inner membrane (Egan *et al.*, 1999; Pfanner & Geissler, 2001). Proteins with a presequence may also contain additional sequences to target them to subcompartments of the mitochondrion. All proteins targeted beyond the mitochondrial outer membrane must traffic across membranes via a series of well-defined protein complexes. We searched the *P. falciparum* database for homologues of these complexes, and our results are summarized in Fig. 2.

All proteins that are targeted beyond the outer membrane must traffic through the Translocase of the Outer Membrane (Tom) complex. *P. falciparum* contains two homologues of Tom complex proteins. Tom40 (PlasmoDB identification number PFF0825c) is a β-barrel protein that serves as the general import pore, translocating virtually all proteins into the mitochondrion, regardless of their targeting signal (Baker *et al.*, 1990; Kiebler *et al.*, 1990; Pfanner & Geissler, 2001). *Plasmodium falciparum* also contains a weak homologue of the Tom22 protein (PFE1230c). To determine whether the putative *Pf* Tom22 is localized to the mitochondrion, we fused a 3XHA construct to the N-terminus of this protein. Immunofluorescence assay indicate that this fusion protein co-localizes with the mitochondrial marker protein Hsp60 (Fig. 1g). It is likely that *Pf* Tom22 is embedded into the outer membrane via a trans-membrane domain (GvD, LS and GIM, unpublished). It functions as a receptor protein that binds mitochondrially destined proteins and transfers them to the Tom40 pore (Kiebler *et al.*, 1993; Pfanner & Geissler, 2001). All eukaryotes studied to date contain Tom22 and Tom40 homologues, which are together thought to form the core of the Tom complex (Meisinger *et al.*, 2001; Macasev *et al.*, 2004). It is probable that the *P. falciparum* Tom complex contains other subunits; the identification of Tom40 and Tom22 may facilitate the identification of these subunits.

After crossing the outer membrane at the general import pore, pre-sequence containing proteins are directed to a complex of the inner mitochondrial membrane known as the Tim23 complex (Pfanner & Wiedemann, 2002). The Tim23 complex translocates proteins across the inner membrane into the mitochondrial matrix. Most of the crucial components of the Tim23 complex have homologues in *P. falciparum* (Fig. 2). These include Tim23 (PF13_0300), Tim17 (PF14_0328) and Tim50 (PF07_0110). After protein translocation through the Tom40 pore, it is believed that the presequence interacts with the intermembrane space domain of Tom22 (Bolliger *et al.*, 1995), a process aided by Tim50 (Geissler *et al.*, 2002; Yamamoto *et al.*, 2002; Chacinska *et al.*, 2005). The presequence then passes to Tim23, which functions as the inner membrane pore through which the protein is translocated. Recent studies in yeast suggest Tim21 is crucial in regulating the translocation of the precursor protein from the Tom to the Tim23 complex (Chacinska *et al.*, 2005; Mokranjac *et al.*, 2005). However, no obvious homologue of Tim21 exists in the *P. falciparum* genome, suggesting novel regulatory mechanisms might exist. Once at the Tim23 channel, the precursor is drawn through into the matrix, a process that requires two energy sources, namely a membrane potential across the inner membrane and ATP (Pfanner & Wiedemann, 2002). The

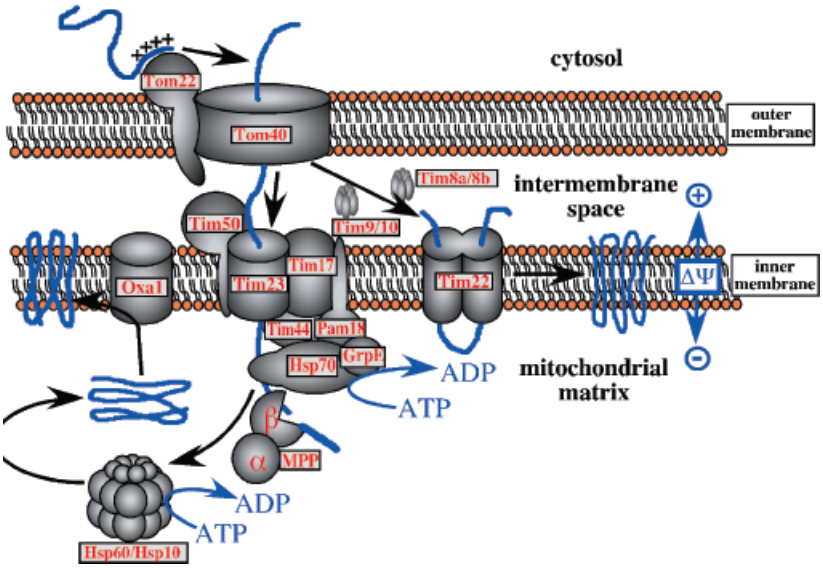


Fig. 2. Summary of the protein translocation components identifiable in the *Plasmodium falciparum* genome. Mitochondrially targeted proteins (blue line) cross the outer membrane via the so-called Tom complex. It is thought that positively charged residues of the N-terminal leader sequences of matrix proteins interact with receptor proteins such as Tom22, which mediate translocation across the outer membrane through the Tom40 pore. Upon translocation into the intermembrane space, proteins are passed through the Tim23 complex of the inner membrane into the matrix. This process is regulated by Tim17 and Tim50, and requires a matrix localized import motor consisting of Hsp70 and its cochaperones Tim44, Pam18 and GrpE. Upon successful translocation, the leader peptide is cleaved by a mitochondrial processing peptidase and the protein folded by chaperones such as Hsp60 and Hsp10. Mitochondrial inner membrane proteins can be targeted to their destination by several pathways. Proteins such as mitochondrial carrier proteins contain internal targeting signals that direct them to the Tim22 complex of the inner membrane, a process aided by so-called tiny-Tim proteins such as Tim8, Tim9 and Tim10. Tim22 functions to laterally insert these hydrophobic proteins into the inner membrane. Proteins may also insert into the inner membrane from the matrix, a process mediated by Oxal.

membrane potential functions in an electrophoretic manner to draw the positively-charged presequence through the Tim23 pore into the negatively charge matrix, while Tim17 functions to recruit an ATP-driven, Hsp70-based motor apparatus to the matrix side of the Tim23 complex, which interacts with the precursor and further functions to translocate the protein into the matrix (Chacinska *et al.*, 2005). Members of this presequence motor that are conserved in *P. falciparum* include mitochondrial Hsp70 (PF11_0351) and its co-chaperones Tim44 (PF11_0265), GrpE (PF11_0258) and Pam18 (PF07_0103; Dolezal *et al.*, 2005). Once in the matrix, a mitochondrial processing peptidase consisting of α- and β- subunits (PFE1155c and PFI1625c; van Dooren *et al.*, 2002) cleaves the precursor from the mature protein, which is folded by a chaperone complex consisting of Hsp60 (PF10_0153) and Hsp10 (Genbank accession no. CAE01413 Sato & Wilson, 2005). Previous studies have localized both *Pf*Hsp60 and *Pf*Hsp10 to the mitochondrion (Sato *et al.*, 2003; Tonkin *et al.*, 2004; Sato & Wilson, 2005).

After translocation into the matrix, some mitochondrial proteins utilize an additional targeting signal that directs them back to the inner membrane or intermembrane space (Pfanner & Geissler, 2001). This targeting mechanism is homologous to YidC-mediated membrane protein insertion in bacteria (Dalbey & Kuhn, 2004) and is mediated by the YidC homologue Oxa1 (MAL81.14) (Fig. 2). Other inner membrane proteins, including metabolite carrier proteins (see later), take a very different route to the inner membrane. These proteins lack a presequence, and instead contain poorly defined internal targeting signals. After passing through the general import pore of the outer membrane, these proteins interact with several small intermembrane space proteins known as 'tiny Tims' (Fig. 2). These include Tim8 (PFL2065c and PF14_0208), Tim9 (PF13_0358) and Tim10 (PFL0430w), and possibly function to prevent self-aggregation of the hydrophobic domains of the inner membrane proteins (Curran *et al.*, 2002a, b). Insertion of these inner membrane proteins is mediated by the Tim22 complex. This complex binds to precursor proteins and, in a step requiring an electrochemical gradient across the inner membrane, inserts it into a pore (Rehling *et al.*, 2003). From here, the protein is laterally inserted into the inner membrane (Fig. 2). The core, pore-forming subunit of this complex is Tim22, which is conserved in *P. falciparum* (PFF1330c). However, there are no clear homologues of other members of this complex, including Tim12, which is a tiny Tim involved in binding of the precursor to the Tim22 complex (Sirrenberg *et al.*, 1998), and Tim18 and Tim54, the exact functions of which are not yet clear.

A functional mitochondrion requires proteins to be targeted to a variety of intra-organellar compartments, including the outer membrane, the inter-membrane space, the inner membrane and the matrix. Protein targeting to these compartments is well understood in model organisms such as yeast. Our bioinformatic analysis indicates that the various translocases involved in mitochondrial targeting are conserved in *P. falciparum*, although there are likely to be subtle differences in the regulation of these processes that warrant further study. Our group and others have shown that proteins containing a presequence are targeted to the mitochondrion (Sato *et al.*, 2003; Sato & Wilson, 2004; Tonkin *et al.*, 2004; McMillan *et al.*, 2005). We have also demonstrated that carrier proteins can be targeted to the mitochondrion (see later), probably via the Tim22 pathway, and that outer membrane proteins such as Fis1 (see above) can be targeted courtesy of hydrophobic transmembrane domains (GvD and GIM, unpublished). Analysis of targeting pathways in *P. falciparum* offers some important insights into the evolution of the Tom and Tim complexes. The evolution of ways to target nuclear-encoded proteins into mitochondria must have occurred early in the acquisition of mitochondria. Since malaria and most other organisms probably split from the well-studied opisthokonts (yeast and animals) early in eukaryotic evolution (Stechmann & Cavalier-Smith, 2002), the fact that the core of the import complexes – namely Tom40/22, the Tim23 complex and Tim22 – are conserved across these lineages suggests that mitochondrial protein import was well established before the major radiation of the eukaryotes. Comparative analysis of different organisms will provide important insights into how import complexes evolved subsequent to the evolution of major eukaryotic lineages. For example, it has been noted that Tom20, a well-conserved receptor protein in opisthokonts, is absent from other eukaryotic lineages (Likic *et al.*, 2005). It is likely that *Plasmodium* similarly contains mitochondrial import components not found in opisthokonts. These components will provide clues as to the evolution of *Plasmodium* and the Apicomplexa as well as providing potentially novel drug targets. The challenge remains to identify these unique proteins.

A (dys)functional tricarboxylic acid cycle?

The tricarboxylic acid (TCA) cycle is the central wheel of mitochondrial metabolism, and has both biosynthetic and catabolic functions. The textbook function of the tricarboxylic acid cycle occurs in aerobic conditions, where pyruvate produced in cytosolic glycolysis is converted to acetyl-CoA, a 2-carbon substrate that feeds the tricarboxylic acid cycle. Acetyl-CoA combines with oxaloacetate to produce citrate, a reaction catalyzed by citrate synthase. Citrate is then broken down in a multi-step pathway that produces CO_2, NADH, $FADH_2$ and GTP, and ultimately regenerates oxaloacetate. Pyruvate produced in glycolysis is converted to acetyl-CoA by a large, multi-enzyme, mitochondrially localized complex called pyruvate dehydrogenase. Acetyl-CoA is also generated through fatty acid catabolism via β-oxidation, a process that can also be mitochondrially localized. While acetyl-CoA provides the major entry point for carbon substrates in the tricarboxylic acid cycle, the breakdown of amino acids produces several others. Amino acids such as glutamine are catabolized to glutamate. Transamination of glutamate produces the keto-acid α-ketoglutarate, which functions as an intermediate in the tricarboxylic acid cycle. Similarly, aspartate can be converted to oxaloacetate, which is also a tricarboxylic acid cycle intermediate. The breakdown of branched-chain amino acids including leucine, valine and isoleucine involves their transamination to a keto-acid followed by a NADH-generating decarboxylase reaction catalyzed by a multi-enzyme complex homologous to pyruvate dehydrogenase called the branched-chain α-ketoacid dehydrogenase. The further breakdown of these amino acids can lead to the production of succinyl-CoA or acetyl-CoA, both of which are intermediates of the tricarboxylic acid cycle. The breakdown of other amino acids can produce fumarate or pyruvate, which all link to the tricarboxylic acid cycle. NADH and $FADH_2$ produced in the tricarboxylic acid cycle supply electrons to the electron transport chain of the inner mitochondrial membrane, which is linked to oxidative phosphorylation (see later). The tricarboxylic acid cycle also has roles in biosynthetic pathways such as haem biosynthesis (see later).

Early studies in *Plasmodium* species demonstrated that glucose is the main energy source during the asexual cycle. However, these studies found that instead of producing pyruvate to feed into the tricarboxylic acid cycle, the endpoint of glycolysis is the production of lactate via lactate dehydrogenase (Bryant *et al.*, 1964; Scheibel & Pflaum, 1970). Similarly, it appears that β-oxidation of fatty acids to produce acetyl-CoA does not occur in *Plasmodium* spp. Other studies have failed to find biochemical evidence for some tricarboxylic acid cycle enzymes such as α-ketoglutarate dehydrogenase (Blum & Ginsburg, 1984). It appears, then, that the tricarboxylic acid wheel of *Plasmodium* spp. is disconnected from the acetyl-CoA producing axle, and that some of the crucial spokes are missing. What light, then, can a bioinformatic approach shed on the functions of what appears to be a very dysfunctional tricarboxylic acid cycle?

The inability of *Plasmodium* spp. to feed glycolytic products into the tricarboxylic acid cycle was explained by the finding that, although *Plasmodium* species contain a pyruvate dehydrogenase enzyme, it is localized to the apicoplast (Foth *et al.*, 2005), where is probably feeds fatty acid biosynthesis (Ralph *et al.*, 2004; Foth *et al.*, 2005). Indeed, mitochondrial pyruvate dehydrogenase appears absent from

the distantly related apicomplexan parasite *Toxoplasma gondii* (Foth *et al.*, 2005), suggesting that apicomplexan parasites lost this protein complex, and consequently the ability to directly feed glycolytic substrates into the tricarboxylic acid cycle, early in their evolution. It is possible that pyruvate dehydrogenase subunits are dual targeted to both the plastid and mitochondrion (perhaps by alternative splicing, a process that has been documented in *P. falciparum* van Dooren *et al.*, 2002), but we think this very unlikely, given western blots reveal only single isoforms of the E1α and E1β subunits (Foth *et al.*, 2005).

Further bioinformatic analysis suggests the presence of all the remaining tricarboxylic acid cycle enzymes in the *P. falciparum* genome. However, closer analysis reveals that the situation isn't quite so straightforward. The first committed step of the tricarboxylic acid cycle is the production of citrate by the enzyme citrate synthase, using oxaloacetate and acetyl-CoA as substrates (Fig. 3). *P. falciparum* contains a clear citrate synthase homologue (PF10_0218), that contains an N-terminal presequence that directs this protein to the mitochondrion (Tonkin *et al.*, 2004). The next step of the cycle is the isomerization of citrate to isocitrate by aconitase. Although *P. falciparum* contains an aconitase-like protein (PF13_0229), aconitases belong to a broader family of proteins that includes iron-responsive element binding proteins (IRPs). Initial studies suggested that the *P. falciparum* aconitase-like protein functions, much like IRPs, in binding to mRNA species, possibly to regulate cellular iron levels (Loyevsky *et al.*, 2001, 2003). A recent study found that this protein also has aconitase activity, and data was presented to suggest this protein may partially localize to the mitochondrion (Hodges *et al.*, 2005). It is possible that, as in the protozoan parasite *Trypanosoma brucei*, this aconitase protein has dual localization in both the mitochondrion and cytosol (Saas *et al.*, 2000), and may also have a dual function in iron regulation and aconitase activity. The role of the next

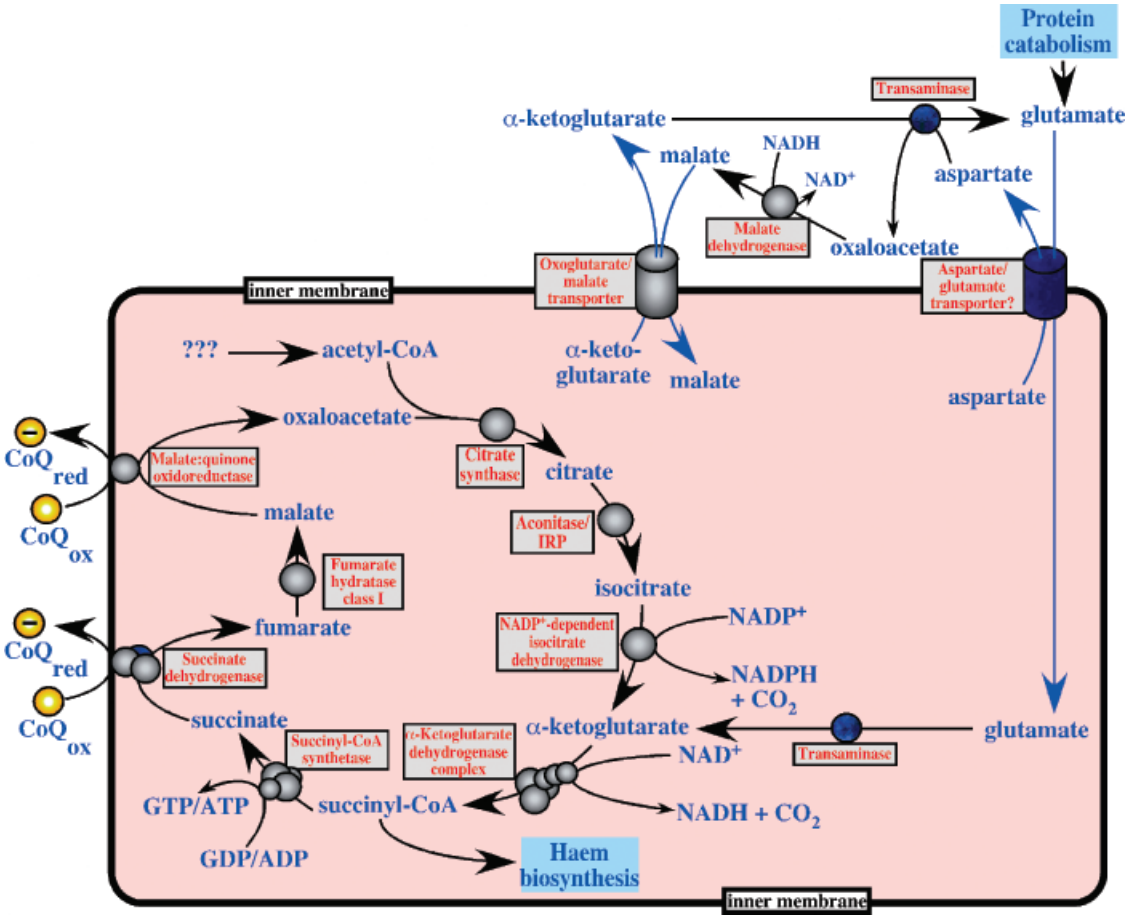


Fig. 3. Schematic depicting the possible TCA cycle in *Plasmodium falciparum* parasites. TCA cycle proteins with clear homologues in *P. falciparum* are depicted as grey spheres, absent proteins as blue spheres and substrates in blue writing. Electrons from substrates generated in this cycle are fed into the electron transport chain (Fig. 4), although it is unclear whether the complete TCA cycle is functional in *P. falciparum*. It is also not known what substrates are fed into the TCA cycle. There is no known source for acetyl-CoA or a clear homologue of one transporter necessary for a complete aspartate-malate shuttle. See text for further discussion.

tricarboxylic acid cycle enzyme in *P. falciparum* is also equivocal. Isocitrate dehydrogenase functions to convert isocitrate to α-ketoglutarate, producing CO_2 and reducing NAD^+ to NADH. Biochemical evidence suggests isocitrate dehydrogenase activity is present during asexual stages of *P. falciparum*, but that it requires $NADP^+$ as a cofactor (Vander Jagt *et al.*, 1989). Indeed, a $NADP^+$-dependent isocitrate dehydrogenase (PF13_0242) was identified in the *P. falciparum* genome (Chan & Sim, 2003; Wrenger & Muller, 2003). This protein contains an N-terminal extension that possibly functions as a mitochondrial presequence. However, it was shown that the *P. falciparum* isocitrate dehydrogenase is upregulated upon oxidative stress (Wrenger & Muller, 2003), suggesting that the NADPH produced in this reaction is necessary as a reducing agent for antioxidant enzymes of the mitochondrion (see later; Wrenger & Muller, 2003; Muller, 2004). The inability of this enzyme to generate NADH suggests that if *Pf*ICDH (isocitrate dehydrogenase) does function in the tricarboxylic acid cycle, it does so only in substrate generation and does not contribute electrons to the electron transport chain. An alternative scenario is that the *Plasmodium* electron transport chain can receive electrons from NADPH (see later).

The remaining steps of the tricarboxylic acid cycle all contain clear homologues in the *P. falciparum* genome, all of which are likely to be mitochondrially targeted (Fig. 3). α-Ketoglutarate is converted to succinyl-CoA by the α-ketoglutarate dehydrogenase complex. This multi-enzyme complex belongs to the keto-acid dehydrogenase family, which also comprises the pyruvate and branch-chain amino acid dehydrogenases mentioned previously. It consists of E1 (PF08_0045), E2 (PF13_0121) and E3 (PFL1550w) subunits. The E3 subunit is shared between all the mitochondrial keto-acid dehydrogenases, and has been shown to target to the mitochondrion of *P. falciparum* (McMillan *et al.*, 2005). The E2 subunit contains a lipoate cofactor, which is probably inserted by the mitochondrial LplA protein (PF13_0083; Wrenger & Muller, 2004). Succinyl-CoA is converted to succinate by succinyl-CoA synthetase, with GTP being generated in the process. Succinyl-CoA synthetase consists of an α- and a β-subunit, both of which have homologues in the *P. falciparum* genome (PF11_0097 and PF14_0295 respectively). Succinate dehydrogenase converts succinate to fumarate, reducing FAD in the process. Succinate dehydrogenase also functions as complex II of the electron transport chain, with electrons from $FADH_2$ passed on to coenzyme Q (see later). The two major subunits of succinate dehydrogenase, the flavoprotein (PF10_0334) and the iron-sulphur (PFL0630w) subunits, both have homologues in *P. falciparum* (Takeo *et al.*, 2000) and have been purified from asexual stage parasites (Suraveratum *et al.*, 2000). The next step of the tricarboxylic acid cycle converts fumarate to malate, and is typically catalyzed by fumarate hydratase. Although *P. falciparum* contains a fumarate hydratase homologue (PFI1340w), it differs substantially from the 'class II' type enzyme found in yeast and mammalian cells. Instead, the *P. falciparum* fumarate hydratase resembles the iron-sulphur-containing 'class I'-type enzymes found in some Bacteria and Archaea (Woods *et al.*, 1988; Flint *et al.*, 1992).

Malate is converted to oxaloacetate to complete the tricarboxylic acid cycle, a reaction that is typically catalyzed by malate dehydrogenase. Malate dehydrogenase has been purified from asexual stages of *P. falciparum* (Lang-Unnasch, 1992), but this enzyme appears not to localize to the mitochondrion (Lang-Unnasch, 1995). Instead, the genome of *P. falciparum* reveals the presence of a malate-quinone oxidoreductase homologue (PFF_0815w) (Uyemura *et al.*, 2004). Malate-quinone oxidoreductase is an enzyme found in some bacteria, and reduces FAD in the generation of oxaloacetate. Electrons from $FADH_2$ are donated to coenzyme Q and the electron transport chain (see later). The addition of both malate and fumarate to asexual stage *Plasmodium yoelii* parasites has been shown to contribute to mitochondrial electron transport, suggesting that both fumarate dehydratase and malate-quinone oxidoreductase are functional in malaria parasites (Uyemura *et al.*, 2004).

Bioinformatically piecing together the tricarboxylic acid cycle of *Plasmodium* parasites raises many questions. The most obvious question is: does *Plasmodium* really require a tricarboxylic acid cycle for energy generation? The answer for asexual stage parasites is apparently no. Biochemical studies demonstrate that *Plasmodium* spp. obtain most of their energy from blood glucose through glycolysis, although small amounts of mitochondrially generated ATP may be required for mitochondrial processes such as protein import (e.g. Fig. 2). However, *Plasmodium* spp. have a complex life cycle, including stages in the mosquito midgut and salivary gland and in human hepatocytes. During some of these stages, it is likely that glucose is less abundant, and the parasite may rely on more than just glycolysis for energy generation. It is interesting to note that transcriptome analysis suggests that several enzymes of the tricarboxylic acid cycle such as citrate synthase and the iron-sulphur subunit of succinate dehydrogenase are upregulated in sporozoites (Le Roch *et al.*, 2003), and proteome analysis suggests citrate synthase, aconitase, isocitrate dehydrogenase, both subunits of the succinyl-CoA synthetase, succinate dehydrogenase and fumarate hydratase all appear upregulated in ookinetes when compared to asexual stages (Hall *et al.*, 2005). This provides strong indirect evidence that mitochondrial energy generation is more important in non-erythrocytic stages, probably as an adaptation to environments where glucose availability is considerably lower than the human bloodstream. Due to technical difficulties, very little biochemical study has been done on mosquito and exoerythrocytic human stages of malaria

parasites. It may be that the role of the tricarboxylic acid cycle in these stages can be elucidated through genetic knockouts, which are feasible in *Plasmodium berghei*, the model parasite for insect and exoerythrocytic stages (Menard *et al.*, 1997; Carvalho *et al.*, 2004). Such genetic studies may also suggest whether tricarboxylic acid cycle enzymes are essential in blood stages of the parasite.

If a complete tricarboxylic acid cycle can function in *Plasmodium*, there must be an available source of acetyl-CoA. With the absence of a mitochondrial pyruvate dehydrogenase complex, and the requirement of apicoplast-generated acetyl-CoA for fatty acid biosynthesis, this source cannot directly be glycolysis. Consistent with this, studies have shown that the addition of pyruvate cannot stimulate oxygen consumption through the electron transport chain in *Plasmodium* parasites (Fry & Beesley, 1991; Uyemura *et al.*, 2000). The presence of citrate synthase in the mitochondrion, however, would suggest that at some point in the malaria life cycle, this initial step of the tricarboxylic acid cycle does occur. In other organisms, acetyl-CoA can be generated by fatty acid oxidation. However, no homologues of enzymes involved in β-oxidation are present in the *P. falciparum* genome. Acetyl-CoA can also be generated through the breakdown of amino acids such as leucine and isoleucine. This initially involves transamination followed by a decarboxylation catalyzed by the branched-chain α-keto acid dehydrogenase complex, a large, multienzyme complex related to pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. *Plasmodium falciparum* contains homologues of all the essential subunits of this complex, including E1α (PF13_0070), E1β (PFE0225w) and E2 (PFC0170c). The E3 or dihydrolipoamide dehydrogenase subunit of branched-chain α-keto acid dehydrogenase complex is shared with the α-ketoglutarate dehydrogenase (McMillan *et al.*, 2005). However, homologues of enzymes involved in the subsequent steps of this catabolic process that lead to acetyl-CoA production (Daschner *et al.*, 2001) appear absent from the *P. falciparum* genome. It is worthy of note that NADH produced by the branched-chain α-keto acid dehydrogenase complex complex may be fed into the electron transport chain (see later), with the breakdown of amino acids thus contributing to energy generation in the mitochondrion. Nevertheless, determining the source of mitochondrial acetyl-CoA is of a high priority in understanding the metabolism of this organelle.

One study found that citrate promotes oxygen consumption in *P. berghei* parasites (Uyemura *et al.*, 2000), suggesting that it may contribute to the tricarboxylic acid cycle. A measure of citrate levels in ring vs. trophozoite stage *P. falciparum* parasites suggests that the concentration of citrate decreases later in the asexual cell cycle (Hodges *et al.*, 2005). This suggests that tricarboxylic acid cycle steps subsequent to the generation of citrate are active at these stages. Further studies are required to determine whether the metabolism of citrate initiates a complete tricarboxylic acid cycle that is linked to oxidative phosphorylation, or, as has been suggested (Hodges *et al.*, 2005), functions in redox regulation through the production of NADPH by NADPH-dependent isocitrate dehydrogenase. It is also possible that the tricarboxylic acid cycle functions in the generation of substrates for biosynthetic pathways (see later) or that an incomplete tricarboxylic acid cycle exists that utilizes amino acids as substrates. For example, glutamate can be transaminated to form α-ketoglutarate (Fig. 3). In such a scenario, α-ketoglutarate can be broken down via subsequent steps in the pathway to ultimately produce oxaloacetate, generating one molecule of NADH, two molecules of $FADH_2$ and one molecule of GTP in the process. It is not clear, however, what would happen to the oxaloacetate that accumulates in this scheme. For efficient channelling of amino acids into an incomplete cycle to occur, such end-product 'intermediates' of the tricarboxylic acid cycle would need to be either transported out of the mitochondrion or diverted for biosynthetic processes.

An interesting comparison can be made between the tricarboxylic acid cycle enzymes of *P. falciparum* and that of the parasite *T. brucei*, which contains all the necessary enzymes for a complete tricarboxylic acid cycle. However, biochemical studies of the procyclic stage that occurs in the midgut of the insect host indicate that, despite the glucose-poor environment, the tricarboxylic acid cycle is not fully functional (van Weelden *et al.*, 2003). Instead, the enzymes of the tricarboxylic acid cycle are predicted to perform biosynthetic roles. For example, citrate produced by citrate synthase is transported to the cytosol of *T. brucei*, where it is catabolized to acetyl-CoA by citrate lyase, with acetyl-CoA consequently used in fatty acid biosynthesis (van Weelden *et al.*, 2005). Such a pathway is not likely to occur in *P. falciparum*, as there is no citrate lyase enzyme apparent in the genome and fatty acid biosynthesis occurs in the apicoplast (Ralph *et al.*, 2004). Nevertheless, the case of the incomplete tricarboxylic acid cycle in *T. brucei* highlights the diverse functions that tricarboxylic acid cycle enzymes can perform and emphasizes the pitfalls of bioinformatic interpretations in the absence of solid biochemical data.

Mitochondrial migration policy: solute gates at the border

The mitochondrion of *Plasmodium*, as that of all other organisms, is integrated into the broader metabolism of the cell. The mitochondrion is commonly a major site of energy production, and has several biosynthetic pathways. The organelle, thus, must be able to contribute necessary metabolites to the rest of the cell. Equally, the mitochondrion is dependent on other metabolic pathways in the cell. In other

organisms, pyruvate from cytosolic glycolysis is fed into the mitochondrial tricarboxylic acid cycle. Although this does not occur in *Plasmodium* (see above), the mitochondrion probably requires numerous other metabolites (amino acids, tricarboxylic acid cycle intermediates, metal ions, etc.) from the cell to carry out its assigned roles. A crucial factor in understanding mitochondrial metabolism is identifying the metabolites that the mitochondrion can import and export. Such information holds the key to piecing together the unusual tricarboxylic acid cycle and several other mitochondrial pathways of *Plasmodium*. The transport of metabolites has been well studied in model yeast, plant and animal organisms, and a considerable amount is known about the proteins that carry out these transport functions.

The outer membrane of mitochondria is semi-permeable to small metabolites. Transport of these metabolites is mediated by a protein known as voltage-mediated anion channel (also known as mitochondrial porin; Blachly-Dyson & Forte, 2001). This is typically the most abundant protein in the outer membrane. It is thought to be a β-barrel protein of around 30–40 kDa that functions as a non-specific pore with a width of around 2–4 nm (Bay & Court, 2002), letting small, predominantly anionic metabolites through. Homology searching reveals no voltage-mediated anion channel homologue in the *P. falciparum* genome. It is not clear whether this implies the absence of the protein from *Plasmodium* mitochondria. The β-barrel structure of voltage-mediated anion channel protein may be more important to protein function than amino acid sequence, in which case sequence identity could be low. Identifying possible voltage-mediated anion channel proteins in the *P. falciparum* may require structurally identifying and characterizing β-barrel proteins in the genome. Mitochondrial carrier proteins are the major protein family that mediate solute transport across the inner mitochondrial membrane. Unlike the general transport that can occur across the outer membrane, mitochondrial carrier proteins transport specific substrates or groups of substrates across the inner membrane (Kunji, 2004). As a result, the inner membrane is considerably less permeable than the outer membrane. Typical carrier proteins are integral membrane proteins approximately 30 kDa in size, comprising three tandem repeats containing two transmembrane domains each (Laloi, 1999; Kunji, 2004). Most organisms contain large families of carrier proteins. Humans are predicted to have 39 carrier proteins, yeast 34 and *Arabidopsis* at least 58, most of which have unknown substrate specificities (el Moualij *et al.*, 1997; Kunji, 2004; Picault *et al.*, 2004). Characterized carrier proteins transport a wide range of solutes into and out of the mitochondrion, including tricarboxylic acid cycle intermediates, amino acids ATP/ADP, protons and other small ions (Kunji, 2004).

We performed a PSI-BLAST search (Altschul *et al.*, 1997) of annotated proteins from the *P. falciparum* genome using the ATP/ADP carrier protein of *S. cerevisiae* as a query sequence. After four iterations, we identified 13 proteins that further analysis showed to contain at least one clear mitochondrial carrier protein repeat domain (see Supplementary Table 1). Based on homology to known mitochondrial carrier proteins, four of these 13 proteins can be confidently assigned to a carrier type and putative function and these are described below.

The mitochondrial synthesis of ATP by the ATP synthase complex (see later) requires a regular supply of ADP and inorganic phosphate (P_i). ADP is imported via the ATP/ADP carrier (PF10_0366) in exchange for a molecule of ATP (Fig. 4). In this way, ATP is transported out of the mitochondrion. P_i is imported into the mitochondrion by the phosphate carrier (PFL0110c), which functions as a proton symporter, concurrently importing protons down their concentration gradient (Fig. 4). The import of P_i is, thus, dependent on the proton gradient generated by the electron transport chain (see later). In mammalian cells, the biochemical link between ATP synthase, the ATP/ADP transporter and the phosphate carrier is structural, with these proteins coming together to form an 'ATP synthasome' (Ko *et al.*, 2003). Both the ADP/ATP transporter and the phosphate carrier have previously been identified in *P. falciparum*. The ADP/ATP transporter is expressed in asexual stages, and immunogold electron microscopy with an antibody against the bovine ADP/ATP transporter suggests the *P. falciparum* homologue may localize to the mitochondrial membrane (Hatin *et al.*, 1992; Hatin & Jaureguiberry, 1995). The *P. falciparum* phosphate carrier is expressed in both asexual and gametocyte stages of the parasite, and has been shown to import into heterologous mitochondria (Bhaduri-McIntosh & Vaidya, 1996,1998), suggesting the mode of import is similar to that seen in other organisms.

The *P. falciparum* genome contains a homologue of the oxoglutarate/malate transporter (PF08_0031), which is localized to the mitochondrion (Fig. 1h). Oxoglutarate/malate transporter functions in the exchanging of dicarboxylates, typically the export of α-ketoglutarate (oxoglutarate) for malate (Fig. 3; Laloi, 1999). In other organisms, it functions in the malate/aspartate shuttle. This shuttle involves the export of aspartate and α-ketoglutarate from the mitochondrion, and the transamination of these compounds to oxaloacetate and glutamate respectively (Fig. 3; Bakker *et al.*, 2001). This is followed by the conversion of oxaloacetate to malate by cytosolic malate dehydrogenase, oxidizing NADH in the process. Malate and glutamate are transported back into the mitochondrion, with mitochondrial malate dehydrogenase converting malate to oxaloacetate, reducing NAD^+ in the process. The net result of this shuttle is that cytosolic NADH is transported into the mitochondrion,

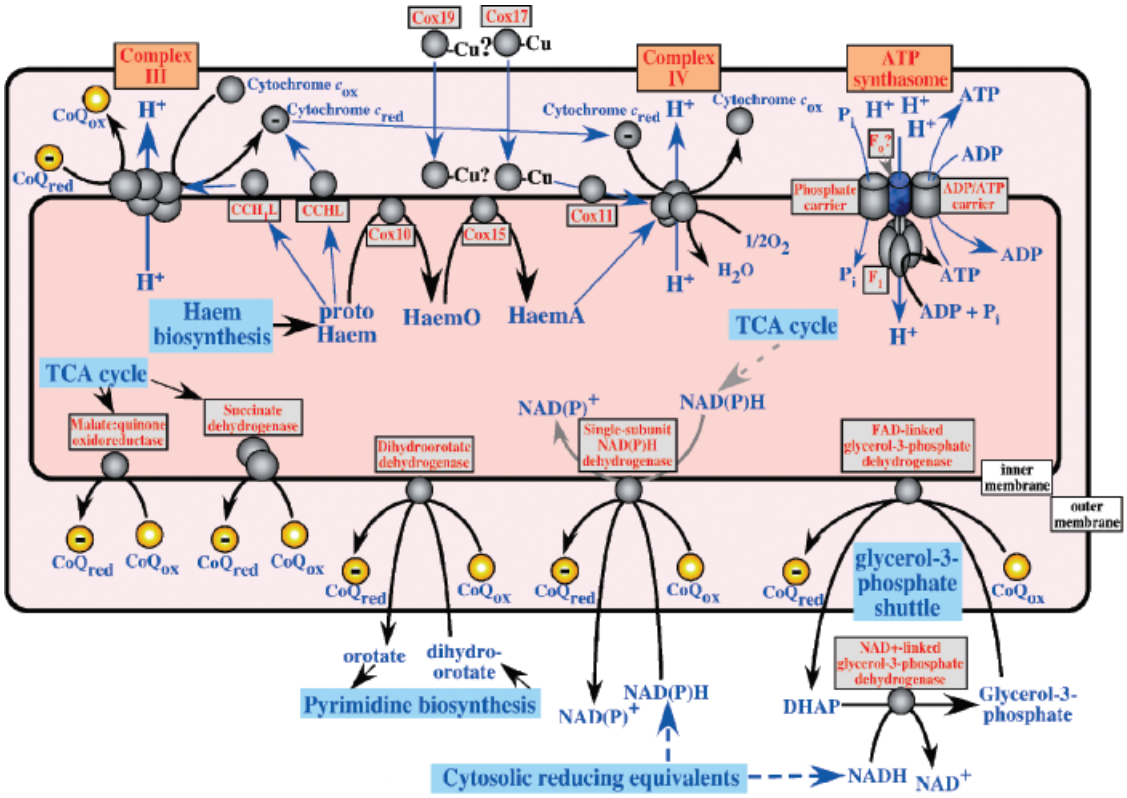


Fig. 4. A putative model for electron transport through the inner membrane of the mitochondrion of *Plasmodium falciparum*. Several proteins and protein complexes localize to the inner membrane of mitochondria, where they function in the accepting and donating of electrons. Several of these enzymes (bottom of diagram) donate electrons to coenzyme Q (CoQ; yellow sphere). Those with clear homologues in *P. falciparum* include succinate dehydrogenase, malate : quinone oxidoreducatase, dihydroorotate dehydrogenase, glycerol-3-phosphate dehydrogenase and NAD(P)H dehydrogenase. It is unclear whether NAD(P)H dehydrogenase oxidizes NAD(P)H derived from the cytosol or from the mitochondrial matrix (see text for discussion). Electrons from coenzyme Q are donated to complex III (cytochrome *c* reductase; top of diagram), which passes electron through to cytochrome *c* and translocates protons (H^+) from the matrix into the intermembrane space. Cytochrome *c* is a soluble intermembrane space protein that donates electrons to complex IV (cytochrome *c* oxidase), with oxygen functioning as the terminal electron acceptor. Complex IV also translocates protons across the inner membrane. The proton gradient generated by complexes III and IV is harnessed by the F_0F_1 ATP synthase complex for the production of ATP. ATP synthase, together with the ADP/ATP and phosphate carrier proteins, may form a multicomponent ATP 'synthasome'. *P. falciparum* contains homologues of numerous proteins involved in the transport of electron transport chain-related solutes, as well as proteins involved in assembling various electron transport chain proteins and complexes (see text for details; CCHL, cytochrome *c* haem lyase; CC_1HL, cytochrome c_1 haem lyase).

where it can be fed into the electron transport chain. It is not clear if this shuttle exists in *Plasmodium*. *Plasmodium falciparum* contains a cytosolic NADH-dependent malate dehydrogenase (PFF0895w) that can convert oxaloacetate to malate (Lang-Unnasch, 1992; Tripathi *et al.*, 2004). Inside the mitochondrion, the equivalent reaction is catalyzed by malate : quinone oxidoreductase, which feeds electrons directly into the electron transport chain (see earlier). This means that in *Plasmodium* such a shuttle would transfer electrons from cytosolic NADH directly into the electron transport chain. The major missing component in the shuttle is the transporter necessary to export aspartate from the mitochondrion. This step is usually performed by an aspartate/glutamate exchanger (Palmieri *et al.*, 2001; Cavero *et al.*, 2003), of which no clear homologue exists in the *P. falciparum* genome. Further study is required to determine whether such a transporter exists.

If *Plasmodium* lacks a malate : aspartate shuttle, what might the function of oxoglutarate/malate transporter be? These proteins closely resemble dicarboxylate/tricarboxylate carriers (Picault *et al.*, 2002), which exchange dicarboxylates such as oxaloacetate and malate for tricarboxylates such as citrate and isocitrate. If oxoglutarate/malate transporter is in fact a dicarboxylate/tricarboxylate carrier, it may be involved in exchange of tricarboxylic acid cycle intermediates to overcome missing steps in this pathway. For example, such a transporter could export oxaloacetate in exchange for citrate, thus bypassing the acetyl-CoA requiring step catalyzed by citrate synthase. Such a scenario would not explain the presence of citrate synthase in the mitochondrion, nor

how citrate is produced in the cytosol. Clearly, characterization of the substrate specificities of oxoglutarate/malate transporter – dicarboxylate/tricarboxylate carrier is required to understand its broader function in cellular metabolism.

The fourth mitochondrial carrier protein with a reasonably clear homology to characterized carrier proteins is a Yhm2 homologue (PFL1145w). Yhm2 was characterized from *S. cerevisiae* as an inner membrane, DNA-binding protein (Cho *et al.*, 1998). It was speculated that Yhm2 tethers mitochondrial DNA to the inner membrane, thus contributing to mitochondrial genome maintenance (Cho *et al.*, 1998). The exact function of Yhm2 in solute transport is not known, but, given its apparent conservation in evolutionarily distinct lineages, it is worthy of further investigation.

Based on BLAST search homologies, the remaining mitochondrial carrier homologues in *P. falciparum* do not clearly match to any one group of carrier proteins. One homologue (PFL2000w) shows some similarities to S-adenosylmethionine carriers, which are involved in importing S-adenosylmethionine into the mitochondrion in exchange for the demethylated S-adenosylhomocysteine (Marobbio *et al.*, 2003; Agrimi *et al.*, 2004). S-adenosylmethionine is a major donor of methyl groups in mitochondria, donating to compounds such as DNA and proteins, as well as to precursors in the synthesis of coenzyme Q (see later; Meganathan, 2001). One *P. falciparum* carrier protein (PFD0367w) has possible homology to a manganese trafficking factor used in the activation of mitochondrial superoxide dismutase (Luk *et al.*, 2003), an enzyme involved in regulating oxidative stress. *Plasmodium falciparum* contains a mitochondrial superoxide dismutase, although this probably contains an iron cofactor (Sienkiewicz *et al.*, 2004). Other mitochondrial carrier proteins identified in our analysis, but without clear homologues in other organisms, include PFI0255c, PFI0425w, PF13_0359, PF08_0093 and PFA0415c. The genes PFA0435w and PF10_0051 are unusual in that they contain only one carrier protein domain. Whether these function in solute transport is unclear.

Although useful for the purpose of identifying candidate mitochondrial transport proteins, phylogenetic searching has its limits in assigning functions to these carrier proteins. Others have noted that carrier proteins with related sequences can have very different substrate affinities (Agrimi *et al.*, 2004). Some biochemical evidence suggests the existence of a mitochondrial uncoupling protein in *Plasmodium*, involved in dissipating the proton gradient across the inner membrane (Uyemura *et al.*, 2000). Our bioinformatic analysis, however, fails to identify an uncoupling protein homologue, but it remains possible that one of the unidentified carrier proteins has an uncoupling function. Recently, it was shown that an *Arabidopsis* carrier protein was targeted to the plastid (Bedhomme *et al.*, 2005), and it is possible that some of the carrier proteins of *Plasmodium* may target to the apicoplast or other locations. Having identified a number of carrier protein homologues, it is now necessary to begin localization and biochemical analysis of these proteins to gain the necessary insights into their undoubtedly crucial role in *Plasmodium* metabolism. Carrier proteins are not the only proteins involved in solute transport across the inner membrane. ATP-binding cassette transporters are a widely distributed group of proteins, some of which are localized to the mitochondrion. Mitochondrial ATP-binding cassette transporters have been implicated in processes such as the export of mitochondrially produced iron-sulphur clusters to the cytosol and the export of degraded peptides into the cytosol (Kispal *et al.*, 1999; Young *et al.*, 2001). The *P. falciparum* genome contains numerous ATP-binding cassette transporters, some of which are known to function in drug resistance (Foote *et al.*, 1990). Homology searches fail to identify clear homologues of mitochondrial ATP-binding cassette transporters. Curiously, the *P. falciparum* multi-drug resistance protein 2 (mdr2; PF14_0455) shows considerable homology to the yeast mitochondrial ATP-binding cassette transporter protein involved in iron-sulphur cluster export. However, the characterization of this and other possible mitochondrial ATP-binding cassette transporters will need to be achieved through localization and biochemical data.

The mitochondrial electron transport chain

If the tricarboxylic acid cycle is the central wheel of mitochondrial metabolism, then the electron transport chain of the inner membrane is the biochemical motor that drives many mitochondrial and cellular functions. The *Plasmodium* electron transport chain has been extensively studied, and is the target of the antimalarial drug atovaquone (for reviews see Fry, 1991; Mi-Ichi *et al.*, 2003; Krungkrai, 2004; Vaidya, 2004). Groups have found different ways of assaying the electron transport chain of *Plasmodium*. Fry & Beesley (1991) partially purified mitochondria and developed a cytochrome *c* reductase assay. Srivastava *et al.* (1997) developed a dye-based flow cytometry assay to measure membrane potential ($\Delta\Psi$) created by the transfer of protons across the inner membrane during electron transport. Uyemura *et al.* (2000, 2004) permeabilized *Plasmodium* cells with digitonin and measured $\Delta\Psi$ by a different dye-based assay. They also measured O_2 consumption in digitonin permeabilized parasites, O_2 being the terminal electron acceptor in the transport chain. Despite the wealth of information gathered from these studies, the functions of the electron transport chain in *Plasmodium* remain controversial and somewhat elusive. In other organisms, the inner membrane contains numerous proteins and protein

complexes that function in the accepting and donating of electrons. The transfer of electrons in some of these protein complexes is coupled to the translocation of protons from the mitochondrial matrix to the intermembrane space, resulting in the formation of a membrane potential ($\Delta\Psi$) and proton gradient (ΔpH) across the inner membrane. This electrochemical gradient is classically harnessed by ATP synthase in the generation of ATP molecules that power many cellular functions. The gradient is also important in protein translocation (see earlier), solute transport and as an electron sink for biosynthetic pathways (Laloi, 1999; Pfanner & Geissler, 2001; Evans & Guy, 2004).

Electrons donated along the electron transport chain are sourced from several biochemical reactions. However, all converge at coenzyme Q (also known as ubiquinol or ubiquinone), a hydrophobic, isoprenoid-containing molecule embedded in the inner membrane. Reduced coenzyme Q donates electrons to complex III (also known as the cytochrome bc_1 complex or cytochrome *c* reductase; Fig. 4). Electrons donated by coenzyme Q are transferred through several complex III proteins to reduce cytochrome *c* ultimately (Crofts & Berry, 1998). All three of the core catalytic subunits of complex III have homologues in the *P. falciparum* genome. These are cytochrome *b* (encoded on the mitochondrial genome), the Rieske iron sulphur protein (PF14_0373) and cytochrome c_1 (PF14_0597). Electrons from coenzyme Q are passed to either the Rieske iron sulphur protein or to the haem prosthetic group of cytochrome *b* (Crofts, 2004). Electrons from the Rieske protein are donated via the haem group of cytochrome c_1 to the haem of cytochrome *c* (Fig. 4), which also has a homologue in the *P. falciparum* genome (PF14_0038). The oxidation of coenzyme Q via this process results in the release of protons from reduced coenzyme Q into the intermembrane space. Electrons donated to cytochrome *b* are used to again reduce coenzyme Q, with protons removed from mitochondrial matrix in the process. This entire cycle of electron transfer, thus, results in the net movement of protons from the mitochondrial matrix to the intermembrane space, contributing to the proton gradient across the inner membrane. Other members of complex III with homologues in *P. falciparum* include a hinge protein (PF14_0248), thought to be involved in facilitating electron transfer between cytochrome c_1 and cytochrome *c* (Braun *et al*., 1994), and the core I and core II subunits. Core I and II subunits are homologous to the two subunits of the mitochondrial processing peptidase (see earlier). In plant, but not in animal, cells, these subunits comprise the functional mitochondrial processing peptidase (Brumme *et al*., 1998). Given that *P. falciparum* contains only one homologue of each of the mitochondrial processing peptidase subunits (van Dooren *et al*., 2002), it is probable that these proteins are also part of complex III, although the exact role of the core I and II subunits in complex III activity are poorly defined (Brumme *et al*., 1998).

Several lines of evidence point to a functional complex III in *Plasmodium* asexual stage parasites. Complex III activity has been detected in *Plasmodium* species (Fry & Beesley, 1991), and typical inhibitors of complex III proteins, such as antimycin, have been shown to inhibit both complex III activity and the consumption of oxygen, which functions as the terminal acceptor of electrons in the electron transport chain (Fry & Beesley, 1991; Uyemura *et al*., 2000, 2004; Krungkrai, 2004) (Fig. 4). Complex III activity was inhibited by the antimalarial compound atovaquone (Fry & Pudney, 1992). It was further shown that mutations in the coenzyme Q binding site of cytochrome *b* correlate with resistance to atovaquone, strongly suggesting that this complex is the major target for atovaquone (Srivastava *et al*., 1999). Consistent with the role of cytochrome *b* in contributing to the inner membrane proton gradient, atovaquone and other complex III inhibitors were shown to collapse membrane potential in *Plasmodium* parasites, supporting the role of a functional, proton-translocating complex III in *Plasmodium* (Srivastava *et al*., 1997, 1999; Uyemura *et al*., 2000, 2004).

Electrons from complex III are donated to cytochrome *c*, a soluble intermembrane space-localized protein (Fig. 4). Cytochrome *c*, like other cytochromes, contains an iron-containing haem prosthetic group that functions as the redox centre where electron transfer takes place. 'C-type' cytochromes, which include both cytochrome *c* and c_1, differ from cytochrome *b* in that the haem group is covalently attached to a conserved CXXCH motif on the protein. Covalent attachment of haem to 'c-type' cytochromes can occur via several mechanisms (Allen *et al*., 2003). In fungal and animal mitochondria, a inner membrane protein known as cytochrome *c* haem lyase is thought to catalyze the covalent attachment of haem to cytochrome *c*. *Plasmodium falciparum* contains homologues of both a cytochrome *c* haem lyase (PFL1185c) and a cytochrome c_1 haem lyase (PFL0180w), suggesting that 'c-type' cytochrome biogenesis involves similar processes to those seen in fungi and animals.

Cytochrome *c* donates electrons to complex IV, which serves as the terminal oxidase of the electron transport chain, ultimately donating electrons to oxygen (Fig. 4). As with complex III, complex IV is able to translocate protons across the inner membrane, thus contributing to $\Delta\Psi$ and the proton gradient. The catalytic core of complex IV consists of three subunits called CoxI, CoxII and CoxIII. *Plasmodium falciparum* contains homologues of all three subunits, with CoxI and CoxIII encoded on the mitochondrial genome and CoxII comprising two nuclear-encoded subunits known as CoxIIa (PF14_0288) and CoxIIb (PF13_0327)(Funes *et al*., 2002). C*ox2* is typically encoded

as a single gene on the mitochondrial genome of most organisms, although the unusual splitting and nuclear relocation of the *cox2* gene is also seen in several green algae (Perez-Martinez *et al.*, 2001). It is not clear why the *cox2* gene would split in such a way. One explanation posits that, were it to be encoded in the nucleus, the hydrophobicity of CoxII would prevent its import into the mitochondrion. Splitting of the *cox2* gene lowers the overall hydrophobicity of the protein and thus enables targeting from the nucleus (Perez-Martinez *et al.*, 2001; Waller *et al.*, 2003). Complex IV is a haem-copper oxidase, with electrons passed in series along molecules containing iron and copper redox centres. Electrons from cytochrome *c* are donated to the copper-containing Cu_A site, which is located on CoxII (Ferguson-Miller & Babcock, 1996). The consequent flow of electrons occurs through iron-containing haem a to a binuclear reaction centre consisting of haem a_3 and a second copper centre known as Cu_B (Ferguson-Miller & Babcock, 1996; Yoshikawa *et al.*, 2000). It is at this haem a_3/Cu_B centre on CoxI that oxygen reduction occurs. The role of CoxIII in the catalytic core is not clear, although it is thought to be involved in the translocation of protons across the inner membrane (Hosler, 2004). Complex IV in other eukaryotes contains several other proteins that function in regulatory roles. *Plasmodium falciparum* contains a homologue of CoxV (PFI1365w), a protein found in numerous other eukaryotes, which possibly functions in regulating the flow of electrons between reactions centres of the complex (Burke & Poyton, 1998). None of the remaining accessory proteins characterized in other organisms is apparent in the *P. falciparum* genome. It is very possible that complex IV of *Plasmodium* contains other regulatory subunits, which may be either novel or not identifiable from simple homology searches of the genome.

Studies in *S. cerevisiae* have identified numerous factors involved in the assembly of complex IV and its various prosthetic groups. Many of these factors are conserved in *P. falciparum*. Cox10 (PFE0970w) and Cox15 (PF14_0331) are involved in the synthesis of haem A from proto-haem (Fig. 4) (Moraes *et al.*, 2004). Cox10 adds a farnesyl group to proto-haem to form haem O (Glerum & Tzagoloff, 1994), with Cox15 then involved in hydroxylating haem O to form haem A (Barros *et al.*, 2001). Cox11 (PF14_0721) is a copper-binding protein that possibly functions in inserting Cu_B into CoxI (Hiser *et al.*, 2000). Cox17 (PF10_0252) is thought to be involved in shuttling copper from the cytosol to the mitochondrion for insertion into complex IV (Glerum *et al.*, 1996; Punter & Glerum, 2003). Like Cox17, Cox19 (PFL0090c) displays dual localization to both the cytosol and mitochondrion of yeast, and may also be involved in shuttling metal ions into the mitochondrion (Nobrega *et al.*, 2002). Curiously, the *P. falciparum* homologue of Cox19 is considerably larger than the yeast version.

Complex IV activity has been detected in *Plasmodium* parasites (Krungkrai *et al.*, 1993), and known inhibitors of complex IV, such as cyanide, reduce oxygen consumption and depolarize $\Delta\Psi$ across the inner membrane (Srivastava *et al.*, 1997; Uyemura *et al.*, 2000). It is clear from these various lines of evidence that, like complex III, complex IV is functional in asexual stage *Plasmodium* parasites, where it contributes to generating a $\Delta\Psi$ across the inner membrane. Some studies have suggested the existence of an alternative terminal oxidase that bypasses complexes III and IV in the consumption of oxygen (Murphy *et al.*, 1997). Alternative oxidases are widely distributed amongst eukaryotes (Chaudhuri & Hill, 1996; Siedow & Umbach, 2000). However, no homologues of an alternative oxidase are apparent in the *P. falciparum* genome, and recent studies indicate that complex IV inhibition by cyanide completely ablates oxygen consumption (Uyemura *et al.*, 2000, 2004), indicating that complex IV is the only terminal oxidase in these parasites. Interestingly, the distantly related apicomplexan parasite *Cryptosporidium* has an alternative oxidase homologue (Putignani *et al.*, 2004; Roberts *et al.*, 2004; Suzuki *et al.*, 2004). This suggests that either the precursor to the Apicomplexa had an alternative oxidase which was later lost in the lineage leading to *Plasmodium*, or that *Cryptosporidium* obtained its alternative oxidase through horizontal gene transfer, a process documented for other *Cryptosporidium* genes (Striepen *et al.*, 2002, 2004).

Although the existence of complexes III and IV in *Plasmodium* is clear, the function of the electron transport chain in a broader context most certainly is not. The remainder of this section will focus on the electron transport that occurs upstream of complex III, as well as on the possible functions of the electron transport chain in these parasites.

Electrons can be donated to coenzyme Q from a variety of inner membrane dehydrogenases. Five such enzymes exist in *P. falciparum*, although the role of several of these is not yet clearly defined. The best characterized is dihydroorotate dehydrogenase, an enzyme involved in pyrimidine biosynthesis, which catalyzes the oxidation of dihydroorotate to orotate, donating electrons to coenzyme Q via a FAD cofactor (Fig. 4). *Plasmodium falciparum* dihydroorotate dehydrogenase (PFF0160c) localizes to the mitochondrion, most probably to the inner membrane (Krungkrai, 1995). Assays measuring both O_2 consumption and complex III activity show that the addition of dihydroorotate can stimulate electron transfer (Fry & Beesley, 1991; Uyemura *et al.*, 2000, 2004; Takashima *et al.*, 2001). Dihydroorotate is synthesized in the cytosol by dihydroorotase and possibly enters the mitochondrial intermembrane space through voltage-mediated anion channel in the outer membrane (Evans & Guy, 2004). Here it can interact with dihydroorotate dehydrogenase, which probably faces the intermembrane space

side of the inner membrane. Orotate probably exits the mitochondrion into the cytosol, where subsequent reactions create pyrimidines. *Plasmodium* species are unable to scavenge pyrimidines from their environment and fully depend on *de novo* production. This makes pyrimidine biosynthesis a major target for antimalarial drugs. A recent study screened for drugs targeting dihydroorotate dehydrogenase and found several with promising activity that had minimal effect on the human version of this enzyme (Baldwin *et al*., 2005). In the biosynthesis of pyrimidines, then, the *Plasmodium* electron transport chain functions as an electron sink.

Two dehydrogenases of the tricarboxylic acid cycle contain a FAD cofactor that, when reduced, can donate electrons to coenzyme Q. Succinate dehydrogenase (also known as complex II) converts succinate to fumarate (see earlier, Fig. 4). In a direct enzyme assay, Takashima and colleagues (Takashima *et al*., 2001) found succinate dehydrogenase activity in partially purified mitochondria from *P. falciparum*. The addition of succinate has furthermore been shown to promote O_2 consumption, to contribute to $\Delta\Psi$ across the inner membrane, and to stimulate complex III activity (Fry & Beesley, 1991; Uyemura *et al*., 2000, 2004). Succinate dehydrogenase has been purified from asexual stage *P. falciparum* (Suraveratum *et al*., 2000), supporting a functional role of this enzyme during this period of the life cycle. Studies have found that the addition of fumarate can inhibit dihydroorotate- and NADH-linked electron transport (Fry & Beesley, 1991; Takashima *et al*., 2001), suggesting that succinate dehydrogenase may function in the reverse reaction to reduce fumarate to succinate, with complex II thus acting as a terminal oxidase for electrons from coenzyme Q. The biological relevance of such *in vitro* reactions remains to be determined.

Malate : quinone oxidoreductase converts malate to oxaloacetate in the tricarboxylic acid cycle, with FAD reduced in the process (see earlier, Fig. 4). The addition of malate promotes oxygen consumption and $\Delta\Psi$ in mouse malaria species (Uyemura *et al*., 2000, 2004), supporting the role of this enzyme complex in the electron transport chain. Curiously, however, one study of *P. falciparum* found that the addition of malate did not promote complex III activity (Fry & Beesley, 1991). It is not clear whether these differences are species specific or due to different sensitivities of the assay techniques. Generally speaking, though, it seems that both succinate dehydrogenase and malate : quinone oxidoreductase can contribute to the electron transport chain of *Plasmodium* parasites. These *in vitro* studies, however, say very little about the biological relevance of these complexes in *Plasmodium*. Due to uncertainties about the tricarboxylic acid cycle in *Plasmodium* (see earlier) it is not known where the electrons that are donated from these enzymes ultimately come from. Are they derived from the acetyl-CoA or from amino acid catabolism? As mentioned above, it is not even clear if complex II is able to donate electrons further along the electron transport chain (Takashima *et al*., 2001). Information about the role of succinate dehydrogenase and malate : quinone oxidoreductase in the *Plasmodium* electron transport chain needs to be placed in the context of what is occurring in the tricarboxylic acid cycle. A challenge for understanding electron transport in *Plasmodium* is to move beyond *in vitro* assays and begin to understand these physiological processes *in vivo*. Reverse genetic approaches recently developed in both *P. falciparum* and the mouse malaria *P. berghei* offer promising means to address these questions (Crabb *et al*., 1997; Menard *et al*., 1997; Carvalho *et al*., 2004; Meissner *et al*., 2005).

NADH dehydrogenases of the mitochondrial respiratory chain oxidize NADH to NAD^+ and donate these electrons to coenyzme Q. In animal cells, NADH reduced during the tricarboxylic acid cycle is oxidized by a multi-enzyme NADH dehydrogenase known as complex I. Complex I donates electrons to coenzyme Q, and couples this process to the translocation of protons across the inner membrane. The function of complex I is significantly inhibited by rotenone. Some eukaryotes, most notably the yeast *S. cerevisiae*, lack complex I. They do, however, retain mitochondrial NADH dehydrogenase activity, a process that is not inhibited by rotenone (Kerscher, 2000). Other organisms such as plants have complex I activity, but inhibition by rotenone reveals that these organisms also have a second, rotenone-insensitive NADH dehydrogenase activity. Characterization of the alternative, rotenone-insensitive NADH dehydrogenase from yeast and plants found it to comprise a single subunit enzyme that is unable to translocate protons across the inner membrane (de Vries & Grivell, 1988; Marres *et al*., 1991; Rasmusson *et al*., 1999). Curiously, multiple isoforms of the single subunit NADH dehydrogenase occur in both plants and fungi. Some isoforms localize to the matrix side of the inner membrane, while others localize to the intermembrane space side (Kerscher, 2000). Some also exclusively accept NADPH as an electron donor, whereas others can receive electrons from both NADH and NADPH (Kerscher, 2000; Carneiro *et al*., 2004). The matrix or 'internal' form oxidizes NAD(P)H from the matrix (e.g. that produced in the tricarboxylic acid cycle), whereas the 'external' form oxidizes cytosolically produced NAD(P)H (e.g. that produced during glycolysis).

Extensive searching of the *P. falciparum* nuclear genome failed to uncover any complex I homologues. Furthermore, several complex I subunits that are almost always encoded on the mitochondrial genome of organisms containing complex I (eg *nad1*, *nad4* and *nad5*; Lang *et al*., 1999) are absent on the *P. falciparum* mitochondrial genome. Although we cannot definitively rule out the presence of complex I in *Plasmodium* parasites, this bioinformatic data

is supported by the finding that NADH-linked electron transport is only inhibited by very high concentrations of rotenone (Fry & Beesley, 1991; Uyemura *et al.*, 2000; Krungkrai *et al.*, 2002), and not at all by lower, more physiologically relevant concentrations (Uyemura *et al.*, 2004). The *P. falciparum* genome does, however, contain a clear 'alternative' NAD(P)H dehydrogenase homologue (PFI0735c; Fig. 4), of a predicted size of around 60 kDa. Fusing the N-terminus of this protein to green fluorescent protein results in localization to the mitochondrion (Fig. 1i), clear evidence that this protein functions in that organelle. A biochemical study that purified NADH dehydrogenase activity found two major protein bands of around 33 kDa and 38 kDa (Krungkrai *et al.*, 2002), entirely inconsistent with the bioinformatic data.

What role might the *Plasmodium* NADH dehydrogenase have in the electron transport chain? O_2 consumption studies in *P. berghei* show that the addition of exogenous NADH cannot stimulate O_2 consumption (Uyemura *et al.*, 2000). However, a very similar assay system in *P. yoelii* found that the simultaneous addition of NADH and NADPH did stimulate O_2 consumption (Uyemura *et al.*, 2004). This data might suggest that the *Plasmodium* enzyme is NADPH specific. However, a complex III activity assay in *P. falciparum* found that NADH could stimulate electron transport (Fry & Beesley, 1991). The substrate specificity of *Plasmodium* NAD(P)H dehydrogenase, thus, requires further confirmation. NADH and NADPH are considered impermeable to the inner mitochondrial membrane. The stimulation of electron transport by addition of exogenous substrate would indicate *Plasmodium* NAD(P)H dehydrogenase is an external enzyme, lying on the intermembrane space side of the inner membrane. Such a scenario requires experimental confirmation, but would suggest this enzyme functions in oxidizing NAD(P)H produced from cytosolic reactions. If this scenario is correct, there would be no obvious enzyme capable of feeding electrons from NAD(P)H produced in the tricarboxylic acid cycle into the electron transport chain. This is contrary to some experimental evidence, which indicates that citrate, glutamate and α-ketoglutarate – substrates typically linked to the NADH and NADPH generating enzymes of the tricarboxylic acid cycle (namely NADPH-specific isocitrate dehydrogenase and NAD^+-reducing α-ketoglutarate dehydrogenase) – can stimulate O_2 consumption (Uyemura *et al.*, 2000).

The NAD(P)H dehydrogenase homologue in *Plasmodium* has no counterpart in humans. This makes the enzyme a good candidate for the development of antimalarial drugs. However, a better understanding of the function and importance of this enzyme is required before such goals can be achieved. Key to this is determining the localization and NADH/NADPH substrate specificities. Another important question is determining the role that the *Plasmodium* NAD(P)H dehydrogenase homologue has in overall cellular metabolism. Does it function to feed electrons from the cytosol or from the mitochondrial matrix into the electron transport chain for ATP generation? Of particular interest is a recent study on the effects of the antimalarial drug artemisinin in a model yeast system (Li *et al.*, 2005). Up-regulation of yeast single-subunit NAD(P)H dehydrogenases was found to increase sensitivity of yeast to artemisinin, whereas ablation of expression lowered the sensitivity. Expression of *P. falciparum* NADH dehydrogenase in yeast cells where native NAD(P)H dehydrogenase had been ablated restored sensitivity to artemisinin. This suggests that mitochondrial electron transport, and the functions of the NAD(P)H dehydrogenase in particular, may be crucial in activating and mediating the effects of this important antimalarial drug.

If *Plasmodium* NAD(P)H dehydrogenase functions as an 'external' enzyme in feeding cytosolic NADH into the mitochondrial electron transport chain, it would seem to be overlapping in function with the fifth of the mitochondrial inner membrane, coenzyme Q-reducing dehydrogenases (and with any malate-aspartate shuttle that may exist – see earlier). FAD-linked, or mitochondrial, glycerol-3-phosphate dehydrogenase forms part of the glycerol-3-phosphate shuttle. This shuttle essentially provides an alternative means of directing electrons from cytosolic NADH to mitochondrial coenzyme Q. The first step in this shuttle involves cytosolic NAD^+-linked glycerol-3-phosphate dehydrogenase, which reduces dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate, oxidizing cytosolic NADH in the process (Fig. 4). Glycerol-3-phosphate is then transported to the mitochondrial intermembrane space, where mitochondrial glycerol-3-phosphate dehydrogenase oxidizes glycerol-3-phosphate back to dihydroxyacetone phosphate, with the electron donated to its FAD cofactor. In turn, this electron is passed into the electron transport chain via coenzyme Q (Fig. 4). The *P. falciparum* genome has homologues of both NAD^+-linked (PFL0780w) and FAD-linked (PFC0275w) glycerol-3-phosphate dehydrogenases and assays indicate that the addition of glycerol-3-phosphate stimulates electron transport through the inner membrane (Fry & Beesley, 1991; Uyemura *et al.*, 2000, 2004).

In summary, bioinformatic and biochemical evidence suggests that electrons are donated to the *Plasmodium* electron transport chain via the FAD-linked tricarboxylic acid cycle enzymes malate : quinone oxidoreductase and succinate dehydrogenase (complex II). Electrons are also donated during pyrimidine biosynthesis via DHODase. The contribution of electrons donated from cytosolic and mitochondrial NADH and NADPH to the electron transport chain requires further elucidation of the functions and localizations of NAD(P)H dehydrogenase and glycerol-3-phosphate dehydrogenase.

Harvesting the mitochondrial proton crop?

In other organisms, the proton gradient generated across the inner membrane by complexes III and IV can be harvested to generate ATP. ATP synthesis at the mitochondrial inner membrane is catalyzed by an intricate complex called ATP synthase, the function and structure of which is known in considerable detail (Capaldi & Aggeler, 2002). ATP synthase consists of two multi-subunit containing components (Fig. 4) that can function in both ATP synthesis and ATP hydrolysis. The F_0 base is embedded in the inner membrane and functions during ATP synthesis to direct protons down their concentration gradient (Capaldi & Aggeler, 2002). This movement of protons is coupled to activity of the matrix localized F_1 knob, which is attached to the base via stalk proteins. The F_1 component functions as a rotary motor, binding ADP and P_i and reacting with these substrates to form ATP before release of the ATP (Capaldi & Aggeler, 2002). ATP is then transported out of the mitochondrion via an ADP/ATP carrier protein (see earlier). F_1 consists of five major subunits, all of which have well conserved homologues in the *P. falciparum* genome. These include the α (PFB0795w), β (PFL1725w), γ (PF13_0061), δ (MAL13P1.47), and ε (MAL7P1.75) subunits. No homologues of the F_0 base are apparent in the genome. It is possible that amino acid conservation of these subunits is insufficient to enable their bioinformatic identification.

It was a long held view that electron transport in *Plasmodium* species was not coupled to ATP production, based largely on the aforementioned studies that showed products of glycolysis were not fed into the tricarboxylic acid cycle. Recently, it was demonstrated that O_2 consumption could be inhibited by the ATP synthase F_1 inhibitor oligomycin (Uyemura *et al*., 2000, 2004). These studies also found that oligomycin affected $\Delta\Psi$, suggesting mitochondrial ATP synthase uses the proton gradient generated by the electron transport chain to synthesize ATP. However, direct evidence for the activity of ATP synthase in *Plasmodium* parasites is lacking, and the importance of mitochondrial ATP synthase in contributing to the cellular ATP pool in asexual stages and in other parts of the life cycle is not yet clear. Indeed, a study looking a cellular ATP levels found that while oligomycin could inhibit growth of *P. falciparum*, it had an apparently negligible effect on overall ATP levels in parasites (Fry *et al*., 1990).

Stressed-out mitochondria

The mitochondrial electron transport chain is notorious for generating reactive oxygen species, major contributors to DNA mutations and other undesirable biochemical reactions. These reactive oxygen species can be generated on both the matrix and the intermembrane space sides of the inner membrane. Mitochondria contain numerous proteins to deal with oxidative stress. The *P. falciparum* mitochondrion also likely contains numerous enzymes involved in nullifying the effects of reactive oxygen species (Muller, 2004). The best characterized of these is a superoxide dismutase known as Sod2 (PFF1130c) (Sienkiewicz *et al*., 2004). Sod enzymes convert superoxide anions to hydrogen peroxide and molecular oxygen, and the N-terminal leader sequence of *P. falciparum* Sod2 was shown to target green fluorescent protein to the mitochondrion (Sienkiewicz *et al*., 2004). Curiously, the N-terminal leader of *Pf* Sod2 closely resembles an apicoplast leader, containing a hydrophobic, signal peptide-like domain, followed by a domain containing a net positive charge (Foth *et al*., 2003; Sienkiewicz *et al*., 2004). This mirrors the situation seen in the Sod2 homologue in the related Apicomplexan *Toxoplasma gondii*, which is also appears to target exclusively to the mitochondrion despite having an apparent apicoplast leader (Brydges & Carruthers, 2003). In yeast, one isoform of superoxide dismutase has been shown to partially localize to the mitochondrial intermembrane space (Okado-Matsumoto & Fridovich, 2001), where complex III of the electron transport chain produces superoxides (Muller *et al*., 2004). One possibility is that the hydrophobic N-terminus of *Pf*Sod2 acts to anchor the protein into the inner membrane, with the protein thus localized in the intermembrane space. Determining the intra-mitochondrial localization of *Pf* Sod2 would address this possibility, although it should be noted that *Plasmodium* also contains a cytosolic Sod protein that could neutralize superoxides produced on the intermembrane space side of the inner membrane (Sienkiewicz *et al*., 2004).

Peroxides produced by Sod2 must be further reduced to form harmless compounds such as water. This is typically achieved by glutathione- or thioredoxin-dependent redox systems. These systems are essentially a cascade of redox reactions that transfer electrons from compounds such as NADPH and ultimately donate them to peroxides (Muller, 2004). A recent study identified a mitochondrially localized thioredoxin-dependent peroxidase (peroxiredoxin) in *P. falciparum* (Yano *et al*., 2005). This suggests the presence of a thioredoxin-based mechanism for reducing peroxides in the *Plasmodium* mitochondrion. Such a system typically donates electrons from NADPH to thioredoxin reductase, and further through thioredoxin to peroxiredoxin, which can then reduce peroxides (Muller, 2004). Müller suggests that the *Plasmodium* mitochondrion lacks thioredoxin reductase, and posits that electrons ultimately donated to peroxides may originally derive from the lipoic acid moiety of the E2 subunit of α-keto acid dehydrogenases found in the mitochondrion (Muller, 2004). Although intriguing, such a scenario requires experimental confirmation.

Electron transport in *Plasmodium*: what's the point?

Based on previous biochemical studies and the bioinformatic analysis presented here, we can propose several possible functions for the electron transport chain in *Plasmodium*. It clearly functions as an electron sink for the dihydroorotate dehydrogenase reaction of the essential pyrimidine biosynthesis pathway, making dihydroorotate dehydrogenase a promising target for drug development (Baldwin *et al.*, 2002, 2005). It is possible that generally inhibiting the electron transport chain may impair pyrimidine biosynthesis. Experimental proof of the dependence of pyrimidine biosynthesis on electron transport would make the electron transport chain a prime target for antimalarial drugs. It is, therefore, of major interest to determine whether drugs that target the electron transport chain (e.g. atovaquone) have any effect on pyrimidine biosynthesis.

It is clear that the electron transport chain of *Plasmodium* functions in generating a $\Delta\Psi$ across the inner membrane. It remains to be determined whether $\Delta\Psi$ has a biologically important role in these parasites. By analogy with other systems, $\Delta\Psi$ may be required for protein import and in the transport of solutes (Laloi, 1999; Pfanner & Geissler, 2001). Interestingly, one study has shown that the addition of calcium ions increases O_2 consumption and depolarizes the $\Delta\Psi$ (Uyemura *et al.*, 2000). This suggests the presence of Ca^{2+} transporters across the inner membrane that are involved in regulating and maintaining cytosolic calcium levels. The $\Delta\Psi$ across the inner membrane, thus, may also serve an important role in cellular calcium homeostasis.

The role of the electron transport chain and proton gradient in ATP generation remains an open question. Based on available evidence, it seems that ATP generation may occur in asexual stage parasites, but possibly at minor levels when compared with the ATP generated by glycolysis, and probably not fed by pyruvate generated in glycolysis. Determining the existence, stage-specificity and importance of ATP generated by the mitochondrion is a priority. It may be that in asexual stage parasites, a minimal amount of ATP is generated, coupled to electrons donated from pyrimidine biosynthesis, and perhaps by the small amount of NADH generated during glycolysis via the glycerol-3-phosphate shuttle and/or an external NADH dehydrogenase. The contribution of the tricarboxylic acid cycle to ATP generation remains unclear. An incomplete tricarboxylic acid cycle, perhaps fed by amino acids, could produce reducing equivalents that lead to the production of ATP. If a complete tricarboxylic acid cycle is possible, more ATP could be generated. Determining the source of acetyl-CoA and answering other outstanding questions regarding the tricarboxylic acid cycle will lead to a better understanding of the importance of oxidative phosphorylation in *Plasmodium*. It is also worth noting that at various stages of the *Plasmodium* life cycle, glucose is presumably not as abundant as in blood stages. It is possible that ATP generated through oxidative phosphorylation is of greater importance at these stages, a finding supported by recent transcriptome and proteomic analyses (Le Roch *et al.*, 2003; Hall *et al.*, 2005; Khan *et al.*, 2005).

We have thus far covered the possible roles of the *Plasmodium* mitochondrion in energy production. The remainder of this review will focus on the biosynthetic functions of this organelle.

The hybrid haem pathway of *Plasmodium*

Plasmodium parasites ingest large quantities of haemoglobin and break it down in their food vacuole. The haem prosthetic group of haemoglobin is considered toxic to parasites, possibly due to the generation of oxygen radicals. *Plasmodium* deals with this threat by polymerizing haem into so-called haemozoin pigment (Slater *et al.*, 1991). Failure to properly polymerize this haem is thought to cause the death of the parasite (Slater & Cerami, 1992). *Plasmodium* requires haem in cytochromes and other components of the electron transport chain (see earlier). Despite the abundance of erythrocyte-derived haem, *Plasmodium* synthesizes its own haem during blood stages. It is unclear why *Plasmodium* does not utilize haemoglobin-derived haem. It is possible that parasites simply never evolved the necessary export mechanisms to obtain haem from their food vacuole. It may also be that the efficiency of host cell haem polymerization is such that obtaining haem from the food vacuole is not possible. Moreover, as several stages of the *Plasmodium* life cycle do not have access to haemoglobin-derived haem, it is likely that a strong selective pressure exists to prevent parasites from losing their *de novo* haem synthesis pathway. Thus, if non-erythrocytic portions of the life cycle make *de novo* haem biosynthesis indispensable, it may be that parasites simply do not require host-derived haem.

Haem itself is a complex molecule with a planar structure known as a tetrapyrrole or porphyrin ring. At the centre of the ring is an iron molecule that can be reduced or oxidized through electron transfer. Such porphyrin rings are also found in chlorophyll molecules, the light harvesting pigments of plant and algal cells. The major difference between chlorophyll and haem is that chlorophyll contains a magnesium ion at the centre of the porphyrin ring. The first committed step of haem biosynthesis (and porphyrin synthesis generally) is the formation of a compound known as δ-aminolaevulinic acid. There are two known mechanisms of its synthesis. Cyanobacteria and plants synthesize δ-aminolaevulinic acid via a two-step enzymatic process from glutamate, with tRNA used as a cofactor. This process is

catalyzed by the enzymes glutamyl-tRNA reductase and glutamate 1-semialdehyde aminotransferase, both of which localize to the plastid of plants. δ-Aminolaevulinic acid can also be synthesized from glycine and succinyl-CoA, a reaction catalyzed by the mitochondrial enzyme δ-aminolaevulinic acid synthase. This process is known to occur in yeast, animals, several protozoa and some bacteria.

In plants, porphyrin synthesis takes place in the plastid, by a series of enzymes labelled HemB through to HemG. Upon production of the porphyrin ring, the pathway splits, with insertion of magnesium into the ring resulting in the biosynthesis of chlorophyll, while insertion of iron by HemH leads to haem production (Grimm, 1998). In plants, plastid-produced haem is utilized in photosynthetic cytochromes. The role of plastid-produced haem in mitochondrial cytochromes is unclear. Some evidence suggests that HemG and HemH, the penultimate and ultimate enzymes of haem biosynthesis, are dually targeted to the mitochondrion and plastid, suggesting the transport of late precursors from the plastid to the mitochondrion and completion of the pathway in the mitochondrion (Grimm, 1998). Recent data, however, indicate HemH may be exclusively localized to the plastid (Masuda *et al.*, 2003), suggesting that the mitochondrial haem molecules are plastid-derived. In animal cells, the same enzymes (HemB through to HemH) catalyze the formation of haem. δ-Aminolaevulinic acid synthesized in the mitochondrion is exported to the cytosol. The steps catalyzed by HemB through to HemE are also catalyzed in the cytosol. The final three steps catalyzed by HemF, HemG and HemH occur across the inner mitochondrial membrane.

Haem biosynthesis in *Plasmodium* parasites presents an unusual scenario. *Plasmodium* has two organelles where δ-aminolaevulinic acid synthesis might occur. However, the plastid does not require chlorophyll or haem for photosynthetic cytochromes, and so there is no apparent reason for the plastid to be involved in haem biosynthesis. How then does *Plasmodium* haem biosynthesis occur? An δ-aminolaevulinic acid synthase homologue (PFL2210w) is present in the *P. falciparum* genome and has been shown to localize to the mitochondrion (Wilson *et al.*, 1996; Varadharajan *et al.*, 2002; Sato *et al.*, 2004) (Fig. 5). No homologues for either of the plastid-type δ-aminolaevulinic acid synthesis enzymes are present, so δ-aminolaevulinic acid synthesis is apparently exclusively mitochondrial. This supports earlier biochemical data showing the incorporation of radiolabelled glycine but not glutamate into haem by *P. falciparum* parasites (Surolia & Padmanaban, 1992). The requirement for the tricarboxylic acid cycle intermediate succinyl-CoA (see earlier) in this reaction points to a biosynthetic role for the tricarboxylic acid cycle.

After the 'animal'-like synthesis of δ-aminolaevulinic acid in the mitochondrion, haem biosynthesis in *Plasmodium* takes a surprising twist, with the enzymes catalyzing subsequent steps localizing to the apicoplast (Fig. 5). δ-Aminolaevulinic acid is converted in four enzymatic reactions, catalyzed by HemB, HemC, HemD and HemE, to coproporphyrinogen III. The *P. falciparum* genome contains clear homologues of HemB (PF14_0381), HemC (PFL0480w) and HemE (PFF0360w). All three enzymes have N-terminal extensions that resemble apicoplast leaders, and the leader sequences of HemB and HemC both target green fluorescent protein to the apicoplast (Sato *et al.*, 2004). The apparent lack of a HemD homologue in the *P. falciparum* genome may result from the generally poor sequence conservation in this protein (Panek & O'Brian, 2002). Identification of *Plasmodium* HemD may require biochemical characterization and purification.

The final three steps of haem biosynthesis are catalyzed by HemF (PF11_0436), HemG (PF10_0275) and HemH (MAL13P1.326), all of which have homologues in the *P. falciparum* genome. The localization of HemF and HemG is unclear (Fig. 5). The N-termini of both proteins lack obvious N-terminal leader targeting sequences. This suggests these proteins are not localized to the apicoplast or the mitochondrial matrix. It is possible that HemF and HemG contain internal targeting signals that direct them to the inner membrane of the mitochondrion, where they are localized in animals (Grandchamp *et al.*, 1978; Deybach *et al.*, 1985). Alternatively, HemF is localized to the cytosol of yeast (Camadro *et al.*, 1986), where it may also localize in *Plasmodium*. HemH, the protein that inserts iron into the porphyrin ring, contains a short N-terminal extension that resembles a mitochondrial targeting sequence (Bender *et al.*, 2003). A recent study, however, presented immunofluorescence data suggesting *Plasmodium* HemH is in fact apicoplast localized (Varadharajan *et al.*, 2004). To test this surprising result, we fused HemH to a short epitope tag and expressed the resulting fusion protein in *P. falciparum* cells. Using immunofluorescence assays to co-label epitope-tagged HemH with either an apicoplast or a mitochondrial marker clearly demonstrated the mitochondrial localization of this enzyme (Fig. 5) (GvD, LS and GIM, unpublished results).

Haem biosynthesis in malaria parasites represents an unusual hybrid pathway shared between the mitochondrion and apicoplast. The pathway commences in the mitochondrion, proceeds in the apicoplast, before concluding in the mitochondrion. Here synthesized haem is presumably used in mitochondrial cytochromes (see earlier). More study is required to identify and localize several enzymes of the pathway. Also unclear is the possible role of host-cell derived HemB in this biosynthetic process (Bonday *et al.*, 1997, 2000), and the source of the substrates required in the initial δ-aminolaevulinic acid synthase-catalyzed reaction.

We and others (Ralph *et al.*, 2004; Sato *et al.*, 2004) have suggested that the sharing of haem biosynthesis might

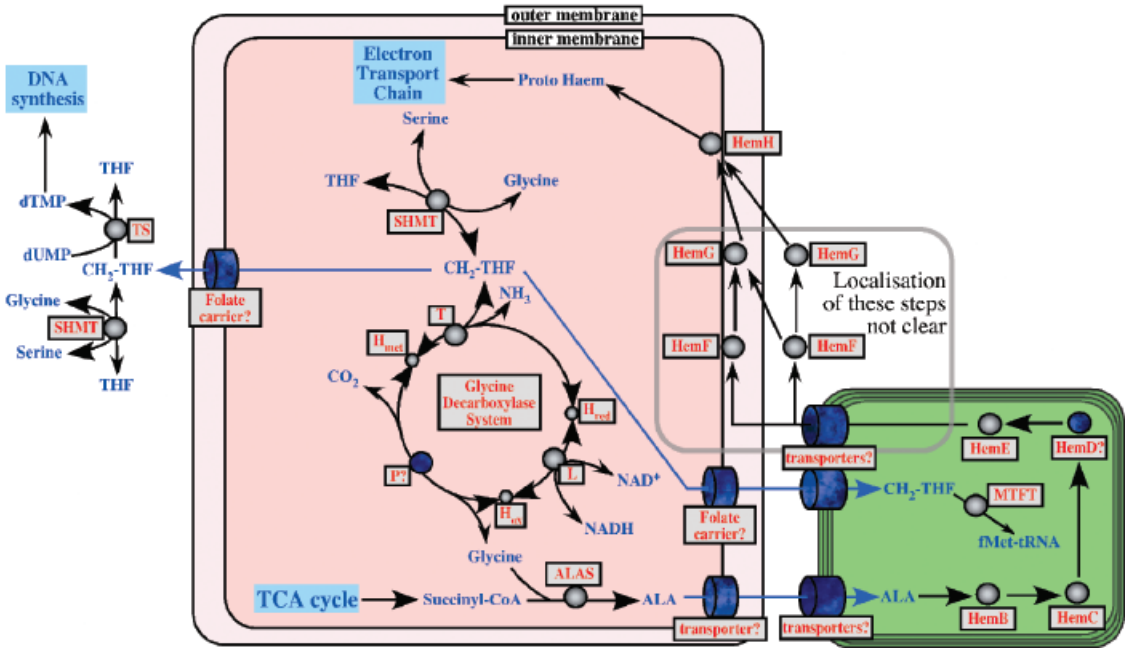


Fig. 5. The hybrid haem biosynthetic pathway of *Plasmodium falciparum*. Haem biosynthesis commences in the mitochondrion, where δ-aminolaevulinic acid synthase (ALAS) reacts succinyl-CoA with glycine to produce δ-aminolaevulinic acid, which must then be transported into the apicoplast by unknown mechanisms, where the enzymatic reactions catalyzed by HemB, HemC and HemE occur. There is no known homologue of the HemD enzyme in the *P. falciparum* genome. The location of the HemF and HemG enzymes is unknown, but is likely to be in either the cytosol or the mitochondrion. The final step of this pathway produces proto-haem, and is catalyzed by HemH in the mitochondrion. Proto-haem can then be further modified before used as a prosthetic group in proteins such as cytochromes of the electron transport chain (see Fig. 4). The mitochondrion also contains homologues of three of the four enzymes that constitute the glycine decarboxylase system (H, L and T, but not P). The glycine decarboxylase system functions in either the breakdown of glycine and production of CH_2-tetrahydrofolate, or in the reverse direction for the synthesis of glycine (perhaps to feed into haem biosynthesis). The H protein contains a lipoamide moiety, which may exist in reduced (H_{red}), oxidized (H_{ox}) or methylaminated (H_{met}) forms. The mitochondrion contains a homologue of serine hydroxymethyl transferase (SHMT), which utilizes CH_2-tetrahydrofolate for the production of serine, or in the reverse direction to produce glycine and CH_2-tetrahydrofolate. CH_2-tetrahydrofolate is necessary for several crucial reactions outside the mitochondrion, such as the synthesis of dTMP (catalyzed by thymidylate synthase, TS) in the cytosol and the synthesis of formylmethionine tRNA (by methionyl-tRNA formyl transferase, MTFT) in the apicoplast. The source of CH_2-tetrahydrofolate for these reactions is unclear, but may derive from the mitochondrial serine hydroxymethyl transferase, the glycine decarboxylase, or the cytosolic isoform of serine hydroxymethyl transferase.

explain the close association between the mitochondrion and apicoplast that is observed in intraerythrocytic stages. We have since argued that the association between the two organelles may function to ensure proper segregation of these organelles into daughter parasites (van Dooren *et al.*, 2005). When considering how such a hybrid pathway may have evolved, it is likely that upon acquisition of the plastid through endosymbiogenesis, the *Plasmodium* ancestor had two haem pathways (Fig. 6; step 1). The linking of organelles during segregation may have placed these organelles in close enough contact to enable them to swap haem biosynthetic metabolites. The cytosolic enzymes of the original pathway then became redundant and were lost (Fig. 6, step 2). Upon loss of photosynthesis, the terminal reactions in the plastid were no longer necessary and were lost, leaving the pathway that we observe today (Fig. 6, step 3). The selective pressure that brought about such a convoluted pathway is not clear, although we might speculate that this enabled the organism to produce haem more efficiently for both the mitochondrion and the plastid from a single pathway. It is also not clear whether close contact between the two organelles would be sufficient to enable them to swap metabolites. Presumably, transporters would be required to enable these compounds to cross the various membranes of the mitochondrion and apicoplast. In the 'proto-*Plasmodium*' cell we describe in Step 1 of Fig. 6, such transporters must already exist to transport δ-aminolaevulinic acid out of the mitochondrion, and late-stage haem intermediates back into the mitochondrion. The scheme we present in Steps 2 and 3, thus, requires the evolution of two transport mechanisms, one to transport δ-aminolaevulinic acid into the plastid and one to transport coproporphyrinogen III (the product of HemE) out of the plastid. No transporters of haem pathway intermediates have yet been identified in other organisms, although they must exist. The identity of these transporters in *Plasmodium* is also unclear, but is certainly worthy of

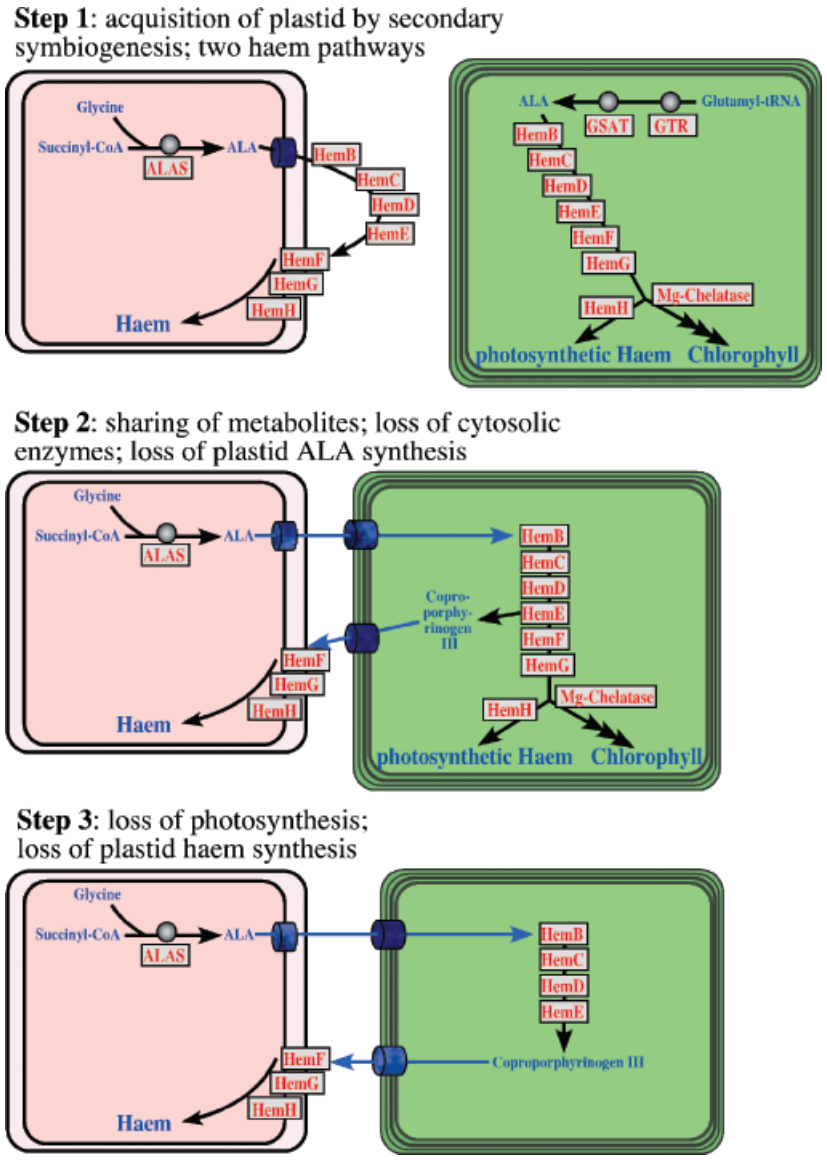


Fig. 6. A putative scheme for the evolution of the unusual haem biosynthesis pathway of *Plasmodium falciparum*. Step 1. Upon acquisition of the plastid through secondary symbiogenesis, the *P. falciparum* ancestor contained two functionally distinct haem biosynthesis pathways. Step 2. The two organelles were able to exchange haem biosynthetic intermediates. This may have resulted from the formation of contact points between the two organelles. The plastid was able to acquire δ-aminolaevulinic acid from the mitochondrion. This initially meant the presence of two mechanisms of synthesizing δ-aminolaevulinic acid, with the plastid version being lost (perhaps because of a greater bioavailability of succinyl-CoA and glycine in the mitochondrion compared to glutamate in the plastid?). The mitochondrion was able to acquire coproporphyrinogen III from the plastid, making redundant the previously cytosolic steps of the biosynthetic pathway, which were consequently lost. This 'intermediate' organism was able to produce proto-haem in both the mitochondrion (where it was used for electron transport) and the plastid (where it was used in photosynthetic cytochromes), as well as being able to produce chlorophyll in the plastid. Step 3. Upon the loss of photosynthesis, the steps catalyzing the production of haem and chlorophyll in the plastid were no longer necessary and were lost, leaving the pathway that is apparent today.

further study. Not only would the identification of transporters provide valuable information about haem biosynthesis, but they may also provide valuable clues about how this unusual haem biosynthetic pathway evolved.

Folate metabolism

The source of glycine for δ-aminolaevulinic acid synthesis in the haem pathway is unclear. It is possible that glycine, perhaps derived from the degradation of host haemoglobin, is imported into the mitochondrion. The mitochondrion also seems to be site of further glycine metabolism, since enzymes involved in the glycine decarboxylase or glycine cleavage system likely occur in the *Plasmodium* mitochondrion. The glycine decarboxylase system catalyzes the breakdown of glycine, yielding CO_2 and NADH, and adding a methylene group to tetrahydrofolate to produce CH_2-tetrahydrofolate (Douce *et al.*, 2001) (Fig. 5). In yeast, this pathway can also function the other way in glycine generation, a process in which CO_2 is fixed (Cossins & Chen, 1997). The glycine decarboxylase system can, thus, function in both glycine synthesis and in glycine catabolism, with the latter process also yielding NADH and CH_2-tetrahydrofolate. Folates such as CH_2-tetrahydrofolate are crucial in the biosynthesis of compounds such as purines, methionine, formylmethionyl-tRNA, and the pyrimidine thymidylate (Cossins & Chen, 1997). *Plasmodium* has homologues of several proteins of glycine decarboxylase, including the H-protein (PF11_0339), the T-protein (PF13_0345) and the L-protein (identical to the E3 subunit of the keto-acid

dehydrogenases mentioned earlier McMillan *et al.*, 2005; Salcedo *et al.*, 2005). However, a clear homologue of the P-protein, the crucial enzyme that initially decarboxylates glycine, is missing (Salcedo *et al.*, 2005). The functions of folate metabolism are poorly understood in *Plasmodium* (Hyde, 2005; Nzila *et al.*, 2005a, b). Parasites are incapable of *de novo* purine biosynthesis, ruling out this major function of folate metabolism. It is thought that a key function of *Plasmodium* folate metabolism is in the production of dTMP from dUMP (Nzila *et al.*, 2005b). This reaction is catalyzed by thymidylate synthase, and requires CH_2-tetrahydrofolate as the methyl donor (Fig. 5). CH_2-tetrahydrofolate may be produced from either the glycine cleavage reaction, or from the reaction catalyzed by serine hydroxymethyl transferase, of which a possible mitochondrial targeted homologue exists in *Plasmodium* (PF14_0534) (Nzila *et al.*, 2005b; Salcedo *et al.*, 2005). Serine hydroxymethyl transferase interconverts glycine and serine, generating CH_2-tetrahydrofolate in the production of glycine. CH_2-tetrahydrofolate is required in other parts of the cell, for instance in the apicoplast during the generation of formylmethionine-tRNA by methionyl-tRNA formyl transferase (MTFT; Nzila *et al.*, 2005b). This suggests that, as in other organisms, the mitochondrion contains a folate transporter, although no clear homologue is identifiable in the genome (see earlier). It is also possible that CH_2-tetrahydrofolate required in the cytosol and apicoplast is sourced from the cytosolic isoform of serine hydroxymethyl transferase, with the mitochondrial pool of CH_2-tetrahydrofolate generated by glycine decarboxylase required for serine synthesis via serine hydroxymethyl transferase (Fig. 5). The lack of understanding of folate metabolism in *Plasmodium* contrasts with the importance of folate metabolism in therapy, with major antimalarial drugs such as pyrimethamine and sulfadoxine targeting this pathway. The role of the *Plasmodium* mitochondrion in folate metabolism and the regulation of glycine levels for haem and serine biosynthesis are not understood; both processes require much further study.

Coenzyme Q biosynthesis

Coenzyme Q is a central component of the electron transport chain that occurs in the mitochondrion of *Plasmodium* (see earlier). The coenzyme Q molecule is composed of two separate parts: a benzoquinone ring that participates in redox reactions, and an isoprenoid tail, the length of which varies between organisms (Turunen *et al.*, 2004). Isoprenoid biosynthesis occurs in the apicoplast via the non-mevalonate (or DOXP) pathway (Jomaa *et al.*, 1999; Ralph *et al.*, 2004; Tonkin *et al.*, 2004) (Fig. 7). This pathway results in the production of isopentenyl pyrophosphate (Ralph *et al.*, 2004). The first requirement of coenzyme Q biosynthesis is the production of the isoprenoid chain. Enzymes known as prenyltransferases join isopentenyl pyrophosphate units together, to form isoprenoid chains of certain lengths. Through radiolabelling experiments using various isoprenoid intermediates, the length of the isoprenoid chain of coenzyme Q in *P. falciparum* was determined to be eight and/or nine isopentenyl pyrophosphate units (de Macedo *et al.*, 2002). The *P. falciparum* genome contains a homologue of the Coq1 protein (PFB0130w), the prenyltransferase involved in the synthesis of the coenzyme Q isoprenoid tail in yeast (Ashby & Edwards, 1990). This protein was recently characterized from *P. falciparum* and found to catalyze the formation of eight isoprenoid unit molecules, and hence designated an octaprenyl pyrophosphate synthase (Tonhosolo *et al.*, 2005). In yeast, Coq1 localizes to the matrix side of the inner mitochondrial membrane (Gin & Clarke, 2005), and it is likely that the *P. falciparum* equivalent also localizes to this compartment (Fig. 7).

The second step in coenzyme Q biosynthesis is the addition of the polyprenyl chain to 4-hydroxybenzote to form polyprenyl-4-hydroxybenzoate (Fig. 7). In *Escherichia coli*, 4-hydroxybenzoate is formed directly from chorismate, whereas in yeast, it can also be derived from tyrosine (Meganathan, 2001; Turunen *et al.*, 2004). The source of hydroxybenzoate in *P. falciparum* is unclear. In yeast, synthesis of polyprenyl-4-hydroxybenzoate is catalyzed by Coq2 (Ashby *et al.*, 1992). *P. falciparum* contains a Coq2 homologue (PFF0370w) and fusion of the N-terminal leader of this protein targets green fluorescent protein to the mitochondrion (Fig. 1j). The remaining steps of coenzyme Q biosynthesis involve modifications of the original benzoate ring (Turunen *et al.*, 2004), not all of which have been fully characterized. Coq3 catalyzes the methylation of hydroxyl groups on the ring, and possibly participates in two steps of the synthesis pathway (Meganathan, 2001). *P. falciparum* contains a possible Coq3 homologue (MAL7P1.130) that also contains a likely mitochondrial-targeting transit peptide. The methyl group in the Coq3-catalyzed reaction is donated by S-adenosylmethionine, a compound that may be imported into the mitochondrion by a carrier protein homologue (see earlier, Fig. 7). Coq5, of which a homologue exists in *P. falciparum* (PFB0220w), catalyzes a different methylation reaction, again using S-adenosylmethionine as the methyl donor. *Plasmodium falciparum* lacks apparent homologues of the Coq6 and Coq7 proteins, both of which are thought to function in hydroxylation of the benzoate ring (Turunen *et al.*, 2004). It is not clear what may catalyze these reactions. Given that most of the coenzyme Q biosynthesis enzymes of *P. falciparum* have low sequence similarity to counterparts in other organisms, it may be that Coq6 and Coq7 simply cannot be detected using simple BLAST searches. *P. falciparum* contains homologues of Coq4 (PF11_0128) and Coq8 (PF08_0098). Both of these proteins

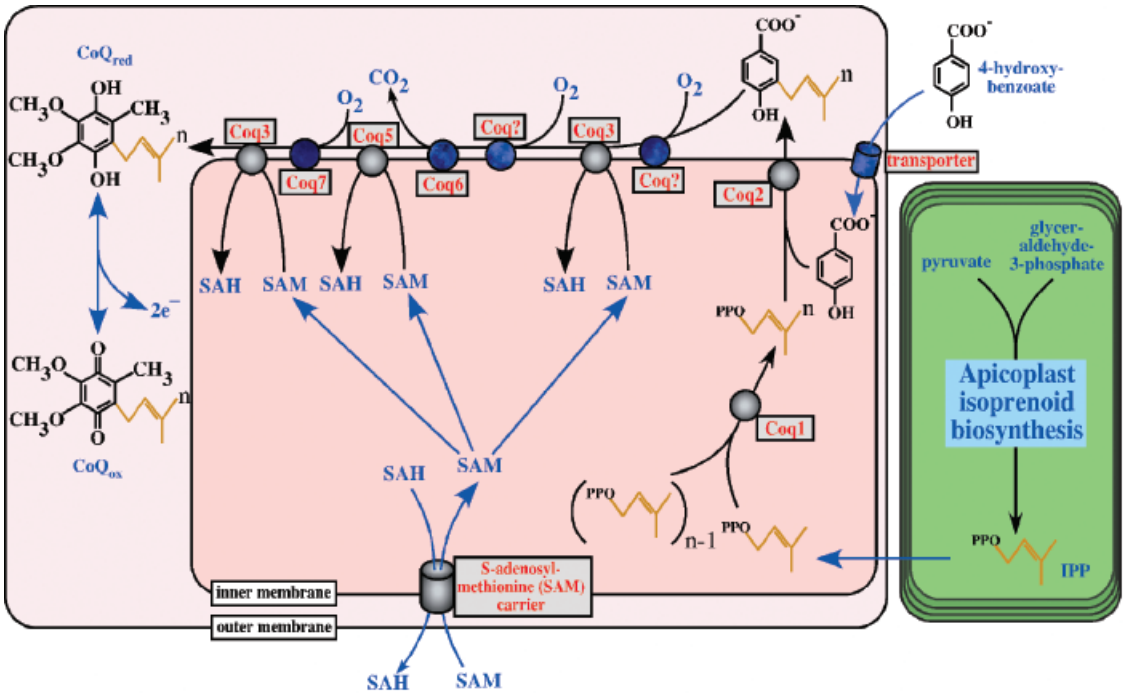


Fig. 7. Schematic for the coenzyme Q biosynthesis pathway in *Plasmodium falciparum*. The isoprenoid tail of coenzyme Q is synthesized as isopentenyl pyrophosphate (IPP) units via the DOXP pathway in the apicoplast. This is then transported to the mitochondrion, where Coq1 adds 8 or 9 isopentenyl pyrophosphate units to 4-hydroxybenzoate. This 4-hydroxybenzoate 'head' group is modified by a series of reactions ultimately to produce coenzyme Q. Enzymes catalyzing some of these reactions have homologues in *P. falciparum* (grey spheres), while others either contain no clear homologues in the genome, or the enzymes required for that particular step are unknown (blue spheres). The Coq2 homologue is known to target to the mitochondrion (see Fig. 1b). The methyl groups added by the Coq3 and Coq5 enzymes are derived from S-adenosylmethionine (SAM), which may be transported into the mitochondrion via a S-adenosylmethionine carrier protein.

have been implicated in coenzyme Q biosynthesis, although their functions are not known.

Plasmodium falciparum appears to contain many of the known proteins for coenzyme Q biosynthesis. As in yeast (Turunen *et al.*, 2004), coenzyme Q biosynthesis probably occurs in the mitochondrion, the organelle where the major function of coenzyme Q is performed. Indeed, blue-native polyacrylamide gel electrophoresis studies in yeast suggest that several coenzyme Q biosynthesis enzymes together form a large, multi-subunit, coenzyme Q-synthesizing protein complex (Gin & Clarke, 2005; Marbois *et al.*, 2005). Recent studies in *Plasmodium* have examined synthesis of the isoprenoid chain of coenzyme Q (de Macedo *et al.*, 2002; Tonhosolo *et al.*, 2005). Promisingly, inhibitors of isoprenoid biosynthesis affect cellular levels of the isoprenoid component of coenzyme Q (de Macedo *et al.*, 2002; Rodrigues Goulart *et al.*, 2004). This is further evidence that apicoplast-derived isoprenoids contribute to the synthesis of coenzyme Q, and suggests that antimalarial drugs targeting isoprenoid biosynthesis in the apicoplast are (at least in part) lethal because they prevent the synthesis of coenzyme Q and ultimately inhibit electron transport. Drugs targeting isoprenoid biosynthesis make promising antimalarial compounds, with the drug fosmidomycin used in recent clinical trials (Jomaa *et al.*, 1999; Missinou *et al.*, 2002). Studies examining possible synergism between isoprenoid-targeting and electron transport chain-targeting drugs like atovaquone will be informative in this regard, although one study found minimal synergy between fosmidomycin and atovaquone (Wiesner *et al.*, 2002).

Iron-sulphur cluster biosynthesis

Iron-sulphur cluster biosynthesis is a process that occurs in the mitochondrion or mitochondrial-like organelle of almost all eukaryotes. It seems to be a major (possibly the only) biosynthetic pathway of highly reduced mitochondria, such as those of the gut parasites *Cryptosporidium* and *Giardia* (LaGier *et al.*, 2003; Tovar *et al.*, 2003). The ubiquity of iron-sulphur cluster biosynthesis in eukaryotes points to its essential and indispensable function. Indeed, some have argued that iron-sulphur biochemistry evolved with the origin of life on earth (Huber & Wachtershauser, 1997). Iron-sulphur clusters serve as prosthetic groups involved in redox reactions on a wide range of proteins. *Plasmodium falciparum* proteins requiring iron-sulphur clusters include

ferredoxin, members of the mitochondrial electron transport chain such as the Rieske protein and a subunit of succinate dehydrogenase, aconitase/IRP, class I fumarate hydratase, the Rli1 protein involved in cytosolic ribosome formation, the IspG and IspH enzymes involved in isoprenoid biosynthesis in the apicoplast, and possibly also HemH (Ralph *et al.*, 2004; Johnson *et al.*, 2005; Lill & Muehlenhoff, 2005). Proteins requiring iron-sulphur clusters often localize to the mitochondrion, but may also be found in other cellular compartments, such as the cytosol and plastid.

Iron-sulphur cluster biosynthesis commences with the sulphur of free cysteine being donated to the thiol group of a cysteine residue on a cysteine desulphurase enzyme. This results in the formation of a persulfide (-S-SH) group (Johnson *et al.*, 2005) (Fig. 8). Nomenclature of iron-sulphur enzymes varies between organisms. The cysteine desulphurase catalyzing this reaction is called Nfs1 in yeast, and SufS, NifS or IscS in bacteria. To avoid confusion, this review follows the nomenclature set out for iron-sulphur cluster biosynthesis enzymes in Apicomplexa by Seeber (Seeber, 2002). The NFS cysteine desulphurase transfers the sulphur-containing group to a cysteine residue on so-called 'scaffold' proteins (Fig. 8). Iron is incorporated into the sulphur groups to form the iron-sulphur clusters upon these scaffolds. Iron is thought to be donated to the scaffold by the iron-binding protein frataxin, although the exact role of this protein remains unclear (Lill & Muehlenhoff, 2005). Two unrelated types of scaffold proteins are thought to occur, known as ISU and ISA (Johnson *et al.*, 2005). An alternative proposal suggests that ISA may function in the acquisition of free iron, and its delivery to ISU (Ding *et al.*, 2004). Most evidence suggests that sulphur binds to the ISU scaffold protein before iron, although the exact mechanisms of Fe-S construction remain unclear (Johnson *et al.*, 2005). Several types of iron-sulphur clusters can be formed, including ones with two iron and two sulphur molecules [2Fe-2S] and one with four of both molecules [4Fe-4S]. The arrangement of these clusters typically features a central iron molecule bound to a tetrahedral arrangement of sulphur molecules (Johnson *et al.*, 2005). Iron-sulphur clusters on scaffold proteins can be transferred to the proteins where they are to function, although mechanisms involved in this transfer are unknown.

NFS and the scaffold proteins ISA and ISU make up the core of the iron-sulphur biosynthesis machinery. Studies in bacteria and yeast have, however, identified several further factors involved in this process. Ferredoxin and its partner ferredoxin reductase, which function as a general, 'mobile' electron carrier system in mitochondria, are proposed to be involved in reducing sulphur on the scaffold protein (Lill & Muehlenhoff, 2005). Chaperones such as Hsp70 (DnaK) and its co-chaperones DnaJ and GrpE are known to be necessary for the maturation of iron-sulphur cluster-containing proteins (Johnson *et al.*, 2005; Lill & Muehlenhoff, 2005). It is possible that these mediate dissociation of iron-sulphur clusters from the scaffold protein and their transfer to the relevant apoproteins (Lill & Muehlenhoff, 2005), although direct evidence for this is lacking. As mentioned earlier, iron-sulphur clusters must be transferred into the cytosol for incorporation into cytosolic iron-sulphur proteins such as IRP and the ribosome biogenesis protein Rli1. This transport may be mediated by the inner membrane-localized ATP-binding cassette transporter Atm1 (Kispal *et al.*, 1999,2005). The intermembrane space sulfhydryl oxidase protein ERV is thought to be essential for biogenesis of cytosolic iron-sulphur cluster proteins, although its exact role is not clear (Lange *et al.*, 2001). Recent studies suggest ERV may be also involved in regulating the import of tiny Tims proteins into the intermembrane space (Mesecke *et al.*, 2005).

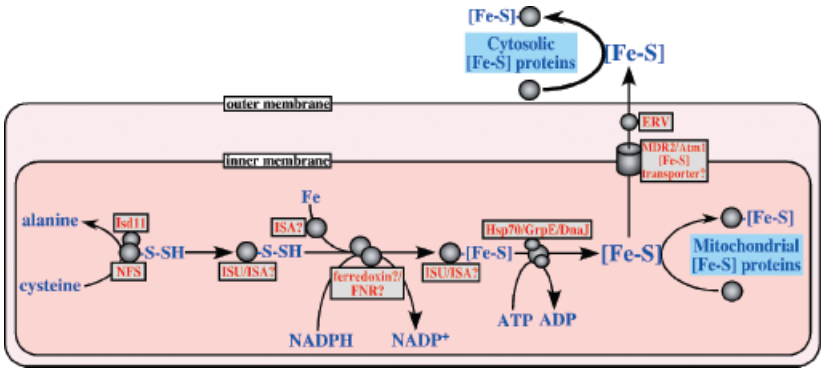


Fig. 8. A putative schematic for the synthesis of iron-sulphur clusters [Fe-S] in the mitochondrion of *Plasmodium falciparum*. A persulfide group is formed on NFS, a process that possibly requires Isd11. This persulfide group is then added to a scaffold protein such as ISU. Iron may then incorporate into the sulphur group, perhaps aided by ISA, with the sulphur groups reduced by ferredoxin/FNR. After assembly of the iron-sulphur cluster on the ISU scaffold, chaperones such as Hsp70 may aid in the transfer of the cluster to the protein where it will serve as a prosthetic group. Iron-sulphur clusters may also be transported from the mitochondrion to be used in iron-sulphur cluster-requiring proteins of the cytosol. This transport may be mediated by ATP-binding cassette-like transporters such as Mdr2. The biogenesis of cytosolic iron-sulphur-containing proteins may require functioning of the intermembrane space protein ERV.

Seeber (2002) performed a bioinformatic search for iron-sulphur cluster biosynthesis genes in a range of Apicomplexa, including *P. falciparum*. He found homologues of the core components of the mitochondrial iron-sulphur biosynthesis machinery, including NFS (MAL7P1.150), ISU (PF14_0518) and two homologues of ISA (PFB0320c and PFC1005c) (Fig. 8). The N-terminal leader sequence of NFS was shown to target green fluorescent protein to the mitochondrion (Sato *et al*., 2003), providing evidence that this process occurs in the *Plasmodium* mitochondrion. Curiously, *P. falciparum* appears to lack a homologue of frataxin, the protein that is thought to deliver iron to the ISU scaffold. It is not clear whether another protein functions in this role in *Plasmodium* (perhaps one of the ISA homologues), or whether the actual function of frataxin (which remains ill-defined) differs from the postulated role of bringing iron to the ISU scaffold. Many of the 'accessory' proteins to iron-sulphur cluster biosynthesis have apparent mitochondrial homologues in *P. falciparum*. These include ferredoxin (PFL0705c), ferredoxin $NADP^+$ reductase (PF11_0407), ERV (PFA0500w), Hsp70, DnaJ and GrpE (see earlier). *Plasmodium falciparum* also has a homologue of the recently identified, eukaryote-specific Isd11 protein (MAL13P1.53). The exact role of Isd11 is not known, but is thought to interact with NFS in the cysteine desulphurase reaction (Adam *et al*., 2006; Wiedemann *et al*., 2006). As alluded to in the section on mitochondrial metabolite transporters, the *P. falciparum* Mdr2 protein may be a homologue of the Atm1 transporter that may transport mitochondrial iron-sulphur clusters into the cytosol, although this requires experimental confirmation. It is very likely that *P. falciparum* requires cytosolic iron-sulphur clusters, based on the presence of several putative cytosolic iron-sulphur cluster proteins. It is possible that the iron-sulphur clusters for these proteins are derived from the mitochondrion. Curiously, however, *Plasmodium* also contains apicoplast-localized iron-sulphur cluster synthesis machinery (Ellis *et al*., 2001; Seeber, 2002). Apicoplast-synthesized iron-sulphur clusters are probably required in the isoprenoid biosynthesis enzymes mentioned earlier, as well as in other apicoplast proteins such as apicoplast ferredoxin and MiaB, an enzyme involved in tRNA modification (Ralph *et al*., 2004). It is unknown whether cytosolic iron-sulphur cluster proteins obtain their iron-sulphur cluster from the mitochondrion or apicoplast. Before the symbiogenic acquisition of the apicoplast, a mechanism to traffic iron-sulphur clusters from the mitochondrion to the cytosol probably already existed. Applying Occam's razor, we would expect that cytosolic iron-sulphur clusters would still be supplied from the mitochondrion, but this speculation obviously requires experimental confirmation.

There are many unknown aspects to iron-sulphur cluster biosynthesis, both in *Plasmodium* and in organisms generally. Perhaps the most pertinent questions regarding this process in *Plasmodium* relate to the role of iron-sulphur clusters in cellular processes. Are the iron-sulphur clusters produced in the *Plasmodium* mitochondrion essential to parasite viability? And if so, what processes are they essential for? Also unknown is the source of iron. *Plasmodium* parasites consume iron-rich haemoglobin protein, but it is not known if this is the source of iron for processes such as haem and iron-sulphur cluster biosynthesis. Iron is required in the mitochondrion for both the HemH catalyzed step of haem biosynthesis and iron-sulphur synthesis. It is not known how iron is imported into mitochondria.

Conclusions and perspectives: completing the jigsaw

We began this review with the aim of gaining insights into the functions of the *Plasmodium* mitochondrion based on a bioinformatic analysis of the genome. We have pieced together several putative biochemical pathways that provide clues to what this enigmatic organelle is really doing. Our bioinformatic analysis supports previous biochemical evidence for the existence of an electron transport chain across the inner membrane. Our analysis into the tricarboxylic acid cycle suggests the presence of most, if not all, steps of this pathway, which is to some degree at odds with the available biochemical data. Our analysis also suggests the existence of pathways for which little or no biochemical data has been gathered, namely haem, folate, coenzyme Q and iron-sulphur cluster biosynthesis.

So, based on the available biochemical data as well as our bioinformatic analysis, what can we conclude about the functions of the *Plasmodium* mitochondrion? One surprising and somewhat paradoxical conclusion is that, during the asexual stages at least, the mitochondrion largely appears to function in self-maintenance. The haem, coenzyme Q and iron-sulphur biosynthesis pathways function in supporting the electron transport chain. The tricarboxylic acid cycle, too, appears to supply some reducing equivalents for electron transport. The electron transport chain, in turn, provides a membrane potential across the inner membrane. This is probably essential for protein import, but these imported proteins are those used in the tricarboxylic acid cycle and haem, coenzyme Q and iron-sulphur biosynthesis. Although this may appear to be a self-defeating cycle of metabolism, we know that disrupting the electron transport chain with drugs like atovaquone kills the parasites. This is strong evidence that the electron transport chain is essential to parasite viability and broader cellular metabolism, and therefore that the *Plasmodium* mitochondrion is not merely a self-maintaining organelle. But what is it that is so important about the malaria mitochondrion?

The only clear link between mitochondrial metabolism and broader cell functions is in pyrimidine biosynthesis, with dihydroorotate dehydrogenase performing an essential reaction in this pathway, while feeding electrons into the electron transport chain. Our bioinformatic analysis further suggests that the mitochondrion may function in other cellular processes. These include ATP generation utilizing the proton gradient across the inner membrane. Although ATP generation appears to be minimal during asexual stages, it is possible that other stages (e.g. those in the insect) rely more on mitochondrially generated ATP. Generating large amounts of ATP in the mitochondrion requires an active tricarboxylic acid cycle. *Plasmodium*, however, lacks mitochondrial pyruvate dehydrogenase, the enzyme traditionally responsible for supplying the tricarboxylic acid cycle with organic molecules. The big unanswered question here, then, is where do these organic compounds come from? It is possible that the *Plasmodium* tricarboxylic acid cycle has a novel source of acetyl-CoA, or that the tricarboxylic acid cycle is incomplete, with a partial cycle, fed by amino acid-derived substrates, fuelling ATP generation. Our analysis of iron-sulphur cluster biosynthesis suggests that cytosolic iron-sulphur proteins may obtain their prosthetic groups from the mitochondrion. This is in line with recent evidence indicating that eukaryotic organisms with highly reduced mitochondria appear to retain this organelle for the sole purpose of making iron-sulphur clusters. It is possible that *Plasmodium*, like all eukaryotes, require their mitochondrion to make iron-sulphur clusters for cytosolic processes. This raises a host of interesting evolutionary questions, including why can't organisms relocate their iron-sulphur cluster synthesis machinery if it would allow them to get rid of an energetically costly organelle such as the mitochondrion?

The bioinformatic approach to analyzing mitochondrial functions is useful in formulating hypotheses about what the roles of this organelle are. It also serves to highlight the enormous gaps in our biochemical and cell biological knowledge of the mitochondrion. To return to the metaphor that opened this review: courtesy of the genome project, we have all the jigsaw pieces necessary to form a complete picture of the *Plasmodium* mitochondrion, but we are only just beginning to fit them together. The focus now must be on developing ways of determining which piece fits with which other pieces, and equally as importantly, to determine which pieces belong in another part of the picture altogether. Reverse genetics provides an untapped resource for studying mitochondrial metabolism. Disrupting genes that our bioinformatic models suggest play crucial functions in the mitochondrion is essential in understanding both the role of that particular gene, and of the mitochondrion in cellular processes. The tools to perform such experiments are close at hand, with the development of homologous recombination techniques, as well as inducible knockout systems in *Plasmodium*. Hand-in-glove with reverse genetics must come new biochemical techniques to assay mitochondrial processes. Already, biochemistry has provided important insights into the functions of the electron transport chain. Now, we must begin to look at other processes in this organelle. This will require overcoming many of the difficulties associated with assaying the biochemical functions of a small, intracellular parasite like *Plasmodium*, such as gaining sufficient yields and purities of parasite mitochondrial material, and allowing for the innate dependence that *Plasmodium* has on the metabolism of its host cell. Another important factor in understanding how mitochondrial metabolism links to the rest of the cell is determining what compounds can be imported into, and exported from, the mitochondrion. This requires a much better knowledge of transport proteins, particularly those of the inner membrane. Finally, the great unknown in *Plasmodium* mitochondrial metabolism regards the stage-specificity of the various biological processes that occur there. Recent studies examining the transcriptome and proteome of *Plasmodium* parasites outside the asexual cycle suggest that many mitochondrial proteins are upregulated in these stages (Le Roch *et al.*, 2003; Hall *et al.*, 2005; Khan *et al.*, 2005). This probably reflects the increased importance of the mitochondrion as it is transferred to the (lower nutrient?) environment of the mosquito. Finding ways to examine mitochondrial functions at these various stages is crucial to completing what is essentially a four-dimensional jigsaw puzzle.

The mitochondrion of *Plasmodium* is a validated drug target. Understanding the biochemical processes of this organelle allows us not only to understand how these existing drugs affect the cell, but also to develop new drugs to target malaria. The bioinformatic analysis presented here is a small step towards achieving these goals. Although our analysis can help make sense of existing data, and point to functions of the mitochondrion, it is important to note that our postulations must be interpreted with caution. Without conclusive localization data, our predicted mitochondrial proteins are merely that. Also, without biochemical data, the metabolic pathways presented here are just theoretical. Indeed, homology in protein sequence does not always correlate to homology in protein function. The challenge for future research is to fill in the gaps, rearrange the pieces where necessary, and complete this fascinating mitochondrial jigsaw puzzle.

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Supplementary material

The following supplementary material is available for this article online.

Table S1. List of the proteins with putative mitochondrial functions identified during our bioinformatic searches.