Respiration and Oxidative Phosphorylation in the Apicomplexan Parasite Toxoplasma gondii

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Toxoplasma gondii is an obligate intracellular protozoan parasite of humans and animals (1). The infection is usually asymptomatic and results in the formation of dormant encysted bradyzoites that remain in the brain and other tissues for life. Only the developing fetus and the immunosuppressed patient are at substantial risk of severe disease (2). T. gondii has emerged as a major opportunistic pathogen of immunocompromised individuals, particularly those infected with the human immunodeficiency virus (3). The parasite is normally found in two forms in mammalian nonfeline hosts, tachyzoites and bradyzoites. The stage that is seen in acutely infected animals is the tachyzoite, the rapidly growing asexual form.

Very little is known about mitochondrial functions in T. gondii. Early histochemical studies suggested the presence of isocitrate dehydrogenase and succinate dehydrogenase (SDH)1 in tachyzoites (4). SDH and NADP+-dependent isocitrate dehydrogenase activities were recently detected biochemically in whole cell homogenates of tachyzoites, whereas bradyzoite homogenates did not show detectable levels of SDH (5). These results were taken as evidence of the presence of a tricarboxylic acid cycle associated with a respiratory chain in tachyzoites and of its absence in bradyzoites (5). We recently showed that oligomycin, an inhibitor of mitochondrial ATP synthesis, caused an increase in cytosolic Ca2+ levels in tachyzoites. This increase suggested a requirement for mitochondrial energy for the regulation of Ca2+ homeostasis in these parasites (6). In addition, incubation of tachyzoites with general mitochondrial inhibitors, rotenone, antimycin, myxothiazol, or hydroxynitrosylcyanide m-chlorophenylhydrazone (CCCP), induced the appearance of bradyzoite-specific markers (7) and differentiation to bradyzoites in vitro (8).

Experiments using rhodamine 123 have suggested that the mitochondria of intracellular tachyzoites do not maintain a pH gradient (9). On the basis of data from the use of 4-hydroxyquinolones (10–12), clopidol (10), and hydroxamic acids (11, 12), it has been proposed that Toxoplasma (12), as well as the related apicomplexan parasites Eimeria tenella (10) and Plasmodium falciparum (11), contains a plant-like alternative oxidase (11, 12). It has also been suggested that the plastid-like organelles that occur in apicomplexan parasites (13–15), which have received considerable attention recently (16, 17), may contain components of a respiratory chain (18), which could contribute to the respiration of the cell (12). Although the importance of the respiratory chain in T. gondii is unknown, it is thought that it is the target of the anti-Toxoplasma activity

1 The abbreviations used are: SDH, succinate dehydrogenase; CCCP, carbonyl cyanide m-chlorophenylhydrazone; TMPD, N,N,N’,N’-tetramethyl-p-phenylenediamine.
of the naphthoquinone atovaquone (19, 20), which is currently used against toxoplasmosis in AIDS patients (19).

In addition to this fragmentary information, most of the current assumptions about T. gondii mitochondrial function have been based on work done in other apicomplexan parasites, such as E. tenella, Plasmodium yoelii, and P. falciparum (21–23). Although mitochondria from these parasites have been isolated, the process of oxidative phosphorylation or their ability to maintain a membrane potential could not be demonstrated (21, 23). A flow cytometry assay has recently been developed to monitor the mitochondrial membrane potential (∆Ψ) of P. yoelii inside erythrocytes and the changes induced by atovaquone (24). It was concluded that malarial mitochondria do not contribute much to the ATP pool and that these parasites lack the machinery for oxidative phosphorylation (24).

Given the lack of information on mitochondrial physiology in T. gondii and the apparent relevance of mitochondrial activity for its differentiation (7, 8) and for the chemotherapy of toxoplasmosis (19, 20), it is extremely important to develop strategies to study the mitochondrial bioenergetics of these cells. In this work we report that the use of tachyzoites permeabilized with digitonin, a procedure previously established to investigate in situ mitochondrial bioenergetics in trypanosomatids (25–28), allowed for the first time the functional characterization of the respiratory chain of an apicomplexan parasite. Our results demonstrate that oxidative phosphorylation occurs in the mitochondria of these parasites and suggest that the parasite respiratory pathway differs from that of the mammalian host.

**EXPERIMENTAL PROCEDURES**

**Culture Methods**—Tachyzoites of the T. gondii RH strain were cultivated according to Moreno and Zhong (6) in bovine turbinate cells (ATCC CRL 1390). Host cells were cultivated in tissue culture flasks using minimum essential medium supplemented with 10% horse serum. Cells were infected with tachyzoites at a final ratio of 1:5 (host/parasite), and parasites were harvested 2–3 days after infection and purified as described before (29). The protein concentration was determined by the biuret assay (30) in the presence of 0.2% deoxycholate.

**Preparation of Rat Liver Mitochondria**—Wistar male rats (200–250 g, Harlan, Inc.) were used in the experiments. They were fed standard rat chow and water ad libitum and were not fasted prior to use. Mitochondria were prepared (31) by homogenizing the livers in 4 volumes of cold sucrose/Hepes/EGTA solution (250 mM sucrose, 10 mM Hepes-KOH (pH 7.2), 1 mM EGTA) and centrifuging the homogenate at 480 × g for 5 min. The supernatant was centrifuged at 9,750 × g for 10 min. The resulting mitochondrial pellet was resuspended to the original volume and resedimented three times, again at 9,750 × g for 10 min. Finally the pellet was suspended in medium containing 250 mM sucrose and 10 mM Hepes-KOH, pH 7.2, to give a 40–50 mg/ml suspension.

**Chemicals**—Minimum essential medium, horse serum, CCCP, valinomycin, oligomycin, ADP, digitonin, antimycin A, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), ascorbate, pentamidine, potassium cyanide, and rotenone were purchased from Sigma. WR-6026 was a gift from the Walter Reed Army Institute, Washington, D.C. Atovaquone and proguanil were from Glaxo Wellcome. All other reagents were analytical grade.

**Measurements of Oxygen Uptake**—Measurements of oxygen consumption were made with a Clark-type electrode fitted to a Gilson oxygraph (Gilson Medical Electronics, Inc., Middleton, WI) in medium containing: 125 mM sucrose, 65 mM KCl, 10 mM Hepes-KOH (pH 7.2), 5 mM MgCl₂, 2 mM potassium phosphate, 0.5 mM EGTA, and, where indicated, 5 mM succinate. Other additions are indicated in the figure legends and Table I. Representative traces from experiments conducted on at least three separate cell preparations are shown in the figures and table.

**Estimation of Mitochondrial Membrane Potential (∆Ψ)**—The mitochondrial membrane potential was measured by the safranine method according to Vercesi et al. (25). The calibrations were made in potassium-free medium containing 200 mM sucrose, 10 mM Na⁺-Hepes buffer (pH 7.0), 1 mM MgCl₂, 2.0 mM sodium phosphate, and 0.25 mM EGTA. Absorption spectra and time-dependent absorption changes of safranine O were recorded with an SLM Aminco DW2000 spectrophotometer. Representative traces from experiments conducted on at least three separate cell preparations are shown in the figures.

**Determination of NADH-fumarate Reductase Activity**—T. gondii tachyzoites (0.2 mg of protein) were incubated in a medium containing 200 mM sucrose, 2.0 mM potassium phosphate, 10 mM Na⁺, pH 7.0, and 24 mM digitonin for 10 min (final volume, 1.5 ml). The suspension was centrifuged at 6,000 rpm for 2 min in an Eppendorf centrifuge (model 5414). The pellet was resuspended in 100 μl of the same medium, frozen in a bath of dry ice plus ethanol, and thawed three times. The NADH-fumarate reductase activity was determined as the rate of NADH oxidation upon addition of 10 mM fumarate at 30 °C (32). The absorbance changes were monitored at 340 nm in an SLM Aminco DW-2000 spectrophotometer.

**RESULTS**

The addition of 16 μM digitonin to a suspension of tachyzoites (0.125 mg/ml) incubated in the standard buffer containing 5 mM succinate was followed by a discrete increase in the rate of oxygen uptake indicating that the plasma membrane became permeable to succinate (Fig. 1A). The subsequent addition of ADP induced the transition from resting (State 4) to phosphorylating (State 3) respiration. A respiratory control (State 3/State 4) of 2.7 was estimated using the value of State 4 respiration after the addition of oligomycin (for nomenclature of respiratory states, see Ref. 33). CCCP addition, at the concentration used (1 μM), partially released the State 4 respiration, which was then completely inhibited by antimycin A. The inclusion of the respiratory substrate system TMPD/ascorbate, which reduces the respiratory complex IV, reinitiated the respiration, which was then inhibited by 2 mM cyanide. The residual respiration observed after KCN addition was due to ascorbate autoxidation, because the addition of KCN in the absence of TMPD/ascorbate completely inhibited respiration (Fig. 1B) as occurred upon the addition of antimycin A (Fig. 1A). These results rule out the presence, as previously postulated (12), of a cyanide-insensitive terminal oxidase in T. gondii tachyzoites. The operation of a phosphorylating site III is suggested by the results shown in Fig. 1C. Addition of ADP to the antimycin A-poisoned preparation after TMPD/ascorbate (in the absence of succinate) stimulated respiration. ADP/O ratios of 0.98 and 1.05 were obtained after the first and second addition of ADP, respectively.

Malate/glutamate, a combination of substrates commonly used to demonstrate the presence of NADH-ubiquinone oxidoreductase (complex I) activity in mitochondrial preparations, was also able to stimulate ADP phosphorylation in permeabilized tachyzoites (Table I). However, this stimulation was rotenone-insensitive (5 μM) (not shown). We therefore tested each substrate separately and found that only malate, but neither glutamate nor 3-oxoglutarate, was able to stimulate ADP phosphorylation under similar conditions (Table I). Malate-stimulated ADP phosphorylation was rotenone-insensitive (5 μM) (not shown), and other potential NADH-dependent substrates, such as pyruvate or isocitrate (Table I), were unable to stimulate ADP phosphorylation. In addition, neither endogenous substrates nor the potential flavoprotein-linked substrates α-glycerol-3-phosphate or dihydroorotase (34) (Table I) were able to stimulate phosphorylation.

We reasoned that malate might be stimulating ADP phosphorylation via conversion into succinate. Malate can generate fumarate through the enzyme fumarase, and fumarate in turn could be converted into succinate through the enzyme NADH-fumarate reductase. In agreement with this hypothesis, a NADH-fumarate reductase activity (8.4 ± 1.0 nmol/min × mg of protein, mean ± S.D., n = 5) was detected in T. gondii mitochondrial extracts. The presence of this pathway has been described in trypanosomatids (35), and it has been postulated to occur in some apicomplexan parasites, such as P. yoelii and P. falciparum (36).
steady state after a period of about 2 min. The
followed by a large increase in absorbance, which reached a
in
A
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A
in
C
is lower in
A
and
B
in
C
(Oligo, 2 μM), CCCP (1 μM), antimycin A (AA, 0.5 μg), ascorbate (0.5 mM) plus TMHP (300 μM in
A
and
B
and 120 μM in
C
(TMHP/ASC), and potassium cyanide (KCN, 2 mM). Note that the scale is different and the amount of TMHP added is lower in
C
allow a better visualization of ADP-stimulated respiration. The numbers in parentheses indicate the rate of oxygen uptake in nmol/min × mg of protein.

**TABLE I**

| Substrate | Rate of respiration |
|-----------|---------------------|
|           | State 3 | State 4 |
| Succinate (10) | 53.9 ± 3.2* | 20.3 ± 1.5 |
| L-Malate (5) + l-glutamate (5) | 49.0 ± 3.5 | 22.7 ± 1.6 |
| L-Malate (10) | 33.5 ± 2.4 | 11.8 ± 0.7 |
| L-Glutamate (10) | 4.1 ± 0.5 | 3.6 ± 0.5 |
| 3-Oxoglutarate (10) | 6.4 ± 1.4 | 5.2 ± 1.6 |
| Pyruvate (10) | 5.2 ± 0.5 | 3.6 ± 0.5 |
| Isocitrate (10) | 3.2 ± 0.4 | 3.6 ± 0.5 |
| sn-Glycerol-3-phosphate (10) | 10.2 ± 0.6 | 9.7 ± 1.2 |
| Dihyroorotate (10) | 10.6 ± 0.4 | 8.5 ± 0.4 |
| TMHP (0.2) + ascorbate (1.5) | 70.6 ± 3.1 | 52.2 ± 2.3 |
| No substrates | 4.9 ± 0.7 | 7.4 ± 1.1 |

* Mean ± S.E. values of four determinations.

Safranine O is a dye whose absorbance spectrum shifts as it binds to and stacks upon increasingly polarized inner mitochondrial membranes. The spectral shift is linearly related to the developed membrane potential (ΔΨ) up to at least 170 mV (37) and can be monitored by the difference in absorbance at the wavelength pair 511–533 nm by using a dual wavelength spectrophotometer. Fig. 2A shows that the permeabilization of the plasma membrane of tachyzoites suspended in the standard buffer containing 5 mM succinate and 10 μM safranine was followed by a large increase in absorbance, which reached a steady state after a period of about 2 min. The dotted line shows that the addition of ADP, after the absorbance had stabilized, was followed by a small downward deflection compatible with the utilization of the electrochemical proton potential to drive ADP phosphorylation by the F_{0}F_{1}-ATP synthase (37). As expected, this absorbance decrease was totally reversed by oligomycin, a known inhibitor of the F_{0}F_{1}-ATP synthase. The inclusion of either CCCP (Fig. 2A) or antimycin A (not shown) promoted a fast downward deflection of the trace compatible with depolarization of the inner mitochondrial membrane and return of safranine to the water phase. It can be observed that the 511–533 nm difference in absorbance did not return to the original value observed at time zero, when digitonin was added to the medium. This is compatible with the distribution of the dye into nonmitochondrial compartments as indicated by the dashed trace showing one experiment in which the cells were permeabilized in reaction medium containing safranine plus antimycin A to prevent inner mitochondrial membrane polarization. Under these conditions, CCCP addition did not cause any additional change in the safranine absorbance (Fig. 2A, dashed line).

Addition of valinomycin, a potassium ionophore, collapsed ΔΨ (Fig. 2B) by facilitating the electrogenic influx of the cation (38). This property of valinomycin allowed for ΔΨ calibration as shown in the experiment depicted in Fig. 2B. In this experiment, the permeabilized cells were suspended in a potassium-free medium, and the addition of valinomycin did not cause any change in absorbance, but the subsequent titration with potassium was followed by the concomitant decrease in membrane potential because of the influx of the cation. Further addition of CCCP completely collapsed ΔΨ. The membrane potential after each K+ addition was calculated according to the Nernst equation (37).

Fig. 3A shows that the addition of cyanide totally collapsed ΔΨ, because the subsequent addition of CCCP did not cause any further change in safranine absorbance. This figure also
shows a significant decrease in ΔΨ induced by the cationic compounds WR-6026 and pentamidine, which have been shown previously to collapse the mitochondrial membrane potential of

Leishmania donovani (26) or rat liver mitochondria (39), respectively. A complete elimination of ΔΨ was obtained by the subsequent addition of CCCP.

It has been proposed that the target in Toxoplasma for atovaquone is the respiratory chain (19, 20, 40) and that this drug promotes the transformation of tachyzoites to bradyzoites by interfering with mitochondrial function (7). Therefore, we investigated its effect on both mitochondrial membrane potential and oxygen consumption. Fig. 3 shows the effect of different inhibitors on the mitochondrial membrane potential of tachyzoites in situ. Experimental conditions were the same as described in Fig. 2A. Potassium cyanide (CN−, 2 mM), pentamidine (PE, 200 μM), WR-6026 (WR, 200 μM), and CCCP (1 μM) were added where indicated. B, atovaquone (ATO, 0.03 μM) and CCCP (1 μM) were added where indicated.

Leishmania donovani (26) or rat liver mitochondria (39), respectively. A complete elimination of ΔΨ was obtained by the subsequent addition of CCCP.
0.1 μM did not have a significant effect on ∆Ψ by tachyzoites, but, in contrast, it did not enhance the ability of atovaquone to collapse ∆Ψ when used in combination (data not shown).

Fig. 5 shows that atovaquone was also able, albeit at higher concentrations, to cause a dose-dependent inhibition of rat liver mitochondrial respiration and, as occurs after addition of antimycin A (Fig. 5A), respiration resumed after addition of TMPD/ascorbate (Fig. 5B). Collapse of rat liver mitochondrial membrane potential was evident with lower concentrations of atovaquone (Fig. 6) than those that caused respiratory inhibition (Fig. 5B). This effect was reversed by the addition of TMPD/ascorbate. Subsequent addition of CCCP collapsed the membrane potential (Fig. 6).

FIG. 4. Effect of atovaquone on the respiration of permeabilized tachyzoites. Experimental conditions were the same as described in Fig. 1C and in the presence of 1 μM CCCP. Atovaquone (ATO), 0.01 μM each addition) and ascorbate (0.5 mM) plus TMPD (300 μM) (TMPD/ASC) were added where indicated.

FIG. 5. Effect of atovaquone on the respiration of rat liver mitochondria. Experimental conditions were as described in Fig. 1A. Additions were: rat liver mitochondria (RLM, 2.7 mg of protein), ADP (300 nmol), oligomycin (OLIGO, 2 μM), CCCP (1 μM), antimycin A (ANT A, 0.5 μg), ascorbate (0.5 mM) plus TMPD (300 μM) (TMPD/ASC), atovaquone (ATO, 1 μM each addition), and potassium cyanide (KCN, 2 mM). The numbers in parentheses indicate the rate of oxygen uptake in nmol/min × mg of protein.

0.35 μM for rat liver mitochondria (0.14 mg of protein/ml), which indicates that T. gondii mitochondrial respiration is at least 23-fold more sensitive to atovaquone than rat liver mitochondrial respiration.

DISCUSSION

This study demonstrates that, as in trypanosomatids (25–28), digitonin can be used to selectively permeabilize the plasma membrane of T. gondii tachyzoites to ions, nucleotides, respiratory substrates, and safranine O without affecting the functional integrity of mitochondria. Indeed, the experiments of Figs. 1 and 2A show that the addition of ADP to digitonin-permeabilized cells was followed by the transition of respiration or ∆Ψ from the resting to the phosphorylating state. This is perhaps the most sensitive test to ascertain mitochondrial intactness (33).
Using these preparations, we studied the effects of various mitochondrial inhibitors on mitochondrial respiration and on the mitochondrial membrane potential. Rotenone did not change the rate of respiration or the extent of $\Delta \Psi$ (not shown) indicating that the NADH-ubiquinone oxidoreductase (complex I) is absent or does not bind rotenone in these cells. In agreement with these results, potential NADH-linked substrates, such as 3-oxoglutarate, glutamate, pyruvate, and isocitrate, were unable to stimulate ADP phosphorylation in these mitochondria in situ. This also agrees with previous reports regarding the possible lack of NADH-ubiquinone oxidoreductase in other apicomplexan parasites, such as *E. tenella* (21) and *P. yoelii* (23, 24). Although rotenone (IC$_{50}$ 0.42 mM) has been shown to increase the expression of bradyzoite-specific proteins as occurs with other mitochondrial inhibitors (7), it is known that when used at high concentrations it can cause partial inhibition of succinate-ubiquinone oxidoreductase, succinate-cytochrome c oxidoreductase, and even cytochrome oxidase (42). We cannot rule out, however, that a rotenone-insensitive NADH dehydrogenase could be present in some developmental stages of *T. gondii* and that the inability of glutamate, 3-oxoglutarate, pyruvate, and isocitrate to stimulate ADP phosphorylation might be because of the lack of permeability of *T. gondii* mitochondria to these substrates. Glycerol 3-phosphate is often taken up by mitochondria, and the lack of respiratory stimulation by this compound is significant; however, *T. gondii* mitochondria simply may not have a transporter for this substrate, which enters the electron transport chain at a point similar to succinate.

The inhibition of respiration and collapse of $\Delta \Psi$ by antimycin A and cyanide support the presence of ubiquinol-cytochrome c oxidoreductase (complex III) and cytochrome c oxidase (complex IV) in the respiratory chain of these cells and the absence of a postulated (12) alternative oxidase. However, we cannot rule out the presence of an alternative oxidase in other developmental or physiological states of the parasite. The sensitivity of respiration and $\Delta \Psi$ to ADP and oligomycin suggests that the machinery for oxidative phosphorylation is similar to that observed in most vertebrate cells. Likewise, the sensitivity of $\Delta \Psi$ to the standard mitochondrial inhibitors and ionophores, such as CCCP and valinomycin, supports the notion that these mitochondria are also similar to vertebrate mitochondria in regard to the generation and utilization of an electrochemical proton gradient. The failure of previous investigators (9) to detect a membrane potential in *T. gondii* using rhodamine 123 could be because of a lack of uptake by, or increased efflux from, the parasites.

Only malate, succinate, and TMPD/ascorbate were able to
stimulate ADP phosphorylation by *T. gondii* mitochondria in situ. The presence of the fumarate reductase system would explain why malate could have such an effect and would imply that a portion of the electron transport chain in *T. gondii* is definitely different from that present in mammalian cells. In this regard, other flavoprotein-linked substrates, such as sn-glycerol-3-phosphate and dihydroorotate (34), were unable to stimulate ADP phosphorylation by these mitochondria in situ. Fig. 8 shows a scheme for the proposed respiratory chain of *T. gondii* tachyzoites.

The ability of mitochondria of *T. gondii* tachyzoites to carry out energy-linked functions such as respiration coupled with generation of a membrane potential sufficient to phosphorylate ADP (Figs. 1–3) supports the hypothesis that inhibitors of the mitochondrial activity stimulate tachyzoite transformation into bradyzoites, because the latter are apparently much less dependent on respiratory energy (7, 8). This might be the reason for the survival of these cells in the presence of antimicrobials that affect mitochondrial function such as atovaquone.

**Fig. 8.** Scheme for the postulated respiratory chain of *T. gondii* tachyzoites. The scheme shows the presence of a ubiquinol-cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV) and the absence of a NADH-ubiquinone oxidoreductase (complex I). A succinate dehydrogenase (complex II) is present. A SDH and fumarate reductase (*FR*) cycle would allow the use of NADH generated from malate that would enter the mitochondria and be transformed into oxaloacetate through the malate dehydrogenase (*MDH*). Two phosphorylating sites (Sites II and III) are shown. *P*, fumarase; *UQ*, ubiquinone.

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Oxidative Phosphorylation in Toxoplasma gondii

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