MITOCHONDRIAL OXYGEN CONSUMPTION IN ASEXUAL AND SEXUAL BLOOD STAGES OF THE HUMAN MALARIAL PARASITE, *PLASMODIUM FALCIPARUM*

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Abstract. The two developmental stages of human malarial parasite *Plasmodium falciparum*, asexual and sexual blood stages, were continuously cultivated *in vitro*. Both asexual and sexual stages of the parasites were assayed for mitochondrial oxygen consumption by using a polarographic assay. The rate of oxygen consumption by both stages was found to be relatively low, and was not much different. Furthermore, the mitochondrial oxygen consumption by both stages was inhibited to various degrees by mammalian mitochondrial inhibitors that targeted each component of complexes I- IV of the respiratory system. The oxygen consumption by both stages was also affected by 5-fluoroorotate, a known inhibitor of enzyme dihydroorotate dehydrogenase of the pyrimidine pathway and by an antimalarial drug atovaquone® that acted specifically on mitochondrial complex III of the parasite. Moreover, antimalarials primaquine and artemisinin had inhibitory effects on the oxygen consumption by both stages of the parasites. Our results suggest that *P. falciparum* in both developmental stages have functional mitochondria that operate a classical electron transport system, containing complexes I–IV, and linked to the pyrimidine biosynthetic pathway.

INTRODUCTION

The malarial parasite's life cycle in human host erythrocytes has both asexual and sexual stages for growth, development and transmission into mosquito vector. Most of the literature on malaria biochemistry contains references only to the asexual stage-parasite. The asexual parasite is known as a microaerophillic organism (Scheibel *et al*, 1979) and is totally dependent on anaerobic glycolytic ATP production. (Sherman, 1979; Scheibel, 1988). A large part of glucose utilization in the malarial parasite results in the formation of organic end products, similar to many other parasitic protozoa and helminths studied in detail. They are aerobic fermenters, capable of decomposing their substrates only partially to fermentation products and unable to oxidize them completely to CO_2 and H_2O. Since the substrates are not completely metabolized, it appears that respiration is a rate limiting step in the asexual parasite.

The mitochondrial function in the asexual parasite appears to be primarily anabolic rather than serving as a significant source of ATP generation (Fry *et al*, 1990). The mitochondrion of the asexual parasite, so far studied, plays an important role in electron disposal from the enzyme dihydroorotate dehydrogenase via the *de novo* pyrimidine biosynthetic pathway (Gutteridge *et al*, 1979; Gero *et al*, 1984; Prapunwattana *et al*, 1988; Krungkrai *et al*, 1991; Krungkrai, 1995). It has been demonstrated that mitochondria of asexual *Plasmodium falciparum* contain no or few cristate structure (Aikawa *et al*, 1966; Aikawa, 1971; Langreth *et al*, 1978; Fry and Beesley, 1991; Krungkrai, 1995), lack many Krebs cycle enzymes (Scheibel, 1988), and appear to lack respiratory complex I of the mitochondrial electron transport system (ETS) (Fry and Beesley, 1991). The aseuxal stage of *P. falciparum* does have an ETS that contains respiratory complexes II-IV (Fry and Beesley, 1991; Krungkrai *et al*, 1997; Murphy *et al*, 1997).

As mentioned earlier, relatively little is known on mitochondrial function in the sexual stage of parasite development in the human erythrocytes. Recently we have shown that the amount of the 6kb mitochondrial DNA is amplified during the asexual stage switch to the sexual stage (Petmitr and Krungkrai, 1995). In addition, the presence of numerous cristae with tubular-like structure, and heterogeneity in mitochondrial morphology have been demonstrated (Aikawa, 1971; Langreth *et al*, 1978). These results indicate that the metabolically active mitochondria are present in the sexual-stage parasite during gametocytogenesis in human erythrocytes.

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In this study, we report the respiratory activity of the sexual-stage parasites taken from *in vitro* continuous cultures by measuring the rate of mitochondrial oxygen consumption using a polarographic assay. Mitochondrial inhibitors, which target each component of the parasite ETS, are used for elucidation of classical ETS containing all four respiratory complexes. The rate of mitochondrial oxygen consumption is determined comparatively in the asexual blood stage-parasite.

MATERIALS AND METHODS

Malarial parasites

P. falciparum (T_9 and KT_3 isolates) were cultivated from frozen samples in the sorbitol-glycerol cryoprotectant (Trigg, 1987) by the candle jar method of Trager and Jensen (1976), using a 5% hematocrit of human erythrocytes group 'O' in the RPMI 1640 medium supplemented with 25 mM N-2-hydroxylethylpiperazine-N´-2-ethanesulfonic acid (Hepes), 32 mM $NaHCO_3$ and 10% human serum group 'O'. The cultures started at low parasitemia (~1-2%) were changed with the medium twice daily until the cultures had ~30% parasitemia and then harvested. Synchrony of the culture to get each stage (ring forms, trophozoites, schizonts) of asexual parasite was performed by the sorbitol procedure of Lambros and Vanderberg(1979). For the sexual blood stages or the gametocytic stages, the KT_3 parasite was used as gametocyte-producing strain and then induced according to Ifediba and Vanderberg (1981). Approximately 3-4% parasitemia of mixed stages' gametocytes was routinely obtained on 10-15-day cultivation after adding the normal fresh erythrocytes. The sexual gametocytic stages were purified by the Percoll step-wise gradient centrifugation according to Knight and Sinden (1982). The parasites were washed at least 3 times in the RPMI 1640 medium before experiments.

Microscopic examination of *P. falciparum* morphology

Transmission electron microscopy (TEM) of the infected-erythrocytes by the asexual and sexual gametocytic stages was performed according to the method of Sinden (1982). The processed samples were examined with a JEOL-100SX transmission electron microscope at the Center of Scientific and Technological Research Equipment of Chulalongkorn University. Light microscopy (LM) of the infected erythrocytes was examined on methanol fixed and Giemsa's stained parasites by a Nikhon labophot-2 microscope.

Measurement of oxygen consumption by *P. falciparum*

The rate of oxygen consumption for a consistent number of *P. falciparum* obtained from both stages of the parasite development were measured in the RPMI 1640 medium (pH 7.4) supplemented with 25 mM Hepes and 32 mM $NaHCO_3$ by using a Clark-type oxygen electrode and YSI oxygen monitor according to the calibration method of Robinson and Cooper (1970). The oxygen consumption, performed in a reacting chamber with a total volume of 3 ml, was kinetically followed and recorded for 3-5 minutes at 37°C with a temperature-controlled circulator. Mitochondrial inhibitors at desired concentrations were tested against the oxygen consumption of the two stages of the parasites by injecting into the reacting chamber. The rate of oxygen consumption in the presence of inhibitors by these parasites was followed for the next 3-5 minutes. The 50% effective concentration (EC_{50}) was defined as the concentration of the inhibitor causing 50% inhibition of the parasites'oxygen consumption, compared to the control parasites'oxygen consumption in the absence of inhibitor.

RESULTS AND DISCUSSION

It has been shown that *P. falciparum* in asexual blood stage is the microaerophillic organism and favors in very low oxygen tension, *eg*, 0.5-3% oxygen, for maximal growth and development in *in vitro* culture (Scheibel *et al*, 1979). The ultrastructural features of mitochondrial organelle in both asexual and sexual blood stages are typically similar to that of other apicomplexan parasites (Aikawa, 1971; Langreth *et al*, 1978; Siddall and Desser, 1992). In this study, the mitochondrial oxygen consumption was measured in both asexual and sexual blood stages' parasites in the presence of various mitochondrial inhibitors and some antimalarial drugs. The parasites used were cultivated *in vitro* and then induced for sexual blood stage or gametocyte production, the T_9 parasite was found to be no longer as a gametocyte-producing line. The KT_3 parasite was still induced for gametocytogenesis to yield ~3-4% parasitemia of sexual stage in the culture (with ~30% parasitemia of mixed asexual and sexual stages). The sexual stage-parasite was purified by the Percoll step-wise gradient as mentioned earlier, and the Giemsa's stained parasite was then examined by LM with low magnification. Fig 1A shows the mixed

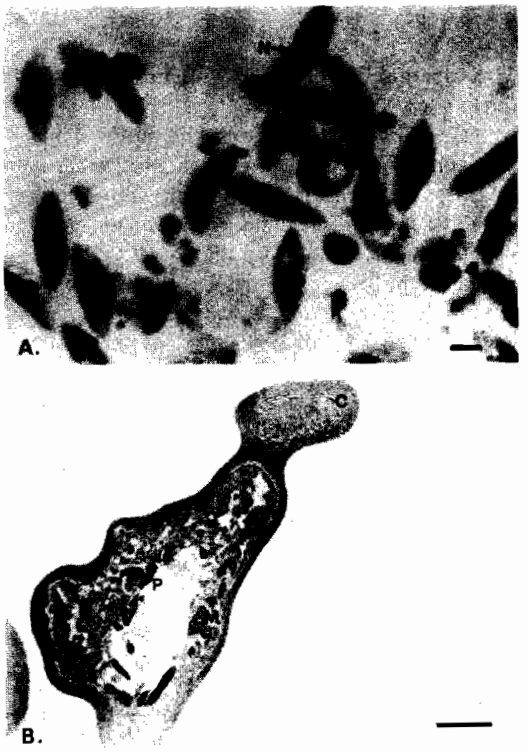

Fig 1–A. Light micrograph of purified sexual gametocytic stage-parasites from *in vitro* cultures of *P. falciparum*. A bar represents 10 μm. N, nucleus.
B. Transmission electron micrograph of *P. falciparum*, taken from a purified parasite of Fig 1A. The parasite contains many mitochondrial organelles (M). The infected erythrocyte is markedly deformed, notable large membranous cleft (C). A bar represents 1 μm. N, nucleus; P, crystalline pigment.

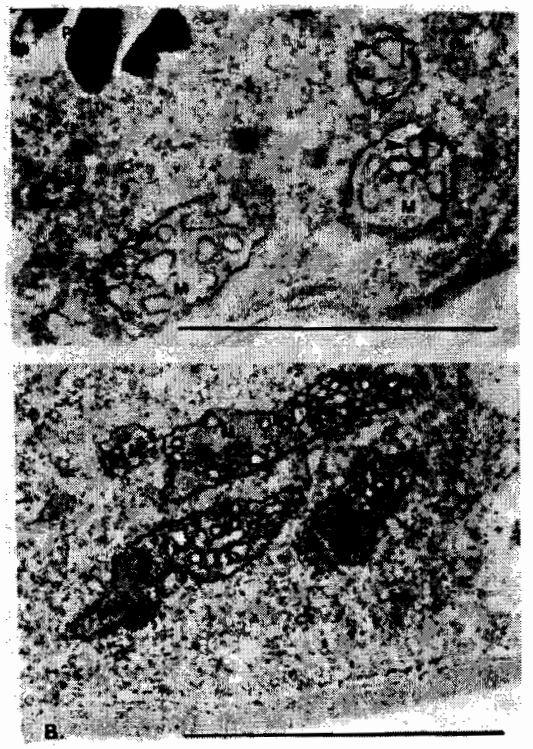

Fig 2–Transmission electron micrograph of higher magnification of mitochondria in the sexual gametocytic stage of *P. falciparum*. Many tubular-like cristae (A) and more numerous with electron dense cristae(B) are noted. A bar represents 1 μm. M, mitochondria. Arrowheads indicate the cristate structure resulting from extensively infoldings of mitochondrial inner membrane.

population of sexual gametocytic stages III-V according to the cytological classification of Hawking *et al* (1971). Higher magnification of the sexual stage-infected human erythrocytes was also performed by TEM for the existence of mitochondrial organelles' integrity (Fig 1B). The host erythrocyte was markedly modified, *eg* large membranous cleft was observed. It is noted that the mitochondria in the sexual stage- parasite are numerous and contain large numbers of tubular cristae with clear intracristal space, notable electron-dense structure, in each organelle (Fig 2 A, B).

To see whether the mitochondria in the sexual gametocytic stages of *P. falciparum* were biochemically active or not, the coupling of mitochondrial electron transport pathway and oxygen consumption through respiratory complex IV (cytochrome c oxidase), well characterized in both *P. berghei* and *P. falciparum* (Krungkrai *et al*, 1993; 1997), was probed for biochemical function of mitochondria in the malarial parasite. Mitochondrial oxygen consumption of these parasites was measured and compared to that of the parasites harboring the defined asexual developmental stages, for instance, ring forms, trophozoites and schizonts. It was found that mitochondrial oxygen consumption of *P. falciparum* from both asexual and sexual blood stages were not much different (Table 1). They were relatively low activities (~ 20-24 nmol/min/10^7 parasites), comparing to the human leukocytes which had an oxygen consumption of ~110 nmol/min/10^7 cells. The human erythrocytes had no detectable mitochondrial oxygen consumption at the density of 10^7 cells in the reaction assay. In marked contrast, *P. falciparum* in the asexual blood stage contain 1-2 mitochondria with no or little tubular cristae structure in each organelle (Aikawa, 1971; Langreth *et al*, 1978; Sinden, 1982). Recently, Murphy *et al* (1997) have mea-

sured the rate of mitochondrial oxygen consumption in the asexual stage of *P. falciparum* and found that it is relatively low activity which is somewhat similar to the results reported here. Based on these findings, it is suggested that the abundance of mitochondria in the sexual gametocytic stage-parasites are still underdeveloped forms with biochemically active, at least in the oxygen consumption.

Moreover, by using the synchronized cultures of the asexual stages' parasites in the same intra-erythrocytic cycle (~42 hours), it was observed that the rate of mitochondrial oxygen consumption by the ring forms stage (~10-15 hour development) was less than that of the trophozoite stage (~25-30 hour development) and the schizont stage (~36-40 hour development with 12-16 merozoites per cell) (Table 1). This observation reported is consistent with two lines of evidence as follows: 1) the structures of mitochondrial organelle in the three developmental stages of the asexual blood parasite is stage-dependent, *eg*, less developed structure in the ring forms than in the maturing trophozoite stage (Aikawa, 1971; Langreth *et al*, 1978); 2) the transcripts of 6 kb mitochondrial element protein-coding genes are least abundant in the ring forms stage and most abundant in the trophozoite and schizont stages (Feagin and Drew, 1995).

Table 1
The rate of mitochondrial oxygen consumption by *P. falciparum*, human erythrocytes and leukocytes.

Stage	Rate of oxygen consumption (nmol/min/10^7 cells)
Asexual stages[a]	20.4±4.4
Ring forms	13.8±2.1
Trophozoites	21.4±1.6
Schizonts	26.0±4.2
Sexual stages[b]	24.2±1.9
Human erythrocytes (n=3)	N.D.[c]
Human leukocytes (n=2)	109.4

[a]Values are mean±SD of 3-5 preparations of all three asexual stages.
[b]Values are mean±SD of 3 preparations of sexual stages.
[c]Not detectable

Interestingly, the mitochondrial oxygen consumption of the parasites from both asexual (mostly trophozoites) and sexual (mostly gametocytes stages III-V, Fig 1A) blood stages were performed with well-known specific inhibitors of all four respiratory complexes as described in detail by Hatefi (1985). The lists of inhibitors and the target site are shown in Table 2. Using cell density at 10^7 parasite in each oxygen consumption reaction, all four mitochondrial inhibitors affecting the respiratory complexes were found to inhibit the oxygen consumption at different degree in both asexual and sexual blood stages (Table 2). It was observed that the sexual blood parasite was more sensitive to rotenone and cyanide, affecting the respiratory complexes I and IV respectively, than the asexual blood parasite. Whereas the parasites from both stages were similarly response to thenoyltrifluoroacetone and antimycin A which affect the respiratory complexes II and III respectively. Furthermore, the mitochondrial oxygen consumption in the three developing forms of the asexual blood stages were found to sensitive to cyanide inhibition at various degree (Fig 3). The ring forms (EC_{50}~ 0.27 mM) and schizont stages (EC_{50}~0.23 mM) showed more sensitive to cyanide inhibition against the oxygen consumption than the trophozoite stage (EC_{50}~ 0.90 mM). The cyanide sensitivity of the sexual stage parasite's oxygen consumption was comparable to that of the asexual stage parasite harboring ring forms and schizont

Table 2
The EC_{50} values of various inhibitors on mitochondrial oxygen consumption.

Inhibitor	Target	EC_{50} (M)	
		Asexual stages	Sexual stages
Rotenone	Complex I	$4x10^{-4}$	$1x10^{-4}$
Thenoyltrifluoroacetone	Complex II	$2x10^{-4}$	$2x10^{-4}$
Antimycin A	Complex III	$3x10^{-4}$	$5x10^{-4}$
Potassium cyanide	Complex IV	$9x10^{-4}$	$2x10^{-4}$
5-Fluoroorotate	DHODase	$1x10^{-7}$	$5x10^{-7}$
Atovaquone®	Complex III	$5x10^{-8}$	$9x10^{-7}$
Primaquine	Complex III	$1x10^{-5}$	$2x10^{-7}$
Artemisinin	Complex IV	$5x10^{-6}$	$1x10^{-5}$

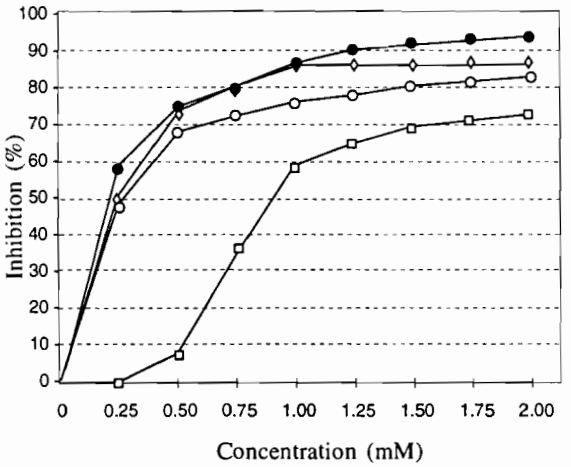

Fig 3–Dose-response of inhibitory effect of potassium cyanide on mitochondrial oxygen consumption of *P. falciparum* in both asexual (ring forms, trophozoites, schizonts) and sexual blood stages. The open symbols are for the three developing stages of asexual blood parasites: o, ring forms; □, trophozoites; ◊, schizonts. The closed circles represent sexual blood parasites.

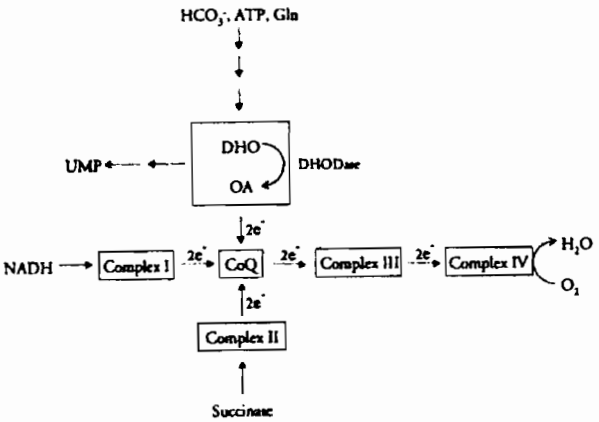

Fig 4–The proposed linkage between the mitochondrial electron transport system (complexes I, II, III, IV)and the *de novo* synthesis of pyrimidine pathway through the enzyme dihydroorotate dehydrogenase (DHO Dase) in the asexual and sexual blood stages of *P. falciparum*.

stages. The differences of the cyanide sensitivity by these stages of parasite remain to be elucidated. In *P. knowlesi*, a monkey malarial parasite, the trophozoite stage has more activity of the complex IV cytochrome oxidase than the ring forms and schizont stages (Scheibel and Miller, 1969). The complex IV activity of *P. falciparum* in the trophozoite stage was found to be ~4 times of the schizont stage parasite, and the enzyme from the trophozoite stage was more refractory to cyanide inhibition (~ 7 times) than that from the schizont stage (Krungkrai and Burat, unpublished results). Cyanide has been shown to exhibit low antimalarial activity against *P. falciparum* growth *in vitro* with IC_{50} values at micromolar concentrations (Ginsburg *et al*, 1986), however, it has strong inhibitory effect against the purified cytochrome c oxidase of both *P. berghei* (Krungkrai *et al*, 1993) and *P. falciparum* (Krungkrai, 1995; Krungkrai *et al*, 1997). It is then concluded that *P. falciparum* in these developmental stages have functional mitochondria consisting of major biochemical activities of classical respiratory ETS complexes I, II, III and IV, and providing their energy metabolism.

Mitochondrial oxygen consumption of the parasites from both stages were performed in the presence of 5-fluoroorotate, a potent inhibitor of enzyme dihydroorotate dehydrogenase (DHODase) of pyrimidine biosynthetic pathway of *P. falciparum* (Krungkrai *et al*, 1992; Krungkrai, 1995), and in the presence of three antimalarial drugs; atovaquone® (Fry and Pudney, 1992; Krungkrai *et al*, 1997) and primaquine (Vaidya *et al*, 1993) that possibly act on complex III, and artemisinin that might target specifically to complex IV (Zhao *et al*, 1986). Table 2 shows the 50% effective concentrations (EC_{50}) of 5-fluoroorotate, atovaquone®, primaquine and artemisinin on the parasites' oxygen consumption in the both stages of development. 5-Fluoroorotate showed strong inhibitory effect on the oxygen consumption by both asexual and sexual stage-parasites (Table 2), suggesting that the parasite in the sexual stage has the linkage of the two metabolic pathways: pyrimidine biosynthesis and mitochondrial ETS through DHODase. The association between the pyrimidine pathway and mitochondrial ETS in the asexual stage-parasite has been recently confirmed (Krungkrai *et al*,1991; Krungkrai, 1995). It is concluded that *P. falciparum* in these developmental stages have functional mitochondria that contribute significantly to *de novo* synthesis of pyrimidine and to the energy metabolism of the parasite. The role of the mitochondria in the sexual blood stages, *eg*, for gametocytogenesis in human host, for exflagellation in mosquito vector, remains to be determined.

The findings on the effects of the antimalarial drugs on the oxygen consumption are well-related to the lines of evidence that these drugs act specifically on the mitochondria of the malarial parasite. Atovaquone® has been shown to inhibit electron transport and depolarize mitochondria of asexual stage *P. yoelii*, a rodent malarial parasite (Srivastava *et*

al, 1997). It has moderate gametocytocidal activity, comparing to its potent blood schizontocidal activity (Krungkrai *et al*, unpublished results), confirming that the atovaquone is indeed gametocytocidal drug (Fleck *et al*, 1996). It has been shown that primaquine (Lanners, 1991) and artemether (Kawai *et al*, 1993), an artemisinin derivative, act specifically on the mitochondria of *P. falciparum* by causing their morphological changes after short exposure. Our results would provide that the mitochondria in the asexual and sexual blood stages may be a putative chemotherapeutic target for new antimalarial development.

Based on these lines of evidence, it is suggested that: firstly, *P. falciparum* in both asexual and sexual blood stages have major biochemically activities of the classical electron transport system consisting of complexes I, II, III, IV and operates in the mitochondria; secondly, the ETS pathway is possibly linked to the pyrimidine biosynthetic pathway through DHODase (Fig 4); and thirdly, *P. falciparum* in both stages has functional mitochondria that contributes significantly to *de novo* synthesis of pyrimidine and to the energy metabolism of the parasites.

ACKNOWLEDGEMENTS

A part of this work was the MSc thesis of D Burat. We thank A Bhumiratana and P Mahannop of Mahidol University for serving as supervisory committee members. We also thank S Vettchagarun and R Kanchanarithisak for their dedicated technical assistance with EM techniques and some experiments on oxygen uptake. The parasites T_9 and KT_3 isolates were kindly provided by S Thaithong of Chulalongkorn University and P Petmitr of Mahidol University, respectively. This work was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. J Krungkrai is a recipient of a career development award from the National Science and Technology Development Agency of Thailand.

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