Respiration, membrane potential, and oxidative phosphorylation of mitochondria of *Plasmodium yoelii yoelii* trophozoites were assayed *in situ* after permeabilization with digitonin. ADP induced an oligomycin-sensitive transition from resting to phosphorylating respiration in the presence of oxidizable substrates. A functional respiratory chain was demonstrated. In addition, the ability of the parasite to oxidize exogenous NADH, as well as the insensitivity of respiration to rotenone and its sensitivity to flavone, suggested the presence of an alternative NADH-quinone (NADH-Q) oxidoreductase. Rotenone-insensitive respiration and membrane potential generation in the presence of malate suggested the presence of a malate-quinone oxidoreductase. These results are in agreement with the presence of genes in *P. yoelii* encoding for proteins with homology to NADH-Q oxidoreductases of bacteria, plant, fungi, and protozoa and malate-quinone oxidoreductases of bacteria. The complete inhibition of respiration by antimycin A and cyanide excluded the presence of an alternative oxidase as described in other parasites. An uncoupling effect of fatty acids was partly reversed by bovine serum albumin and GTP but was unaffected by carboxyatractyloside. These results provide the first biochemical evidence of the presence of an alternative NADH-Q oxidoreductase and a malate-quinone oxidoreductase and confirm the operation of oxidative phosphorylation in malaria parasites.

Intraerythrocytic stages of mammalian malaria parasites have been considered for a long time to rely primarily on anaerobic glycolysis for energy production and to possess mitochondria that lack oxidative phosphorylation and a functional tricarboxylic acid cycle (1). A study using a flow cytometry assay developed to monitor the mitochondrial membrane potential of *Plasmodium yoelii yoelii* inside erythrocytes concluded that the malaria mitochondria did not contribute much to the ATP pool and that these parasites lack the machinery for oxidative phosphorylation (2). However, these conclusions were in contrast to the relevance of mitochondrial activity for the chemotherapy of malaria. The antimalarial drug atovaquone has been shown to inhibit electron transport at the bc1 complex (3) and to collapse the mitochondrial membrane potential in *P. yoelii yoelii* (2), and this effect has been shown to be enhanced by another antimalarial agent, the drug proguanil (4).

More recent studies using the rodent malaria parasite *Plasmodium berghei berghei* have demonstrated the capacity of mitochondria in trophozoites to sustain oxidative phosphorylation and calcium transport in the presence of tricarboxylic acid cycle intermediates (5). With the almost total completion of the malaria genome project, the relevance of mitochondrial function in *Plasmodium falciparum* (6) and *P. yoelii* (7) has been re-evaluated. The genes necessary for a complete tricarboxylic acid cycle were identified. However, it was indicated that it remains unclear whether the tricarboxylic acid cycle is used for the full oxidation of products of glycolysis or to supply intermediates for other biosynthetic pathways (6). Despite the finding of almost all the subunits of the ATP synthase in these parasites, the contribution of mitochondria to the ATP pool was considered to be minimal (6).

In this work, we report that the use of *P. yoelii* trophozoites permeabilized with digitonin, a procedure previously established to investigate *in situ* mitochondrial bioenergetics in trypanosomatids (8–11) and in the apicomplexan parasites *Toxoplasma gondii* (12) and *P. berghei* (5), allowed the demonstration of the presence of oxidative phosphorylation in these parasites. In contrast to the results previously obtained with *P. berghei* (5), no evidence of the presence of a Complex I was found in *P. yoelii*. Biochemical evidence was found of the presence of a malate-quinone oxidoreductase similar to those present in bacteria (13–17) and of an alternative NADH-quinone (NADH-Q)1 oxidoreductase, similar to those present in plants, bacteria, and fungi (18). Both enzymes had been predicted to be present by the genome project (6, 7). The mitochondria of *P. yoelii* could also be uncoupled in the presence of fatty acids as occurs with *P. berghei* mitochondria (5).

**EXPERIMENTAL PROCEDURES**

*P. yoelii yoelii*—*P. yoelii yoelii* (strain 17XL, a gift of Akhil B. Vaidya) was maintained *in vivo* in male Balb/c mice by weekly transfer infection. Blood was collected in citrate-glucose buffer in the ascending phase of parasitemia and was checked for the presence of reticulocytes, which was minimal. Red blood cells were washed twice in Dulbecco’s...
Phosphorylation in *P. yoelii* yoelii Mitochondria in Situ

PBS by centrifugation at 4 °C at 1,500 g for 5 min. Washed erythrocytes were diluted 1:1 in PBS and passed over a powdered cellulose column (CF1; Whatman, Clifton, NJ) to remove leukocytes and platelets (19). Contamination of the resulting preparations with white blood cells was always less than 5% as determined by using a Neubauer chamber and Giemsa staining. Red blood cells depleted of leukocytes and platelets were washed once as described above and resuspended in PBS. Infected erythrocytes were enriched using the Percoll method (20). Infected erythrocytes within the 70% Percoll interphase were collected and contained predominantly trophozoites (85–90% of the red blood cells, the rest containing ring stage and schizont forms), which were identified by examination of Giemsa-stained thin blood smears. To isolate the parasites, infected erythrocytes were lysed with 0.1 mg/ml saponin in PBS at room temperature for 5 min. After centrifugation at 1,500 × g for 5 min at 4 °C to remove red blood cell membranes, the parasites were washed five times in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 5.5 mM glucose, and 50 mM Heps, pH 7.2). The parasites were resuspended at 1 × 10^6 cells/ml in the same buffer. Contamination of the preparation with red blood cells was negligible. Contamination with white blood cells (mostly lymphocytes) was always less than 1% as determined using a Neubauer chamber and Giemsa staining (typically we obtained about 1.2–1.3 × 10^7 trophozoites contaminated with 1.0–1.15 × 10^7 white blood cells/mouse).

Control experiments were also done with blood enriched in reticulocytes. Reticulocytosis was induced by intraperitoneal injection of 0.06 ml (0.13 mg of sodium hydrochloride/g of weight) to Balb/c mice. This procedure increased the level of reticulocytes to 10–20% after 3 days of inoculation (21). After 3 days, the reticulocytes were collected using the same procedure employed to isolate *P. yoelii*-infected red blood cells. To isolate possible contaminating mitochondria, enriched reticulocytes were then submitted to the same procedures used to isolate trophozoites (lysis with saponin (except where indicated) and five washes with buffer A). The final pellet, containing reticulocyte mitochondria, was resuspended in buffer A at an equivalent or higher protein concentration of the isolated trophozoites described above.

**Chemicals**—ADP, antimycin A, ascorbate, ATP, atracyloside, digitoxin, carbonyl cyanide m-trifluoromethoxyphenylhydrazide (FCCP), GTP, oligomycin, potassium cyanide, rotenone, N,N,N′,N′-tetramethyl-1,3-propylene diamine (TMPD), and valinomycin were purchased from Sigma. Carboxyatractyloside was from Calbiochem. All other reagents were analytical grade.

**Oxygen Uptake Measurements**—Oxygen uptake was measured with a Clark-type electrode (22) fitted to a Gilson oxygraph (Gilson Medical Electronics, Inc., Middleton, WI) in 1.5 ml of standard incubation medium containing 125 mM sucrose, 65 mM KCl, 10.5 mM Hepes-KOH, pH 7.2, 5 mM MgCl2, 2 mM potassium phosphate, and 0.5 mM EGTA at 30 °C. Other additions are indicated in the figure legends.

**Determination of Mitochondrial Membrane Potential (ΔΨ)**—The mitochondrial membrane potential was determined by measuring the fluorescence of 4′,6-diamidino-2-phenylindole (DAPI) as a fluorescence spectrophotometer, at 30 °C. The excitation and emission wavelengths were 495 and 586 nm, with a slit of 5 mm. To evaluate the effect of exogenous substrates, trophozoites were kept at 4 °C for 1–2 h to deplete them of endogenous substrates. The valinomycin-induced K^+^ diffusion potential was used to perform a calibration curve, in a potassium-free medium containing 200 mM sucrose, 10 mM Na^+^-Heps buffer, pH 7.2, 5 mM MgCl2, 20 mM sodium phosphate, and 0.5 mM EGTA. Energized mitochondria were incubated with safranine O in the presence of valinomycin, and titration with K^+^ was performed. The ΔΨ decay caused by the electrogenic influx of the cation determined by the Nerst equation (23) is linearly correlated to the increase in the fluorescence intensity of safranine O (24). The fluorescence intensity (see “Experimental Procedures”). The addition of ADP was nevertheless able to stimulate respiration. This was probably due, despite several washes, to the permeabilization of the plasma membrane of trophozoites by the saponin treatment of the infected red blood cells performed to isolate the parasites (see “Experimental Procedures”). The addition of ADP was nevertheless able to stimulate respiration (Fig. 1B, trace a). To rule out the possibility that the residual respiration observed after KCN was due to ascorbate autoxidation, we performed a similar assay in the absence of TMPD/ascorbate, and the respiration was completely inhibited (Fig. 1A, inset).

Because *P. yoelii*, as well as other *Plasmodium* species, has a marked predilection for invading reticulocytes (28) and reticulocytes possess mitochondria, it was important to rule out the possibility that the respiratory activity measured was due to the presence of contaminating mitochondria in the preparations of isolated trophozoites. We therefore induced reticulocytosis in mice by intraperitoneal injection of phenylhydrazine
and, after 3 days of inoculation, submitted the blood from these mice to the same procedures used to isolate trophozoites to isolate possible contaminating mitochondria. At higher protein concentrations (2 mg of protein) than those used for isolated trophozoites (0.6 mg of protein), the respiratory activity of these preparations was very low (Fig. 1B, trace b). Only the addition of TMPD/ascorbate increased respiration, which was only partially sensitive to KCN and possibly caused by ascorbate autoxidation.

**Presence of a NADH-Q Oxidoreductase**—Genes encoding for putative alternative NADH-Q oxidoreductases have been reported to be present in the *P. falciparum* (6) and *P. yoelii* (7) genomes. We therefore tested the ability of exogenous NADH to stimulate respiration of genomes. We therefore tested the ability of exogenous NADH to stimulate respiration of these preparations was very low (Fig. 1B, trace b). The additions were digitonin (DIG, 2 μM), ADP (400 nM), oligomycin (OLIGO, 2 μM), AA (0.5 μM), FCCP (1.0 μM), ascorbate (0.5 mM), TMPD (300 μM), and KCN (2 mM). B, trace a, trophozoites (0.6 mg of protein); trace b, reticulocyte fraction (2 mg of protein). Inset in A, the medium was as above in the presence of 400 nM of ADP. Digitonin (DIG, 2 μM), and 2 mM KCN were added where indicated by the arrows. The numbers in parentheses indicate the rate of oxygen uptake in nmol/min × mg of protein.

**Fig. 1. Oxygen consumption by digitonin-permeabilized trophozoites and reticulocyte fractions.** The test system (final volume, 1.6 ml; 30 °C) contained 125 mM sucrose, 65 mM KCl, 10 mM Hepes-KOH, pH 7.2, 5 mM MgCl2, 2.5 mM potassium phosphate, 0.5% BSA, and 0.5 mM EGTA in the presence of 10 mM malate (A) or 10 mM succinate and 5 μM rotenone (B). The additions were digitonin (DIG, 2 μM), ADP (400 nM), oligomycin (OLIGO, 2 μM), AA (0.5 μM), FCCP (1.0 μM), ascorbate (0.5 mM), TMPD (300 μM), and KCN (2 mM). B, trace a, trophozoites (0.6 mg of protein); trace b, reticulocyte fraction (2 mg of protein). Inset in A, the medium was as above in the presence of 400 nM of ADP. Digitonin (DIG, 2 μM), and 2 mM KCN were added where indicated by the arrows. The numbers in parentheses indicate the rate of oxygen uptake in nmol/min × mg of protein.

The deduced amino acid sequence of the gene encoding the putative NADH-Q oxidoreductase (accession number EAA22988) of *P. yoelii* has 52, 26–24, and 20% identity and 65, 42–43, and 42% similarity to the sequences of *P. falciparum* (accession number CAD51833), *S. cerevisiae* (NP013861.1-NP013865.1), and *Trypanosoma brucei* (AAM95239) NADH-Q oxidoreductases, respectively. Translation of the open reading frame yielded a polypeptide of 569 amino acids with a predicted molecular mass of 64.4 kDa. A CLUSTALW alignment (25) of NADH-Q oxidoreductase peptide sequences from bacteria, fungal, plant, and protozoa was then used as the basis for the generation of a phylogenetic tree. The results (data not shown) suggested that *P. yoelii* and *P. falciparum* enzymes shared a most recent common ancestor. *T. brucei*, fungal, and plant NADH-Q-oxidoreductases were the closest out groups to the Plasmodium sequences.

**Determination of the Mitochondrial Membrane Potential (ΔΨ) in Situ**—Δψ is a very sensitive indicator of the energy-coupling condition of mitochondria. The dye safranine O-electrotransported into the mitochondria in response to Δψ; the uptake/release of the dye by the organelle is linearly correlated to Δψ up to at least 150 mV. Permeabilization of the membrane of trophozoites in the standard buffer containing succinate was followed by a large decrease in the fluorescence response, reaching a steady state after 3 min (Fig. 3, trace c). The addition of ADP was followed by a small upward deflection compatible with utilization of the electrochemical proton gradient to drive ADP phosphorylation by ATP synthase (Complex V). As expected, this fluorescence decrease was completely reversed by oligomycin, a known inhibitor of the enzyme. The addition of antimycin A (AA) promoted a fast upward deflection compatible with depolarization of the inner mitochondrial membrane and return of safranine to the water phase. Preparations obtained from reticulocyte-enriched blood, either treated (Fig. 3, trace d) or untreated (Fig. 3, traces b and c) with saponin, and at protein concentrations higher (Fig. 3, traces b and c) than those used for isolated trophozoites (Fig. 3, trace a) were unable to generate a large mitochondrial membrane potential under similar conditions, and when generated it decreased very rapidly.

The extent of Δψ respiring on succinate, α-glycerophosphate (which enters the electron transport chain at a point similar to succinate), or fumarate was about 150 mV (Fig. 4A, traces a and b); all other substrates, such as pyruvate (Fig. 4A, trace c), glutamate (Fig. 4A, trace d), or endogenous substrates (Fig. 4A, trace e), generated lower Δψ. In contrast, maleate was able to generate a high Δψ (Fig. 4B), which was collapsed by the addition of antimycin A. Further addition of TMPD/ascorbate was able to generate ΔΨ, which was then collapsed by addition
Uncoupling of Mitochondria by Fatty Acids—Previous studies in *P. berghei* suggested the presence of an uncoupling protein in malaria parasites (5). The uncoupling of mitochondria is regarded as an unwanted pathological effect or an isolation artifact. However, regulated uncoupling mediated by uncoupling proteins or other anion carriers could play an important role in several mitochondria where a sudden or transient cut-off of efficient ATP production is required (34). Linoleic acid exerted an uncoupling effect on *P. yoelii* trophozoite mitochondria *in situ* increasing their resting respiration with succinate as substrate (Fig. 7). Bovine serum albumin (BSA), which removes free fatty acids, reversed this effect, which was further weakly reversed by GTP, a known inhibitor of the uncoupling protein of brown adipose tissue (34) (Fig. 7). GTP also increased ∆ψ, which was further increased by BSA (Fig. 8A), with the same pattern being observed when the order of additions was reversed (Fig. 8B). Fig. 9 shows that incubation of these preparations with carboxyatractyloside, an inhibitor of the ADP/ATP antiporter, had no effect on linoleic acid uncoupling effect.

Immunoblotting of a total homogenate of *P. yoelii* and potato mitochondrial preparations with antibodies against the PUMP (35) allowed immunological detection of a 32-kDa protein in both preparations (Fig. 10). Additional bands of lower molecular mass were detected in the *P. yoelii* preparations, probably because of proteolysis or because of the presence of additional cross-reacting proteins. These same antibodies have also been shown to have cross-reactivity with proteins of similar size in the free living protozoa *Acanthamoeba castellani* (36), in the slime mold *Dictyostelium discoideum* (37), and in *P. falciparum* and *P. berghei* (5), and the presence of these bands has been taken as evidence for the presence of one or more uncoupling proteins in these cells.
Reticulocyte Contamination—To verify that our preparations of infected red blood cells were free of reticulocytes, we performed rhodamine staining experiments. Rd123, like other related lipophilic dyes, distributes readily across polarized membranes and has been used before to localize the single mitochondrion of *P. yoelii* (38). As described in *P. falciparum* (38), each daughter merozoite shown in Fig. 11A has a single mitochondrion, which extends almost its entire length. The addition of a mixture of inhibitors (azide + oligomycin + KCN + atracyloside) resulted in the release of the dye (Fig. 11B). No rhodamine staining was detected in the red blood cells. Mitochondria from normal reticulocytes are easily identified (Fig. 11C).

**DISCUSSION**

Digitonin has been used to permeabilize selectively the plasma membrane of a wide variety of cells without significantly affecting the gross structure and function of intracellular organelles such as endoplasmic reticulum and mitochondria. This study demonstrates that digitonin can be used to permeabilize the plasma membrane of *P. yoelii* trophozoites to ions, nucleotides, oxidizable substrates, and safranine O without affecting the functional integrity of mitochondria, as it has been reported before for *P. berghei* (5).

The effects of various inhibitors on respiration and ΔΨ of mitochondria of *P. yoelii* were evaluated. Malate (Fig. 4B) was able to generate a significant mitochondrial membrane potential in these mitochondria in situ, although its stimulation of respiration was rotenone-insensitive (Fig. 1A and 2C). Malate has been shown to stimulate rotenone-insensitive respiration and ADP phosphorylation in the related apicomplexan parasite *T. gondii* (8), and genes encoding for a malate-quinone oxidoreductase have been reported in the *P. falciparum* (6) and *T. gondii* (8) genomes. Malate-quinone oxidoreductase is a FAD-dependent membrane-associated protein that catalyzes the oxidation of malate to oxalacetate. The electrons are donated to quinones of the electron transfer chain, and NAD will not be accepted as electron donor (13). Genes encoding for this enzyme have only been reported in the bacteria *Corynebacterium glutamicum* (13), *Helicobacter pylori* (14), *Escherichia coli* (15), and *Pseudomonas aeruginosa* (16), although the enzyme activity was found before in several Gram-positive and Gram-negative bacteria (17). The presence of this enzyme also explains the generation of a significant ΔΨ by fumarate (Fig. 4A, trace b), which is converted into malate by the action of a fumarase.

**Fig. 4. Generation of a mitochondrial membrane potential in permeabilized trophozoites in the presence of different substrates.** The reaction medium (2 ml, 30°C) contained 125 mM sucrose, 65 mM KCl, 10 mM Hepes-KOH, pH 7.2, 5 mM MgCl₂, 2.5 mM potassium phosphate, 0.5% BSA, 0.5 mM EGTA, 2 μM digitonin, 5 μM safranine O, and 0.44 mg of protein (Py). A was with different substrates: 10 mM succinate (trace a); 10 mM α-glycerophosphate or 10 mM fumarate (trace b); 10 mM pyruvate (trace c); 10 mM glutamate (trace d); and without oxidizable substrate (trace e). Traces for α-glycerophosphate and fumarate were superimposable. In B the substrate was 10 mM malate. 300 μM TMPD plus 0.5 mM ascorbate (ASC), AA (0.5 μg), and 1.5 mM KCN were added where indicated. The other conditions are as in Fig. 3. RFU, relative fluorescence units.

**Fig. 5. Effect of rotenone and flavone on the mitochondrial membrane potential of digitonin-permeabilized trophozoites.** Trophozoites (Py, 0.6 mg protein) and a substrate mixture (5 mM α-glycerophosphate + 5 mM malate + 5 mM glutamate + 0.2 mM NADH/NADPH) were used. All other conditions are as described in the legend to Fig. 3. Rotenone (ROT, 30 μM), flavone (FLAV, 0.5 mM), and FCCP (10 μM) were added where indicated. (trace a). RFU, relative fluorescence units.
lized trophozoites. Trophozoites (0.78 mg) were added to a reaction medium (2 ml, 30 °C) containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes-KOH, pH 7.2, 5 mM MgCl₂, 2.5 mM potassium phosphate, 0.5% BSA, 0.5 mM EGTA, 10 mM succinate, 2 μM digitonin, 5 μM safranine O, and 0.4 mg of protein (Py). 1.5 μM FCCP (A) or 2 μM KCN (B) were added where indicated. The other conditions are as described in the legend to Fig. 3. RFU, relative fluorescence units.

A gene encoding a putative fumarase has been shown to be present in the genome of malaria parasites (6, 7). Three groups of NADH-Q oxidoreductases have been described (18). They are the proton-translocating NADH-Q oxidoreductase (designated complex I in mitochondria and NDH-1 in bacteria), the Na⁺-translocating NADH-Q oxidoreductase reported only in bacteria, and the NADH-Q oxidoreductase lacking an energy coupling site (18). Mammalian mitochondria are believed to contain only complex I as the NADH dehydrogenase in the respiratory chain. The mammalian complex I is composed of at least 43 subunits and has a very intricate structure. This enzyme complex is sensitive to rotenone. In contrast to mammalian mitochondria, mitochondria of *S. cerevisiae* lack complex I but instead have two rotenone-insensitive NADH-Q oxidoreductases. These two NADH-Q oxidoreductases do not function as proton-translocating enzymes; one faces the intermembrane space (referred to as external rotenone-insensitive NADH-Q oxidoreductase), and the other faces the mitochondrial matrix (designated internal rotenone-insensitive NADH-Q oxidoreductase). The Ndi1 enzyme of *S. cerevisiae* mitochondria is a single polypeptide enzyme and is inhibited by flavone but not by rotenone (26). The *P. falciparum* and *P. yoelii* genomes seem to lack genes encoding components of a rotenone-sensitive NADH-Q oxidoreductase or complex I (6, 7). Instead, a single subunit NADH-Q oxidoreductase gene with homology to other rotenone-insensitive NADH-Q oxidoreductases has been found in the genomes of *P. falciparum* (6) and *P. yoelii* (7). Our results show that mitochondria of permeabilized trophozoites of *P. yoelii* can oxidize exogenous NADH and that this process is not inhibited by rotenone, even at very high concentrations, but is inhibited by flavone. Although high concentrations of rotenone could inhibit complex III and other respiratory enzymes (40), we used very high concentrations of this inhibitor because it has been shown that some NADH dehydrogenases are less sensitive to rotenone. For example, in rat liver (41) and human platelet mitochondria (42) complex I activity determined with coenzyme Q as electron acceptor was only inhibited 30% by rotenone. On the other hand, flavone derivatives have been shown to inhibit the external NADH-quinone oxidoreductase of plants (43). These results support the presence of an external rotenone-insensitive NADH-Q oxidoreductase in *P. yoelii* mitochondria. However, flavone is known to inhibit other dehydrogenases as well (18) and was able to inhibit respiration (Fig. 2) and the generation of ΔΨ (Fig. 5) even in the presence of substrates that can enter the electron transport chain at a point similar to succinate (α-glycerophosphate; Fig. 5). Thus, additional specific inhibitors would certainly be valuable tools for further studies on this enzyme.

In previous work (5) we detected that rotenone (>1 μM) was able to collapse the mitochondrial membrane potential of digitonin-permeabilized *P. berghei* trophozoites. Rotenone (1 μM) was also shown to release Rh123 from the mitochondria of *P. falciparum*-infected erythrocytes (38). Because high concentrations of rotenone (>1 μM) have been shown to cause partial inhibition of the succinate-ubiquinone oxidoreductase, the succinate-cytochrome *c* oxidoreductase systems, and even the cytochrome oxidase (40), we cannot rule out the possibility that those effects were nonspecific. Completion of the *P. berghei* genome project will reveal the presence or absence of a typical complex I in these parasites.

Potential flavoprotein-linked substrates such as succinate were able to stimulate ADP phosphorylation by *P. yoelii* mitochondria in situ (Figs. 1 and 3), and succinate-dependent respiration was sensitive to antimycin A, indicating the presence...
of complex II in its respiratory chain. The presence of an $H^+/H_1$ conductance in trophozoites similar to that produced by a mitochondrial uncoupling protein and the large amounts of free fatty acids known to be present in malaria parasites (39, 44–46) would explain the weak stimulatory effect of ADP on respiration observed in Fig. 1. The results showing inhibition of respiration by antimycin A and cyanide (Fig. 1) support the presence of the ubiquinol-cytochrome $c$ oxidoreductase (complex III), and cytochrome $c$ oxidase (complex IV) and the absence of an alternative oxidase (6). The results showing the inhibition of state 3 respiration by oligomycin (Figs. 1 and 3) support the presence of an ATP synthase (complex V). Although genes encoding all subunits of the catalytic F1 portion of the ATP synthase, the protein that confers oligomycin sensitivity, and the gene that encodes the proteolipid subunit $c$ for the F0 portion of ATP synthase were detected in the $P. falciparum$ and $P. yoelii$ genomes, the $a$ and $b$ subunits of the ATP synthase have not been detected (6). However, because parts of the genome sequences are incomplete, the presence of the $a$ and $b$ subunits and therefore a functional ATP synthase could not be ruled out (6). Our results are in favor of the presence of a functional ATP synthase in the mitochondria of $P. yoelii$.

It has been demonstrated that uncoupling proteins and other carriers found in mitochondria of mammalian brown adipose tissue and some nonthermogenic tissues, plants, fungus, and some protozoa are able to mediate free fatty acid-induced uncoupling (34). Fatty acids anions are ejected from the matrix by uncoupling proteins or other anion carriers, and protonated fatty acids return to the matrix. This protonophore cycle dissipates the $H^+/H_1$ transmembrane electrochemical gradient and bypass ATP synthase. The addition of free fatty acids such as linoleic acid to mitochondria of permeabilized trophozoites of $P. yoelii$ resulted in uncoupling, whereas the presence of BSA, which removes free fatty acids, and of GTP, which inhibits uncoupling proteins (34), recoupled respiration increasing $\Delta \Psi$ in situ (Figs. 7 and 8)

![Fig. 8. Effect of GTP and BSA on the mitochondrial membrane potential of digitonin-permeabilized trophozoites.](image)

The conditions were the same as described in the legend to Fig. 3 in the presence of 2 $\mu$g of oligomycin and 10 mM succinate. The additions were 1 mM GTP, 0.5% BSA, and 1 $\mu$g of AA. RFU, relative fluorescence units.

![Fig. 9. Lack of inhibition by carboxyatractyloside of the uncoupling effect of linoleic acid.](image)

The reaction medium (2 ml, 30 $^\circ$C) contained the incubation medium described in the legend to Fig. 3, plus 10 $\mu$g carboxyatractyloside. Trophozoites (Py, 0.6 mg of protein), linoleic acid (LA, 1.0 $\mu$M), and KCN (1.5 mM) were added where indicated. RFU, relative fluorescence units.

![Fig. 10. Immunological identification of proteins cross-reacting with antibodies against PUMP in trophozoites.](image)

Primary antibodies were raised against potato PUMP. Reticulocyte fraction (lane 1), $P. yoelii$ trophozoite lysates (lane 2), and potato crude mitochondria (lane 3) (30 $\mu$g) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The PUMP antibody recognized a polypeptide with an apparent molecular mass of 32 kDa in potato mitochondria and $P. yoelii$ lysates. Additional smaller bands appeared in the trophozoite preparation probably because of proteolysis or the presence of other cross-reacting proteins.
boxyatractyloside (Fig. 9), although it could have been responsible for the cross-reaction with PUMP antibodies (Ref. 5 and Fig. 10). Because malaria parasites are known to contain large amounts of free fatty acids (39, 44–46), the presence of such a H+ conductance could explain the low stimulation by ADP observed in Fig. 1. An uncoupling protein or an anion carrier with the properties of an uncoupling protein could provide a cellular defense system preventing formation of superoxide anion by the malaria mitochondria as it has been postulated to occur in animal cells (34).

Our preparations of the 17X strain of P. yoelii (instead of the 17X strain that inhibits reticulocytes) in its parasite stage (2) revealed minimal reticulocyte contamination. In addition, reticulocyte-enriched preparations obtained following the same protocols used for trophozoite isolation revealed minimal mitochondrial activity (Figs. 1B, trace b, and 3, traces b–d). Together, these results rule out the possible contamination of our preparations with reticulocyte mitochondria.

The results obtained using Rd123 (Fig. 11) are compatible with the generation of a mitochondrial membrane potential and its dependence on the respiratory chain. Only a mixture containing electron transport inhibitors was able inhibit Rd123 accumulation by P. yoelii mitochondria. Azide and KCN are able to inhibit completely the electron transport chain, and oligomycin and atractyloside prevent membrane potential generation by ATP hydrolysis.

In conclusion, the respiratory chain and oxidative phosphorylation are functional in P. yoelii. The presence in malaria mitochondria, as demonstrated by the biochemical and genetic evidence available, of enzymes absent in mammalian cells, such as the malate-quinone oxidoreductase and the alternative NADH-Q oxidoreductase, provides exceptional targets for drug design.

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REFERENCES
1. Vaidya, A. B. (1998) in Malaria, Parasite Biology, Pathogenesis and Protection (Sherman, I. W., ed) pp. 355–368, American Society for Microbiology, Washington, D. C.
2. Srivastava, I. K., Rottenberg, H., and Vaidya, A. B. (1997) J. Biol. Chem. 272, 29661–29666.
3. Fry, M., and Pulley, M. (1992) Biochem. Pharmacol. 43, 1545–1553.
4. Srivastava, I. K., and Vaidya, A. B. (1999) Antimicrob. Agents Chemother. 43, 1334–1339.
5. Uyemura, S. A., Luo, S., Moreno, S. N. J., and Docompo, R. (2000) J. Biol. Chem. 275, 9709–9715.
6. Gardiner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M. S., Nene, V., Shalonn, S. J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haib, D., Mathe, M. W., Vaidya, A. B., Martin, D. M., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W., Fraser, C. M., and Barrell, B. (2002) Nature 419, 498–511.
7. Carlton, J. M., Angiuoli, S. V., Suh, B. H., Keji, T. W., Pertea, M., Silva, J. C., Ernoladse, D., Allen, J. E., Selengut, J. D., Koo, H. L., Peterson, J. D., Pop, M., Kosack, D. S., Skurnik, M. F., Badwell, S. L., Shalonn, S. J., van Aken, S. E., Riedmuller, S. B., Feldlybun, T. Y., Cho, K. J., Quackenbush, J., Sedgah, M., Shafihi, A., Cummings, L. M., Flores, L., Yates, J. R., Raine, J. D., Sin, E. E., Harris, M. A., Cunningham, D. A., Preiser, P. R., Bergman, L. W., Vaidya, A. B., van Lin, L. H., Jansse, C. J., Waters, A. P., Smith, H. O., White, O. R., Salzberg, S. L., Venter, J. C., Fraser, C. M., Hoffman, S. L., Gardner, M. J., and Carucci, D. J. (2002) Nature 419, 512–519.
8. Vercesi, A. E., Bernardes, C. F., Hoffmann, M. E., Gadilha, F. R., and Docompo, R. (1991) J. Biol. Chem. 266, 14431–14434.
9. Vercesi, A. E., and Docompo, R. (1992) Biochem. J. 284, 463–467.
10. Vercesi, A. E., Docompo, R., and Moreno, S. N. J. (1992) Mol. Biochem. Parasitol. 56, 251–258.
11. Vercesi, A. E., Moreno, S. N. J., Bernardes, C. F., Meinicke, A. R., Fernandes, E. C., and Docompo, R. (1993) J. Biol. Chem. 268, 8564–8568.
12. Vercesi, A. E., Rodrigues, C. O., Uyemura, S. A., Zhong, L., and Moreno, S. N. J. (1999) J. Biol. Chem. 274, 31040–31047.
13. Molenaar, D., van der Rest, M. E., and Petrovic, S. (1998) Eur. J. Biochem. 254, 395–403.
14. Kowarik, B., Stirling, K., Van der Rest, M. A., and Molenaar, D. (2000) J. Biomed. Chem. 182, 3204–3209.
15. Van der Rest, M. E., Frank, C., and Molenaar, D. (2000) J. Biol. Chem. 182, 6892–6899.
16. Kreitshelf, U., Bürckt, A., Jeong, J.-H., and Girisch, H. (2002) Microbiology 148, 3839–3847.
17. Cohn, D. V. (1958) J. Biol. Chem. 233, 299–304.
18. Kerscher, S. (2000) Biochim. Biophys. Acta 1509, 274–283.
19. Homewood, C. A., and Neame, K. D. (1976) Ann. Trop. Med. Parasitol. 70, 249–251.
20. Dziukawski, A. R., Ling, T. T., Rangachari, K., Bates, P. A., and Wilson, R. J. M. (1984) Trans. R. Soc. Trop. Med. Hyg. 78, 622–624.
21. Viens, P., Chevalier, J. L., and Sonea, S. (1971) Can. J. Microbiol. 17, 257–261.
22. Akerman, K. O. and K. F. Wikstrom, K. F. (1976) FEBS Lett. 68, 191–197.
23. Beavis, A. D., and Vercesi, A. E. (1992) J. Biol. Chem. 267, 3079–3087.
24. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4500–4505.
25. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680.
26. Plessenster, J. (1989) Cladistics 5, 164–166.
27. Saitou, N., and Nei, M. (1987) Mol. Biol. Evol. 4, 406–425.
28. Monis, P. (1990) Blood Cells 16, 299–312.
29. de Vries, S., and Grivell, L. A. (1988) Eur. J. Biochem. 176, 377–384.
30. Kitajima-Ihara, T., and Yagi, T. (1998) FEBS Lett. 421, 57–40.
31. Yagi, T., Liao, D., Di Bernardo, S., and Matsuno-Yagi, A. (1998) Biochim. Biophys. Acta 1364, 125–133.
32. Smith, I. F., Plant, D. L., Doyle, J. P., Skinner, R. A., Pearson, H. A., and Peers, C. (2003) J. Neurochem. 85, 1109–1116.
33. Sensi, S. L., Ton-That, D., Sullivan, P. G., Jonas, E. A., Gee, K. K., Kazmarchek, L. K., and Weiss, J. H. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 6157–6162.
34. Skulachev, V. P. (1998) Biochim. Biophys. Acta 1363, 100–124.
35. Zee, P., Engstová, H., Zábokvá, M., Vercesi, A. E., Costa, A. D. T., Arruda, P., and Garlid, K. D. (1998) Biochim. Biophys. Acta 1365, 319–327.
36. Jarmuszkiewicz, W., Sluse-Goffart, C. M., Hryniewiecka, I., and Sluse, F. E. (2002) FEBS Lett. 532, 459–464.
| Page | References |
|------|------------|
| 38   | Divo, A. S., Geary, T. G., Jensen, J. B., and Ginsburg, H. (1985) *J. Protozool.* 32, 442–446 |
| 39   | Holz, G. G. (1977) *Bull. World Hlth Org.* 55, 237–248 |
| 40   | Teeter, M. E., Baginsky, M. L., and Hatefi, Y. (1969) *Biochim. Biophys. Acta* 172, 331–333 |
| 41   | Lenaz, G., Fato, R., Genova, M. L., Formiggini, G., Parenti Castelli, G., and Bovina, C. (1995) *FEBS Lett.* 366, 119–121 |
| 42   | Merlo-Pich, M., Bovina, C., Formiggini, G., Cometti, C. G., Parenti Castelli, G., Genova, M. L., Marchetti, M., Semeraro, S., and Lenaz, G. (1996) *FEBS Lett.* 380, 176–178 |
| 43   | Ravanel, P., Creuzet, S., and Tissut, M. (1990) *Phytochemistry* 29, 441–445 |
| 44   | Wallace, W. R. (1966) *Am. J. Trop. Med. Hyg.* 15, 811–813 |
| 45   | Holz, G. G., Beach, D. H., and Sherman, I. W. (1977) *J. Protozool.* 24, 566–574 |
| 46   | Beach, D. H., Sherman, I. W., and Holz, G. G. (1977) *J. Parasitol.* 63, 62–75 |