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Mitochondrial Dehydrogenases in the Aerobic Respiratory Chain of the Rodent Malaria Parasite *Plasmodium yoelii yoelii*

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In the intraerythrocytic stages of malaria parasites, mitochondria lack obvious cristae and are assumed to derive energy through glycolysis. For understanding of parasite energy metabolism in mammalian hosts, we isolated rodent malaria mitochondria from *Plasmodium yoelii yoelii* grown in mice. As potential targets for antiplasmodial agents, we characterized two respiratory dehydrogenases, succinate:ubiquinone reductase (complex II) and alternative NADH dehydrogenase (NDH-II), which is absent in mammalian mitochondria. We found that *P. y. yoelii* complex II was a four-subunit enzyme and that kinetic properties were similar to those of mammalian enzymes, indicating that the *Plasmodium* complex II is favourable in catalysing the forward reaction of tricarboxylic acid cycle. Notably, *Plasmodium* complex II showed IC_{50} value for atpenin A5 three-order of magnitudes higher than those of mammalian enzymes. Divergence of protist membrane anchor subunits from eukaryotic orthologs likely affects the inhibitor resistance. Kinetic properties and sensitivity to 2-heptyl-4-hydroxyquinoline-*N*-oxide and aurachin C of NADH: ubiquinone reductase activity of *Plasmodium* NDH-II were similar to those of plant and fungus enzymes but it can oxidize NADPH and deamino-NADH. Our findings are consistent with the notion that rodent malaria mitochondria are fully capable of oxidative phosphorylation and that these mitochondrial enzymes are potential targets for new antiplasmodials.

Key words: complex II, inhibitor, mitochondria, NDH-II, rodent malaria.

Abbreviations: AC, aurachin C; DCIP, 2,4-dichlorophenolindophenol; DHO, dihydroorotate; DHOD, DHO dehydrogenase; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; hrCNE, high-resolution clear-native electrophoresis; IC_{50}, the 50% inhibitory concentration; NBT, nitro blue tetrazolium chloride; NDE, NDH-II bound to the outer surface of the mitochondrial inner membrane; NDI, NDH-II bound to the matrix side of the mitochondrial inner membrane; NQR, NADH:quinone reductase; Q_n, ubiquinone-n; SDH, succinate dehydrogenase; SQR, succinate:quinone reductase; TCA, tricarboxylic acid.

INTRODUCTION

Malaria remains one of the main global health problems, causing more than 1 million deaths per year, with about 90% of deaths and 60% of cases occurring in Africa, south of the Sahara (*1*). Mortality associated with malaria is mainly caused by the erythrocytic stage cells of human malaria *Plasmodium falciparum*. The emerging resistance against established drugs in *Plasmodium* populations (*2*) emphasizes the urgent need for the development of new antiplasmodial drugs.

Energy metabolism of *Plasmodium* is quite different from that of mammalian hosts. Intraerythrocytic stages of parasites have been considered for a long time to rely on incomplete oxidation of glucose with secretion of end products such as lactate and pyruvate (*3*) and to possess mitochondria that lack oxidative phosphorylation and a functional tricarboxylic acid (TCA) cycle (*4*, *5*). *Plasmodium* spp. lacks genes coded for the proton-translocating NADH dehydrogenase (NDH-I, complex I) present in mammalian mitochondria (*6*, *7*) and uses a rotenone-insensitive single-subunit NADH dehydrogenase (NDH-II) (*8*), which is assumed not to oxidize deamino-NADH (*9*). Succinate:ubiquinone oxidoreductase (complex II, succinate dehydrogenase (SDH)) is a membrane-bound TCA cycle enzyme and consists of four subunits: a flavoprotein subunit (Fp, SDH1) and an iron–sulphur subunit (Ip, SDH2) form a soluble heterodimer, which binds to a membrane anchor *b*-type cytochrome [CybL (SDH3)/CybS (SDH4) heterodimer]. The *Plasmodium SDH1* and *SDH2* genes have been cloned by homology probing (*10*) while SDH3 and SDH4 appear highly divergent from orthologs and are still not annotated in the current database (*6*, *7*). Membrane bound subuniuts *a* and *b* of ATP synthase also remain unidentified (*6*, *7*), and thus complete mitochondrial ATP synthase was assumed to be absent in *Plasmodium* spp. (*4*, *5*, *11–13*). Recently, Painter *et al*. (*13*) claimed that

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the mitochondrial respiratory chain is required only for the regeneration of an oxidized form of ubiquinone, which serves as the electron acceptor for type 2 dihydroorotate dehydrogenase (DHOD), an essential enzyme for pyrimidine biosynthesis. It is widely accepted that the majority of the parasite's ATP demand is met through glycolysis (*11*).

On the contrary, atovaquone, an inhibitor for ubiquinol:cytochrome *c* reductase (complex III) (*14*), showed the antiplasmodial activity for *P. falciparum* with the 50% inhibitory concentration (IC_{50}) of 1 nM (*15*) and collapsed the mitochondrial membrane potential in *P. yoelii yoelii* (*16*). Uyemura *et al*. (*8*, *17*) demonstrated oxidative phosphorylation and succinate respiration in trophozoites of rodent malaria parasites. These observations suggest that *Plasmodium* mitochondria possess all subunits for canonical complex II and ATP synthase and are fully capable of oxidative phosphorylation.

It was shown recently that metabolism in *P. falciparum* parasites grown in human patients is affected by varied oxygen and substrate levels and by host-parasite interactions (*18*). The authors found the induction of gene sets associated with oxidative phosphorylation including respiratory enzymes. For understanding energy metabolism in malaria parasites, the isolation of active mitochondria from parasites, which have been adapted to host environments, is essential. In this study, *P. y. yoelii* mitochondria were isolated from parasites grown in mouse erythrocytes and enzymatic properties of complex II and NDH-II were characterized. Two-dimensional PAGE analysis supports the presence of membrane anchors in *Plasmodium* complex II. These findings indicate that *Plasmodium* mitochondria are fully capable of succinate-dependent oxidative phosphorylation as suggested by previous observations (*8*, *17*). Because the difference in the inhibitor sensitivity of complex II between *Plsamodium* and mammalian enzymes and the absence of NDH-II in mammalian mitochondria, these two enzymes are promising targets for new antimaralials.

MATERIALS AND METHODS

Parasite Culture—Animal care and experimental procedures were performed according to the Guidelines for Animal Experimentation, the University of Tokyo. *P. y. yoelii* strain 17XL was a kind gift of H. Otsuki (Ehime University). This strain can rapidly propagate without cerebral malaria and does not infect reticulocytes. About 3.0×10^7 parasites were injected intraperitoneally to 8-week-old female BALB/c mice, and the developmental stage and parasitemia were monitored by examination of Giemsa-stained thin blood smears. About 7.5 ml of the blood was collected from 10 mice by cardiac puncture 130–140 h after infection. To remove leukocytes and platelets, the blood was mixed with 0.5 ml of heparine and passed over a powdered cellulose column (CF11; Whatman, Clifton; 0.5 ml/ml blood), which has been equilibrated with 20 ml of PBS (*19*). Erythrocytes were eluted with 30 ml of PBS and collected by centrifugation at 4°C at $800 \times g$ for 5 min. In control experiments with uninfected mice, microscopic observations and examination of complex II and dihydroorotate dehydrogenase (DHOD) activities excluded the possible contamination of mouse leukocytes in the eluate. Erythrocytes were washed three times with RPMI-1640 medium (Gibco) and then transferred to RPMI-1640 medium supplemented with 10% AlbuMax I (Gibco) at the hematoclit of 3%. Then erythrocytes were incubated at 37°C for 2 h under conditions of 90% N_2, 5% O_2 and 5% CO_2, and trophozoite-rich parasites were recovered by centrifugation as above.

Preparation of Mitochondria—To isolate parasites, infected erythrocytes were lysed for 10 min on ice with 0.1% (w/v) saponin and the lysate was centrifuged at 4°C at $2,380 \times g$ for 10 min to remove erythrocyte membranes. Parasites were washed twice with PBS by centrifugation at 4°C at $5,800 \times g$ for 10 min and resuspended with 10–20 ml of buffer A [225 mM mannitol, 75 mM sucrose, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 5 mM HEPES, 1 mM EGTA (pH 7.4)], supplemented with 0.1% (w/v) fatty acid-free bovine serum albumin (PAA Cell Culture Co.), 1 mM phenylmethanesufonyl fluoride (Sigma) and $1 \times$ Protease Inhibitor Cocktail for general use (Sigma). Parasites were disrupted by N_2 cavitation at 1,200 psi for 20 min with 4639 Cell Disruption Bomb (Parr, USA) (*20*). Lysate was centrifuge at 4°C at $700 \times g$ for 8 min, and the resultant precipitate containing unbroken parasites was resuspended with 10 ml of buffer A and disrupted as above. This procedure was repeated twice to improve the parasite yield. Crude mitochondria were recovered from the supernatant by centrifugation at 4°C at $10,000 \times g$ for 8 min and suspended in buffer A at ~5 mg protein/ml. Rat liver mitochondria were prepared as described by Johnson and Lardy (*21*).

Enzyme Assay—Enzyme assay was performed at 25°C with V-660 double monochromatic spectrophotometer (JASCO, Tokyo, Japan; <0.00005 Abs noise) or UV-3000 double wavelength spectrophotometer (Shimadzu Corp., Kyoto, Japan), and reactions were started by addition of substrates (electron donors). Succinate:quinone reductase (SQR) activity was determined as quinone-mediated succinate:2,4-dichlorophenolindophenol (DCIP) reductase in 50 mM potassium phosphate (pH 8.0) containing 10 mM potassium succinate, 100 μM ubiquinone-2 (Q_2) and 45 μM DCIP ($\varepsilon_{600} = 21\,mM^{-1}\,cm^{-1}$) in the presence of 2 mM KCN. NADH:ubiquinone reductase (NQR) activity was measured in 50 mM potassium phosphate (pH 8.0) containing 200 μM NADH ($\varepsilon_{340} = 6.22\,mM^{-1}\,cm^{-1}$) and 100 μM ubiquinone-1 (Q_1) in the presence of 10 μM atovaquone and 2 mM KCN (*15*). DHOD activity was measured as DHO:DCIP reductase in 30 mM Tris–HCl (pH 8.0) containing 500 μM DHO, 100 μM Q_2 and 45 μM DCIP in the presence of 2 mM KCN (*20*). DHO:cytochrome *c* reductase activity was determined with 20 μM horse cytochrome *c* ($\varepsilon_{550} = 19\,mM^{-1}\,cm^{-1}$) in place of 45 μM DCIP (*20*). For inhibition studies, the reaction mixture was preincubated for 5 min in the presence of 0.1% (w/v) sucrose monolaurate (Mitsubishi-Kagaku Foods Co., Tokyo, Japan) to disperse hydrophobic substrates and inhibitors. Kinetic analysis and the estimation of the 50% inhibitory concentration (IC_{50}) were performed as described previously (*22*).

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Clear-Native Electrophoresis and Activity Staining—Mitochondria were precipitated at 4°C at 20,400 × *g* for 5 min and resuspended at 6 mg protein/ml in 10 mM Tris–HCl (pH 7.4) containing 1% sucrose monolaurate, 1 mM sodium malonate and Protease Inhibitor Cocktail by brief sonication. After 20 min incubation at 4°C with rotating, the mixture was centrifuged at 4°C at 107,000 × *g* for 30 min and supernatant was concentrated at 4°C at 4,000 × *g* with Nanosep ultrafiltration devices (MWCO 100,000, Pall Life Science). Solubilized mitochondrial proteins were subjected to high resolution clear-native electrophoresis (hrCNE) (*23*) with 3–12% Novex gels (Invitrogen) using 0.02% dodecylmaltoside and 0.05% sodium deoxycholate for the cathode buffer additives. Gels were incubated at 25°C for 10 min in 30 mM Tris–HCl (pH 7.4) containing 20 mM potassium succinate and 0.5 mM nitro blue tetrazolium chloride (NBT), and then complex II band was visualized by 1 h incubation in dark in the presence of 0.2 mg/ml phenazine methosulphate. Protein bands were stained with GelCode (Pierce).

Analysis of Membrane Anchor Subunits of Complex II—Complex II bands identified as succinate:NBT reductase in hrCNE were cut out from gels and equilibrated with an equal amount of 2× SDS–PAGE sample buffer. Gel pieces were applied to 10–20% Supersep gels (Wako Pure Chemicals, Tokyo, Japan) and SDS–PAGE analysis was carried out. Protein bands were visualized by silver staining.

Miscellaneous—Protein contents of mitochondria and solubilized membrane proteins were determined with BIO-RAD and BCA protein assay reagent (Pierce), respectively, using bovine serum albumin as standard. Western blot analysis was carried out using anti-*P. falcifaum* (Pf) Fp and anti-PfIp rabbit antiserum and cross-reacted bands were visualized by alkaline phosphatase-conjugated anti-rabbit IgG (Bio-Rad) *(24)*.

RESULTS

Preparation of Plasmodium Mitochondria—After infection of mice with rodent malaria parasites, we monitored amounts of erythrocytes and parasitemia and found that the number of parasites decreased sharply 140 h after infection as the number of the erythrocyte decreased. Thus, we collected the infected blood 130 to 140 h after infection. Leukocyte-free washed erythrocytes were incubated at 37°C for 2 h in RPMI-1640 medium supplemented with 10% AlbuMax I to adjust the developmental stage to trophozoites (trophozoite : ring : schizont = 7 : 2 : 1). Then the parasites were released from infected erythrocytes with 0.1% saponin and disrupted by the N_2 cavitation method (*20*).

Yield of Plasmodium Mitochondria—SQR activity and DHOD activity of *P. y. yoelii* mitochondria were 5- and 3-fold, respectively, higher than those of the axenic cultured *P. falciparum* (*20*). Furthermore, yields of mitochondrial proteins (5.5 ± 1.3 mg protein) and total activities of complex II (56 ± 14 mU) and DHOD (132 ± 18 mU) after preparation from ten infected mice were much greater than those of *P. falciparum* mitochondria [1 mg protein, 2 mU (*25*), and 7 mU (*20*), respectively, from the 360-ml *in vitro* culture]. Thus, in terms of the yield and specific activity, *P. y. yoelii* mitochondria are suitable for biochemical studies on mitochondrial enzymes of malaria parasites.

Table 1. **Enzymatic properties of *P. y. yoelii* mitochondria.**

Enzyme	Specific activity (mU/mg protein)	
	P. y. yoelii[a]	Rat liver
Succinate:DCIP reductase (complex II)	2.66 ± 0.02	188
NADH:Q_1 reductase[b]	42.2 ± 0.3	152
NADH:Cyt *c* reductase[c]	18.6 ± 1.6	ND[d]
DHO:DCIP reductase (DHOD)	10.5 ± 1.3	2.6
Q_1H_2 oxidase (complex III + complex IV)	19.4 ± 0.2	166

[a]Values were mean ± SD. Freshly prepared *P. y. yoelii* mitochondria showed SQR, NQR and DHOD activities of 10.2 ± 0.1, 63.2 ± 10.1 and 24.1 ± 3.9 mU/mg protein (*n* = 6), respectively. Enzyme activities were reduced to about one half after freeze-thaw of mitochondria preparations, which have been stored at −80°C. [b]NDH-II of *P. y. yoelii* or NDH-I of rat liver mitochondria were analysed. [c](NDH-II of *P. y. yoelii* or NDH-I of rat liver mitochondria) + complex III were analysed. [d]ND, not determined.

Comparison of Mitochondrial Enzymes from P. y. yoelii and Rat Liver—When comparing with rat liver mitochondria, SQR (complex II), NQR (NDH-II) and Q_1H_2 oxidase (complex III *plus* complex IV) activity of *P. y. yoelii* mitochondria were 1.4%, 28% and 12%, respectively, of rat liver mitochondria whereas DHOD activity was 4-fold higher than that of rat liver mitochondria (Table 1). Rotenone [IC_{50} = 13 nM for bovine complex I (*26*)] inhibited rat liver mitochondria complex I 95–97% at 1 μM while the inhibition of the *P. y. yoelli* NQR activity by 10 μM rotenone was only 20%. Since NQR activity of *P. y. yoelii* mitochondria followed a simple Michaelis–Menten kinetics (see below), we concluded that the enzyme activities are not due to contaminated mouse mitochondria derived from leukocytes or platelets.

Enzymatic Properties of Plasmodium Complex II—SQR activity of *P. y. yoelii* mitochondria displayed Michaelis–Menten kinetics (Fig. 1). Apparent K_m values for succinate and Q_2 were estimated to be 49 and 0.17 μM, respectively, which are close to 20 and 0.5 μM, respectively, of bovine complex II (*27*). Apparent K_m value for Q_1 was found to be 1.6 μM. Differences in K_m value (9-fold) and V_{max}/K_m ratio (19-fold) between Q_1 and Q_2 indicate that the 6-polyprenyl tail of the ubiquinone ring contributes to the binding affinity and that Q_2 is better substrate than Q_1.

Then effects of the quinone-binding site inhibitors on the SQR activity were examined. Atpenin A5 and carboxin are known inhibitors for bovine complex II with IC_{50} values of 4 nM and 1 μM, respectively (*28*) and plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) has been reported to inhibit *P. falciparum* complex II (IC_{50} = 5 μM) and the growth (IC_{50} = 0.27 μM) (*29*). At 100 μM Q_2, we found that IC_{50} values for atpenin A5 and carboxin were 4.6 and 3.6 μM, respectively, in *P. y. yoelii* mitochondria and 7.1 nM and 3.8 μM, respectively, in rat liver mitochondria (Fig. 2). The inhibition by plumbagin was only 50% even at 100 μM.

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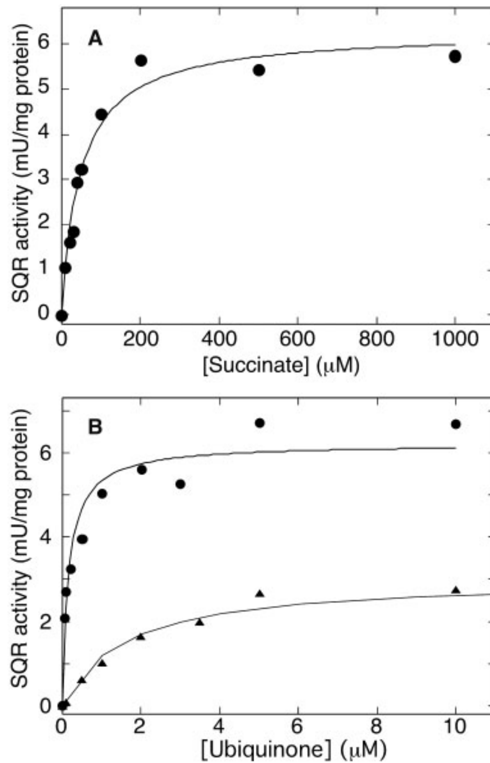

Fig. 1. **Kinetic analysis of SQR activity of *P. y. yoelii* mitochondria.** (A) As a function of the succinate concentration, SQR activity was examined at 6 μg mitochondrial protein/ml in the presence of 0.1 mM Q_2. Data points were averages from three independent preparations (6.19 ± 0.93 mU/mg protein with 1 mM succinate and 0.1 mM Q_2). Data were fitted to Michaelis–Menten kinetics with apparent K_m and V_{max} values of 49.3 ± 7.0 μM and 6.26 ± 0.27 mU/mg protein, respectively. (B) As a function of the Q_1 (circles) or Q_2 (triangles) concentration, SQR activity was examined in the presence of 10 mM succinate. Data points were averages from two independent preparations (6.25 ± 0.87 mU/mg protein with 1 mM succinate and 0.1 mM Q_2). Data were fitted to Michaelis–Menten kinetics with apparent K_m and V_{max} values of 1.61 ± 0.20 μM and 3.03 ± 0.12 mU/mg protein, respectively, for Q_1 and 0.17 ± 0.04 μM and 6.20 ± 0.30 mU/mg protein, respectively, for Q_2.

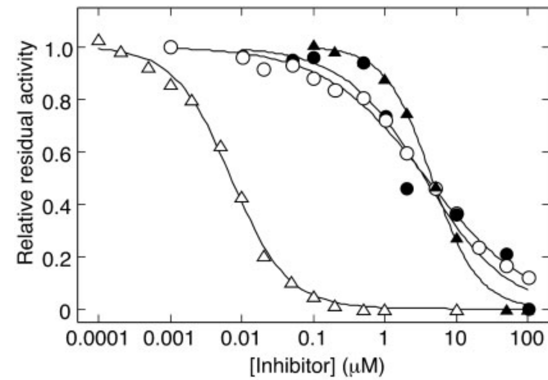

Fig. 2. **Inhibition of SQR activity of *P. y. yoelii* mitochondria by atpenin A5 and carboxin.** SQR activity of *P. y. yoelii* (closed symbols) and rat liver (open symbols) mitochondria was determined with 10 mM potassium succinate and 0.1 mM Q_2 in the presence of atpenin A5 (triangles), and carboxin (circles). Data points were average values from two independent preparations. IC_{50} values were determined to be 4.6 ± 0.2 μM for atpenin A5 and 3.6 ± 1.0 μM for carboxin in *P. y. yoelii* mitochondria and 7.1 ± 0.3 nM for atpenin A5 and 3.8 ± 0.1 μM for carboxin in rat liver mitochondria. Control activitiy of *P. y. yoelii* mitochondria was 2.68 ± 0.03 mU/mg protein.

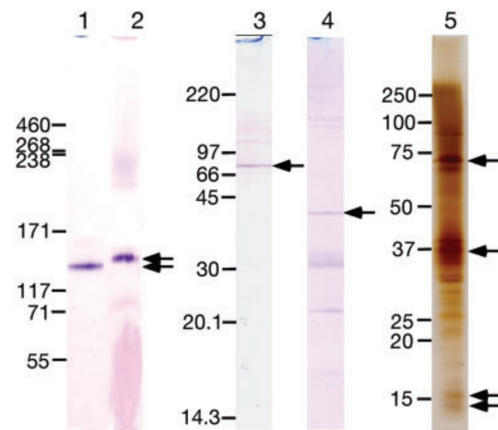

Fig. 3. **Electrophoresis analysis of complex II in *P. y. yoelii* mitochondria.** Solubilized mitochondrial proteins were subjected to hrCNE, and complex II of bovine (lane 1, 2.4 μg protein) and *P. y. yoelii* (lane 2, ~0.4 mg protein) mitochondria were visualized by SDH activity staining. Arrows indicate complex II bands. For Western blot analysis, 10 μg of mitochondrial proteins were subjected to 15% SDS–PAGE and Fp (lane 3) and Ip (lane 4), indicated by arrows, were identified by anti-PfFp and anti-PfIp rabbit antisera, respectively. For identification of *P. y. yoelii* complex II subunits, complex II bands in hrCNE were excised from gels and subjected to 10–20% SDS–PAGE, followed by silver staining (lane 5). Putative subunits of *P. y. yoelii* complex II are indicated by arrows. HiMark Prestained High Molecular Weight Protein Standard (Invitrogen), Rainbow Colored Protein Molecular Weight Marker (High molecular weight range) (Amersham Pharmacia Biotech), and Precision Plus Protein Standard (Bio-Rad) were used as molecular weight standards for lanes 1 and 2, lanes 3 and 4, and lane 5, respectively.

Membrane Anchor Subunits of Plasmodium Complex II—For reduction of ubiquinone, *Plasmodium* complex II should have a quinone-binding pocket provided by Ip and the CybL/CybS heterodimer (*30–32*). For the examination of subunit structure of *Plasmodium* complex II, we first determined the molecular weight of *P. y. yoelii* complex II by hrCNE, followed by in-gel activity staining as phenazine methosulphate-mediated succinate : NBT reductase. An apparent molecular weight of *P. y. yoelii* complex II was estimated to be 135 kDa (Fig. 3, lane 2), which is comparable to 130 kDa of bovine and yeast complex II (*33*). Western blot analysis identified Fp and Ip as the 70- and 35-kDa proteins, respectively (Fig. 3, lanes 3 and 4), indicating that a sum of molecular weights of membrane anchor subunits is about 30 kDa. Subsequently, the 135-kDa bands in hrCNE were excised from gels and subjected to SDS–PAGE analysis. Due to an extremely low activity of *Plasmodium* complex II (~1% of mammalian mitochondria) and the diffusion of a reduced product of NBT, it was difficult to cut out the complex II band but we were able to identify 70, 35, 16 and 14 kDa bands as putative subunits of the 135-kDa complex (Fig. 3, lane 5).

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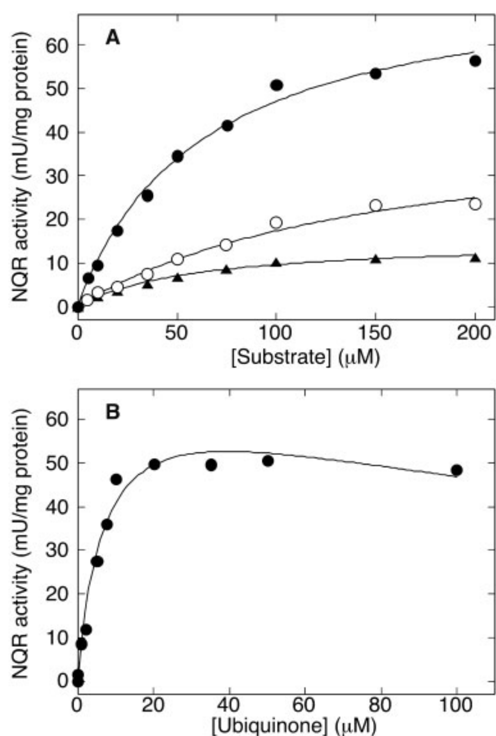

Fig. 4. **Kinetic analysis of NQR activity in *P. y. yoelii* mitochondria.** (A) As a function of the concentration of NADH (closed circle), NADPH (open circle) or deamino-NADH (closed triangle), NQR activity was examined at 6 μg protein/ml in the presence of 0.1 mM Q_1. Data points were averages from two independent preparations (44.9 ± 4.8 mU/mg protein with 0.2 mM NADH). Data were fitted to Michaelis–Menten kinetics with apparent K_m and V_{max} values of 63.2 ± 6.9 μM and 76.7 ± 3.4 mU/mg protein, respectively, for NADH, 157 ± 33 μM and 44.4 ± 5.4 mU/mg protein, respectively, for NADPH, 58.4 ± 5.7 μM and 15.1 ± 0.6 mU/mg protein, respectively, for deamino-NADH. (B) As a function of the concentration of Q_1, NQR activity was examined in the presence of 0.2 mM NADH. Data points were average values from two independent preparations [48.6 ± 7.9 mU/mg protein at 0.1 mM Q_1). Data were fitted to substrate inhibition kinetics with apparent K_m, V_{max} and K_{is} values of 7.2 ± 1.7 μM, 71.8 ± 7.6 mU/mg protein, and 218 ± 97 μM, respectively, using the equation $v = V_{max}$ S/(K_m + S + S (1+S/K_{is})].

Enzymatic Properties of Plasmodium NDH-II—*Plasmodium* spp. lacks genes encoding complex I (*6*, *7*) and uses a single-subunit NADH dehydrogenase (NDH-II) (*8*, *15*). Upon permealization of mitochondria with 30 μg/ml alamethicin, which forms pores large enough to permit the rapid diffusion of NADH (*34*), NQR and SQR activities increased 32% and 27%, respectively, indicating that *Plasmodium* NDH-II is likely located at the matrix side of the inner membrane.

When reactions were started by addition of NADH, NQR activity showed a simple Michaelis–Menten kinetics with apparent K_m and V_{max} values of 63 μM for NADH and 77 mU/mg protein, respectively (Fig. 4A). K_m value for NADH was closer to 31 μM of *Saccharomyces cerevisiae* internal NDH-II (NDI1) (*35*) and 34 μM of *E. coli* NDH-II (*36*) than 15 μM of yeast *Yarrowia lipolytica* external NDH-II (NDE) (*37*). In contrast,

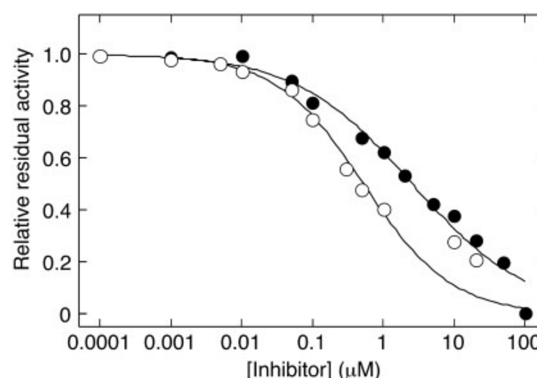

Fig. 5. **Inhibition of NQR activity of *P. y. yoelii* mitochondria by HQNO and Aurachin C1-10.** NQR activity of *P. y. yoelii* mitochondria was determined with 0.2 mM NADH and 0.1 mM Q_1 in the presence of HQNO (closed circle) or aurachin C1-10 (open circle). Data points were average values from two independent preparations. Control activity of *P. y. yoelii* mitochondria was 45.6 ± 1.3 mU/mg protein with 0.1 mM Q_1. IC_{50} values for HQNO and aurachin C1-10 were estimated to be 2.5 ± 0.4 and 0.47 ± 0.03 μM, respectively.

Q_1-started NQR activity showed substrate inhibition kinetics with K_m and K_{is} values of 7 and 218 μM, respectively, for Q_1 (Fig. 4B). Unlike *E. coli* NADH-II (*36*) and *Y. lipolytica* NDE (*37*), *P. y. yoelii* NDH-II can oxidize deamino-NADH (K_m = 58 μM, V_{max} = 15 U/mg protein) and NADPH (K_m = 157 μM, V_{max} = 44 mU/mg protein) (Fig. 5A). V_{max}/K_m ratios indicate that *Plasmodium* NDH-II is more specific to NADH compared to NAD(P)H dehydrogenases from red beet root mitochondria [NDI (*38*) and NDE (*39*)].

Since mammalian hosts lack NDH-II, this enzyme is a promising target for new antiplasmodial agents. However, inhibitors for NDH-II are rare and mostly unspecific (*34*). Fry *et al*. (*11*) examined effects of inhibitors on ATP level in erythrocytic *P. falciparum* and found that 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and 5-hydroxy-2-methyl-1,4-naphthoquinone (plumbagin) showed antimalarial activities with IC_{50} values of 4.0 and 3.5 μM, respectively. In yeast, quinolone analogues HQNO and aurachin C 0-11 were shown to inhibit NDI1 with the IC_{50} values of 8 and 0.2 μM, respectively (*40*). In this study, we examined effects of HQNO and aurachin C 1-10 (*41*) on NADH:Q_1 reductase activity and determined IC_{50} values to be 2.5 and 0.5 μM, respectively (Fig. 5). Our data indicate that the quinolone analogues are potent inhibitors for *Plasmodium* NDH-II. Trifluoroperazine, the uncompetitive inhibitor in terms of Q_2 for *Mycobacterium tuberculosis* NDH-II (IC_{50} = 12 μM) (*42*), reduced the NADH:Q_1 reductase activity to 26% of the control at 100 μM.

DISCUSSION

Properties of Plasmodium Complex II—Parasitic nematodes adapted to hypoxic host environments, have modified respiratory chain, where isoforms of complex II serve as fumarate reductase (*43*, *44*). Kinetic properties of *P. y. yoelii* complex II are similar to those of mammalian enzymes and thus suitable for catalysing the

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Table 2. **Effects of quinone-binding site inhibitors on SQR activity of *P. y. yoelii* mitochondria.**

100 μM inhibitor	*P. y. yoelii* mitochondria	Rat liver mitochondria
Control	100%	100%
Atpenin A5	<0.4	<0.05
Carboxin	<0.4	<0.05
Flutoranil	58	22
TTFA	80	12
HQNO	54	94
Plumbagin	52	98
DNP-17	67	99

Control activities (mean ± SD) were 2.66 ± 0.02 (*P. y. yoelii*) and 180 ± 5 (rat liver) mU/mg protein.

forward reaction of TCA cycle (i.e. the oxidation of succinate). It should be noted that *Plasmodium* complex II was more resistant to known quinone-binding site inhibitors for mammalian complex II (Table 2), probably due to the divergence of membrane anchor subunits of *Plasmodium* complex II.

From the whole cell lysate of *P. falciparum*, Suraveratum *et al.* (*28*) purified complex II as the Fp/Ip heterodimer with an apparent molecular weight of 90 kDa and claimed that it has a much lower K_m value (3 μM) for succinate and plumbagin-sensitive SQR activity. However, the concentration (0.2%) of octyl glucoside used for the isolation of *P. falciparum* complex II was not enough for the solubilization of membrane proteins (i.e. critical micell concentration of octyl glucoside is 0.73%). Octyl glucoside likely dissociates the Fp/Ip dimer from the membrane anchor and the aerobic isolation of the Fp/Ip dimer would damage the iron–sulphur clusters in Ip. Thus, SQR activity of such preparations need to be carefully examined.

Plasmodium CybL and CybS are still not annotated in the current database (*6*, *7*), likely due to the divergence from ortholog sequences. However, 2D–PAGE analysis (Fig. 3), SQR activity (Fig. 1, refs. *20*, *25*) and the structure of quinone-binding site in complex II (*30–32*) support the presence of these membrane anchor subunits in *Plasmodium* spp. In membrane anchors of complex II, "$Rx_{16}Sx_2HR$" (helix I) and '$YHx_{10}D$' (helix II) motifs in CybL and '$LHx_{10}DY$' (helix II) motif in CybS are conserved for quinone/haem binding. And only such motifs are conserved in protist membrane anchors (*45*). One candidates for *P. y. yoelii* CybL (accession no. XP_731082, 10,086 Da) and one candidate for CybS (accession no. XP_726783, 10,379 Da) can be identified from 3,310 ORFs shared by *P. falciparum* and *P. y. yoelii* on the basis of the size (<200 amino acid residues), the presence of transmembrane segments (⩽3), and the quinone/haem-binding motifs. PyCybL and PyCybS have two transmembrane regions and contain the quinone/haem-binding motifs, '$Rx_{14}Sx_2HY$' and '$YYx_{10}DY$' motifs and '$Yx_{10}G$' motif, respectively. In *S. cerevisiae* strain S288C (Baker's yeast), CybS (accession no. NP_010463) uses the $Yx_{10}DY$ motif, and the His-to-Tyr mutant of the CybL $YHx_{10}D$ motif retained a half of the enzyme activity and haem (*46*). Thus, in *Plasmodium* CybL and CybS, Tyr could also substitute the role of the conserved His residue in membrane anchor subunits. Although it has to be tested by protein chemically in future studies, our data support that the subunit structure of *Plasmodium* complex II is similar to that of mammalian complex II.

Properties of Plasmodium NDH-II—Previously, Krungkrai *et al.* (*47*) isolated mitochondrial complex I from *P. falciparum* and *P. berghei* as a 130-kDa complex containing 38- and 33-kDa subunits. They claimed that NADH:ubiquinone-8 reductase activity was sensitive to rotenone ($IC_{50} = 12\,\mu M$) and plumbagin ($IC_{50} = 6\,\mu M$). However, NDH-I is not encoded by the *Plasmodium* genomes (*6*, *7*) and concentrations of *n*-octyl glucoside used for the solubilization and purification were below its critical micelle concentration (CMC) where *n*-octyl glucoside cannot serve as a detergent. Alternative NADH dehydrogenase NDH-II is a rotenone-insensitive single-subunit enzyme (*15*, *34*) and the apparent molecular weights and subunit structure of *P. falciparum* (acc. no. XP_001352022 and MW 61,670) and *P. y. yoelii* (acc. no. XP_731423, MW 66,156) NDH-II are totally different from those reported by Krungkrai *et al.* (*47*). The IC_{50} value of mouse liver mitochondria for rotenone (8.4 μM; Table 3 in ref. 47) was three orders of magnitude higher than the IC_{50} reported for mammalian enzymes (*26*). Recently, Biagini *et al.* (*15*) used the whole cell lysate of *P. falciparum* and claimed that PfNDH-II was inhibited by diphenylene iodonium chloride (DPI, IC_{50} of 15–25 μM) and diphenyl iodonium chloride (IDP, $IC_{50} = 66\,\mu M$). As pointed out by Vaidya *et al.* (*48*), the IC_{50} for the enzyme was 100- and 10-fold higher than those for the growth inhibition and other NADH oxidases in the lysate may contribute to the activity. Very recently, it was reported that purified recombinant PfNDH-II was not inhibited by known NDH-I inhibitors and flavoenzyme inhibitors (DPI and IDP) (Dong, C., Patel, V., Clardy, J., and Wirth, D., personal communication). Thus, previous studies on *Plasmodium* NDH-II need to be reexamined. Our data indicate that *Plasmodium* NDH-II is a member of internal NDH-II (Ndi), which reoxidizes NADH in the mitochondrial matrix. Recently, Saleh *et al.* (*49*) demonstrated the antiplasmodial activity ($IC_{50} = 14\,nM$) of 1-hydroxy-2-dodecyl-4(1*H*)quinolone (HDQ), which has been identified as the potent inhibitor for *Y. lipolytica* NDE ($IC_{50} = 0.2\,\mu M$) (*50*), demonstrating that *Plasmodium* NDH-II is a promising target for new drugs.

Oxidative Phosphorylation in Plasmodium Mitochondria—For a long time, it has been assumed that *Plasmodium* mitochondria cannot carry out oxidative phosphorylation (*4*, *5*) because of a lack of membrane anchor subunits of ATP synthase (*9*, *11*). Oxidative phosphorylation, succinate respiration (*8*, *17*), and effects of respiratory complex inhibitors on the generation of membrane potential (*16*) in rodent malaria mitochondria support the notion that *Plasmodium* mitochondria are fully capable of oxidative phosphorylation. Careful analysis of current genome databases (*6*, *7*) with partial subunits sequences of *Crithidia fasciculata* (*51*) and *Leishmania tarentolae* (*52*) could identify ten subunits of *P. falciparum* F_oF_1-ATP synthase, including membrane anchor subunits *a* (XP_001347344) and *b* (XP_001348969) (Mogi, T. and Kita, K., unpublished

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results), which are found to be highly divergent from eukaryotic and bacterial counterparts. Thus, all canonical subunits of complex II and ATP synthase are present in *Plasmodium* spp., and malaria parasites can yield energy *via* oxidative phosphorylation. The *in vivo* expression profiles of parasites derived from infected patients showed the up-regulation of these enzymes under conditions similar to starvation in yeast (*18*).

CONCLUSION

We isolated active mitochondria from rodent malaria *P. y. yoelii* from infected mouse erythrocytes and characterized complex II and NDH-II. *Plasmodium* complex II is the four-subunit enzyme but its quinone-reduction site in the membrane anchor subunits seems structurally different from that of mammalian enzyme. *Plasmodium* NDH-II showed enzymatic properties similar to those of NDI and quinolones were found to be potent inhibitors. Alternative respiratory enzymes, which are absent in mammalian mitochondria, are as promising targets for new antibiotics (*53*, *54*). We hope that our findings will help understanding of energy metabolism in malaria parasites and the development of new antimalarial drugs.

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CONFLICT OF INTEREST

None declared.

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