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Biochimica et Biophysica Acta 1457 (2000) 200–210

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Oxidative phosphorylation supported by an alternative respiratory pathway in mitochondria from *Euglena*

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Received 17 November 1999; received in revised form 3 February 2000; accepted 11 February 2000

Abstract

The effect of antimycin, myxothiazol, 2-heptyl-4-hydroxyquinoline-*N*-oxide, stigmatellin and cyanide on respiration, ATP synthesis, cytochrome *c* reductase, and membrane potential in mitochondria isolated from dark-grown *Euglena* cells was determined. With L-lactate as substrate, ATP synthesis was partially inhibited by antimycin, but the other four inhibitors completely abolished the process. Cyanide also inhibited the antimycin-resistant ATP synthesis. Membrane potential was collapsed (<60 mV) by cyanide and stigmatellin. However, in the presence of antimycin, a H^+ gradient (>60 mV) that sufficed to drive ATP synthesis remained. Cytochrome *c* reductase, with L-lactate as donor, was diminished by antimycin and myxothiazol. Cytochrome bc_1 complex activity was fully inhibited by antimycin, but it was resistant to myxothiazol. Stigmatellin inhibited both L-lactate-dependent cytochrome *c* reductase and cytochrome bc_1 complex activities. Respiration was partially inhibited by the five inhibitors. The cyanide-resistant respiration was strongly inhibited by diphenylamine, *n*-propyl-gallate, salicylhydroxamic acid and disulfiram. Based on these results, a model of the respiratory chain of *Euglena* mitochondria is proposed, in which a quinol-cytochrome *c* oxidoreductase resistant to antimycin, and a quinol oxidase resistant to antimycin and cyanide are included.

Keywords: ATP synthesis; Antimycin; Cyanide-resistant respiration; *Euglena*

1. Introduction

Mitochondria isolated from dark-grown *Euglena gracilis* have respiratory components that are resistant to antimycin and cyanide [1–3]. The cyanide-resistant respiratory pathway is inhibited by diphenylamine (DPA) [2,4], preferentially oxidizes L-lactate [4,14], and builds up a small, uncoupler-sensitive membrane potential [4,5], that supports the energy-dependent uptake of Ca^{2+} [6]. This pathway is partially inhibited by salicylhydroxamic acid (SHAM) [4,7], a potent inhibitor of alternative respiratory pathways in plant mitochondria [8]. Cell growth in the presence of antimycin [1,2,9], cyanide [10] or ethanol [10] as carbon source, induces an increase in the content of a *b*-type cytochrome, that reacts with carbon monoxide. This last observation has been interpreted in terms of an adaptable enhancement of an alternative oxidase, which is resistant to the stress conditions of the culture [1,2,10]. However,

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0005-2728/00/$ – see front matter
PII: S0005-2728(00)00102-X

the semipurified antimycin-sensitive cytochrome *b* also reacts with carbon monoxide [10] and, hence, such an interpretation should be further evaluated.

Based on studies of respiratory inhibition by antimycin and cyanide, and of spectral characteristics of *Euglena* mitochondria, Buetow [3] proposed a respiratory chain in which L-lactate oxidation could occur through both an alternative terminal oxidase and the classical pathway (cytochromes bc_1 and aa_3), by transferring electrons from the dehydrogenase to either the quinone pool or directly to cytochrome *c*. In an effort to elucidate the site of branching for the alternative pathway, the effect of several inhibitors of the mammalian cytochrome bc_1 complex [11] on the rates of respiration, ATP synthesis, cytochrome *c* reductase and quinol oxidase was determined. The effect of SHAM [8], DPA [2,4], *n*-propylgallate [12], and disulfiram [13], inhibitors of alternative respiratory pathways in plant mitochondria, on the cyanide-resistant respiration was also examined.

2. Materials and methods

2.1. Materials

Antimycin, myxothiazol, SHAM, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), L-lactic acid, D-lactate, duroquinone, decylbenzoquinone (DBQone), *N*,*N*,*N*′,*N*′, tetramethyl-*p*-phenylene diamine (TMPD), disulfiram, fatty acid-free bovine serum albumin, hexokinase, cytochrome *c*, oligomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2,6-dichlorophenol indophenol (DCPIP), and phenazine methosulfate (PMS) were purchased from Sigma. Stigmatellin was from Fluka, *n*-propyl gallate (nPG) from ICN, DPA from Aldrich, and $^{32}P_i$ and ^{3}H-tetraphenylphosphonium (^{3}H–TPP^+) from New England Nuclear.

2.2. Cell culture and preparation of mitochondria

E. gracilis Klebs (a Z-like strain), kept in the dark in liquid medium for several months, was reactivated and axenically grown as described [4,5,14]. The cells were grown in the dark in the Hutner's acidic organotrophic medium with glutamate+malate as carbon source [15,16] at 25 ± 1°C under orbital agitation (125 rpm). Cells were harvested after 82–86 h, in the late exponential phase of growth, by centrifugation at 1000×*g* for 10 min at 4°C and washed once in SHE medium (250 mM sucrose, 10 mM HEPES, 1 mM EGTA, pH 7.3).

The procedure previously described [4,5,10] for isolation of mitochondria by sonication of cells was used with slight modifications. Cells were resuspended at a density of 2×10^9 cells in 25 ml of SHE medium, supplemented with 0.4% (w/v) fatty acid-free bovine serum albumin. The cell suspension was sonicated in ice with a microprobe of 12 mm tip diameter for 10 s three times, with 1 min resting period, at 50–60% of maximal output in a Branson sonifier. The sonicate was diluted with 2–3 volumes of SHE medium and centrifuged at 600×*g* for 10 min at 4°C. The supernatant was centrifuged at 8500×*g* for 10 min at 4°C. The mitochondrial pellet was carefully resuspended in 2–3 ml of SHE medium supplemented with 0.2% fatty acid-free albumin, 1 mM ADP, and incubated for 10 min in ice with occasional agitation. The mitochondrial suspension was diluted with 10–15 volumes of fresh SHE medium and centrifuged at 7800×*g* for 10 min at 4°C. The pellet was resuspended in SHE medium (+0.2% fatty acid-free albumin) to a final concentration of 40–70 mg protein/ml. The usual yield was 45–60 mg protein per l of culture. The respiratory control and ADP/O ratio values, with 10 mM L-lactate as oxidizable substrate, were 2.0 ± 0.1 (14) and 1.1 ± 0.1 (14) (mean ± S.E.M., *n*), respectively. Mitochondrial protein was determined by the biuret method as described previously [4].

2.3. Oxygen uptake and ATP synthesis

The rate of respiration of *Euglena* mitochondria was measured at 30°C, with a Clark-type oxygen electrode, in an air-saturated standard medium that contained 120 mM KCl, 20 mM MOPS, 1 mM EGTA, 5 mM K-phosphate, 1 mM $MgCl_2$, of pH 7.25. For ATP synthesis, mitochondria were incubated at 30°C in 1 ml of standard medium which also contained $^{32}P_i$ (0.3–0.6 μCi/μmol), 10 mM glucose, and five units hexokinase. At predetermined times the reaction was stopped with ice-cold 5% (w/v) trichloroacetic acid. After centrifugation of denatured protein, an aliquot was withdrawn for $^{32}P_i$

extraction from the aqueous phase by reaction with ammonium molybdate/sulfuric acid, and using acetone plus *n*-butyl acetate as organic phase [17]. The $^{32}P_i$-free aqueous phase was used for determination of $^{32}P_i$ incorporated into ATP and glucose-6-phosphate by measuring the Cerenkov radiation in water.

2.4. Quinol and TMPD oxidases

These activities were determined at 30°C by measuring the rate of 0_2 uptake stimulated by 0.25 mM duroquinol, and 5 mM ascorbate plus 2.5 mM TMPD, respectively [10]. Irradiation of mitochondria (25–30 mg protein/ml) with ultraviolet light was made in SHE medium supplemented with 1 mM ADP, 2 mM $MgCl_2$ and 0.2–0.5% (w/v) fatty acid-free albumin at 4°C, under occasional stirring. The lamp (Mineralight, UVG-54, 254 nm) was placed at about 1.5–2 cm from the suspension. Extraction of quinones was made according to Ding et al., [18]. Aliquots of 50 mg of mitochondrial protein were freeze-dried, resuspended in 100 ml iso-octane and incubated for 1 h at room temperature under gentle orbital shaking. The supernatant was discarded and the pellet was extracted five more times with iso-octane. The final mitochondrial extract was brought to dryness using a stream of N_2 and resuspended in SHE medium.

2.5. Cytochrome c reductase and NAD-independent L-lactate dehydrogenase (iLDH) activities

Euglena mitochondria (0.08–0.14 mg protein/ml) were incubated at 30°C in SHE medium plus 1 mM ADP, 5 mM K-phosphate, 1 mM $MgCl_2$ and 30 μM oxidized horse heart cytochrome *c*. The reaction was started by addition of either 10 mM L-lactate, 10 mM succinate, or 60 μM decylbenzoquinol (DBQ), which was reduced according to Rieske [19]. The rate of cytochrome *c* reduction was followed by the increase in the absorbance difference at 550 minus 540 nm in a dual wavelength SLM-Aminco DW-2000 spectrophotometer; an extinction coefficient of 21.1 mM^{-1} cm^{-1} was used in the calculations [20]. For iLDH activity determination, mitochondria (0.05–0.1 mg protein) were incubated at 30°C in 1 ml of standard medium, which also contained 0.2 mM DCPIP and 0.25 mM PMS. The rate of variation in the absorbance at 600 nm was measured after addition of 10 mM L-lactate. The extinction coefficient for DCPIP was taken as 21.3 mM^{-1} cm^{-1} at pH 7.5 [21].

2.6. Membrane potential

The distribution of ^3H–TPP^+ (0.06–0.07 μCi/nmol) was used to estimate the difference of electrical potential across the inner mitochondrial membrane, following the formulations proposed by Rottenberg [22], which involve corrections for nonspecific binding to the external and internal faces of the inner mitochondrial membrane.

3. Results

We previously reported that inhibition of respiration by cyanide in *Euglena* mitochondria was lower with L-lactate than with NADH or succinate as substrate [4,10]. This observation suggested that the alternative respiratory pathway preferentially oxidized L-lactate. In an attempt to elucidate the respiratory components involved in L-lactate oxidation, the effect of several inhibitors of the mammalian cytochrome

Table 1
Inhibition of oxidative phosphorylation in *Euglena* mitochondria

Addition	Rate of ATP synthesis (nmol/min/mg protein)	
	L-lactate	Succinate
None	205.5 ± 11 (12)	111 ± 15 (8)
+1 mM NaCN	4.2 ± 1 (8)	6.2 ± 1.4 (3)
+0.5–1 μM Antimycin	91 ± 13 (9)	19 ± 4 (6)
+12.5 μM Oligomycin	2.5 ± 0.1 (5)	1.3
+2.5 μM CCCP	2.1 ± 0.5 (7)	–

Euglena mitochondria (0.35–0.5 mg protein/ml) were incubated as described in Section 2 in the presence of the indicated substrates and inhibitors. After 2 min, 1 mM ADP was added; the reaction was stopped 2 min later and the incorporation of $^{32}P_i$ into ATP was determined. The rates of state 3 respiration for the same mitochondrial preparations were 177 ± 21 (12) and 83 ± 19 (8) ng atoms oxygen/mg protein/min for 10 mM L-lactate and 10 mM succinate, respectively. The data shown are mean ± S.E.M., with the number of preparations assayed between parentheses.

bc_1 complex on respiration and ATP synthesis was assayed.

3.1. ATP synthesis

The rates of ATP synthesis and state 3 (ADP-stimulated) respiration (Table 1) were two-fold higher with L-lactate than with succinate. However, the P/O ratio was identical with the two substrates: 1.25 ± 0.09 (12) for L-lactate and 1.23 ± 0.26 (6) for succinate (mean ± S.E.M.; *n*). With D-lactate, the rates of state 3 respiration reached values of 300–400 ng atoms oxygen/min/mg protein, although the rates of ATP synthesis were similar to those attained with L-lactate (data not shown). Determination of the cell levels of lactate revealed that the L-isomer was at a concentration of 3.8 ± 1.2 mM (7), considering a water intracellular volume of 2.11 μl/10^7 cells, whereas the D-isomer concentration was negligible (< 0.1 mM). Therefore, L-lactate, but not D-lactate, was used throughout the rest of this study.

Oxidative phosphorylation was sensitive to cyanide, oligomycin and the uncoupler CCCP, but partially resistant to a high antimycin concentration, in particular with L-lactate as substrate (Table 1). This observation was made several years ago by our group [10]. The rate of antimycin-resistant ATP synthesis supported by L-lactate oxidation was also fully blocked by 2.5 μM oligomycin, 5 μM CCCP or 1 mM cyanide (data not shown).

3.2. Cytochrome c reductase activities

To explore the existence of a cytochrome bc_1 complex with an unusual low sensitivity to antimycin, as occurs in mitochondria from *Tetrahymena pyriformis* [23] and yeast mutants [24], the activity was directly measured (Table 2). Cytochrome bc_1 complex in *Euglena* mitochondria efficiently reduces added horse heart cytochrome *c*; this is in contrast to *Euglena* cytochrome *c* oxidase, which interacts only with endogenous cytochrome *c* [14]; 30 μM oxidized cytochrome *c* and 60 μM DBQ as substrates sufficed to reach maximal rates of bc_1 complex activity.

Thus, with DBQ as artificial electron donor, the activity of cytochrome bc_1 complex was strongly inhibited by antimycin. With succinate, cytochrome *c* reductase activity was also extensively inhibited by antimycin (Table 2), indicating that electron transfer from succinate flowed preferentially through the cytochrome bc_1 complex. It should be noted that, although the concentrations of antimycin used for inhibition of oxidative phosphorylation and cytochrome *c* reductase were similar, the relation inhibitor/protein was 3–5 times higher for the assays of cytochrome *c* reductase activity. Despite such a high antimycin concentration, cytochrome *c* reductase activity was only partially inhibited when L-lactate was the electron donor. This suggested the presence of an electron flow pathway from quinone to cytochrome *c* that by-passes the cytochrome bc_1 complex.

Myxothiazol, another specific inhibitor of mammalian cytochrome bc_1 complexes [11], exerted a weak effect on *Euglena* cytochrome bc_1 activity using DBQ or succinate as electron donors. Cytochrome bc_1 complexes from closely related trypanosomatids and from *Paramecium* are also resistant to myxothiazol [23]. In contrast, myxothiazol significantly diminished cytochrome *c* reductase activity when L-lactate was the electron donor. This observation indicated the presence of a myxothiazol-sensitive quinol-cytochrome *c* oxidoreductase activity, different to that of the cytochrome bc_1 complex (antimycin-sen-

Table 2
Cytochrome *c* reductase activity

Donor	Activity (nmol cytochrome *c*/mg/min)
60 μM DBQ	96.4 ± 18 (5)
+0.16–0.6 μM Antimycin	0.3 ± 0.2 (5)
+20 μM Myxothiazol	79 ± 24 (3)
10 mM L-Lactate	84.3 ± 8 (6)
+0.16–0.6 μM Antimycin	43 ± 7 (8)
+20 μM Myxothiazol	20.5 ± 5 (3)
+0.16 μM Antimycin+ 20 μM Myxothiazol	6.8 ± 2 (4)
10 mM Succinate	15.6 ± 1.5 (5)
+0.16–0.6 μM Antimycin	2.4 ± 0.4 (5)
+20 μM Myxothiazol	13.4 ± 0.9 (3)

Euglena mitochondria (0.08–0.14 mg protein/ml) were incubated as described in Section 2. After 1–2 min, DBQ, succinate or L-lactate were added. The initial rate of cytochrome *c* reduction was corrected for by the rate of reduction attained in the presence of 1 μM stigmatellin (non-enzymatic reduction) and by the activity due to endogenous substrates, in the same experimental conditions. Mean ± S.D. (*n*).

sitive, myxothiazol-resistant), that is preferentially fed by iLDH, an enzyme that is located in the inner mitochondrial membrane and which does not require pyridine nucleotides ([3]; R. Jasso-Chávez et al., unpublished data). Stigmatellin, a third specific inhibitor of mammalian cytochrome bc_1 complexes [11], fully abolished the activity of cytochrome *c* reductase with the three electron donors (data not shown). This indicated that both quinol-cytochrome *c* oxidoreductases had similar binding sites for this inhibitor.

3.3. Antimycin, HQNO and cyanide

Titration with antimycin of L-lactate-supported state 3 respiration (Fig. 1) and ATP synthesis (Fig. 2A) revealed the presence of an antimycin-resistant respiratory component, which was able to drive oxidative phosphorylation with a lower thermodynamic efficiency; with 500 nM antimycin (1000–1250 pmol antimycin/mg protein), the P/O ratio diminished from 1.25 in control mitochondria to 0.44 (Fig. 2A). Inhibition of succinate oxidation by antimycin (Fig. 1) also exhibited an antimycin-resistant respiratory component. Accordingly, this antimycin-resistant pathway generated a H^+ gradient of a sufficient magnitude (−94 mV; Table 3) able to drive oxidative phosphorylation. It is noted that a H^+ gradient of about 60–80 mV (negative inside) has been determined as threshold value for mitochondrial [25,26] and bacterial [27,28] ATP synthesis.

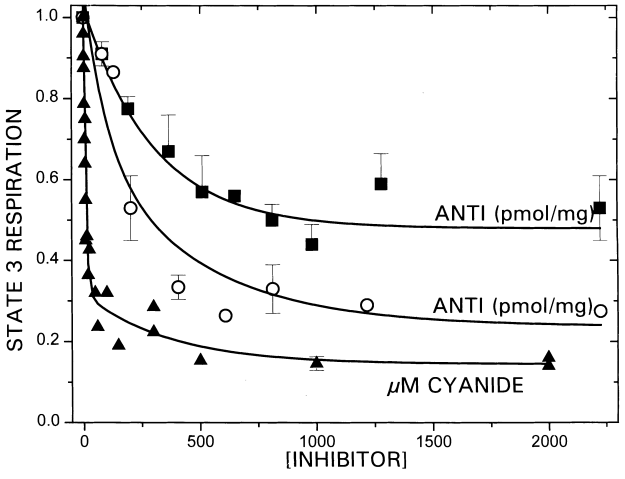


Fig. 1. Inhibition of state 3 respiration by antimycin and cyanide. *Euglena* mitochondria (0.5–1 mg protein/ml) were incubated as described in Section 2 in the presence of 2 mM ADP, 10 mM L-lactate (filled symbols) or 10 mM succinate (empty circles) and the indicated concentrations of antimycin (ANTI) or cyanide. The values shown represent the mean ± S.E.M. of titrations with antimycin of 4–12 different preparations. The experimental values with cyanide are from three different preparations. The rates of state 3 respiration in the absence of inhibitors were 237 ± 17 (33) and 82 ± 12 (14) ng atoms oxygen/mg protein/ min for L-lactate and succinate, respectively. The solid lines represent the best-fit to a second order exponential decay.

The larger inhibition of L-lactate oxidation (Fig. 1), complete suppression of L-lactate dependent oxidative phosphorylation (Fig. 2A), and collapse of the H^+ gradient (Table 3) by cyanide, indicated that a fraction of the electron transfer, resistant to antimycin, required the activity of cyanide-sensitive cytochrome *c* oxidase. This interpretation accounts for the lowering in the P/O ratio in the presence of antimycin, in which only one site of energy conservation participates. In consequence, the respiratory component that by-passes the cytochrome bc_1 complex would not be able to drive ATP synthesis.

The cyanide concentration required to attain half-maximal inhibition (IC_{50}) of ATP synthesis, in the absence (Fig. 2A) or in the presence of 0.5 μM antimycin (IC_{50} = 7.6 μM; data not shown), were similar to K_i values previously reported for inhibition of TMPD oxidase [10,14]. Hence, it appears that cytochrome *c* oxidase is the common terminal oxidase for both, antimycin-sensitive and antimycin-resistant phosphorylating pathways.

HQNO, an inhibitor of alternative quinol oxidases in bacterial respiratory systems [29] and of cytochrome bc_1 complexes [11], was able to abolish oxidative phosphorylation in *Euglena* mitochondria (Fig. 2B) with IC_{50} values in the absence (2.3 μM) and in the presence of 0.5 μM antimycin (14.3 μM) similar to those required for half-maximal inhibition of cytochrome *bo* from *Escherichia coli* [29].

3.4. Myxothiazol and stigmatellin

Myxothiazol completely inhibited L-lactate supported ATP synthesis, but it was partially effective with succinate as substrate (Fig. 3); the respective IC_{50} values were 1.1 and 32.8 μM myxothiazol for L-lactate and succinate, respectively. The respiratory rates with the two substrates were diminished in 85–

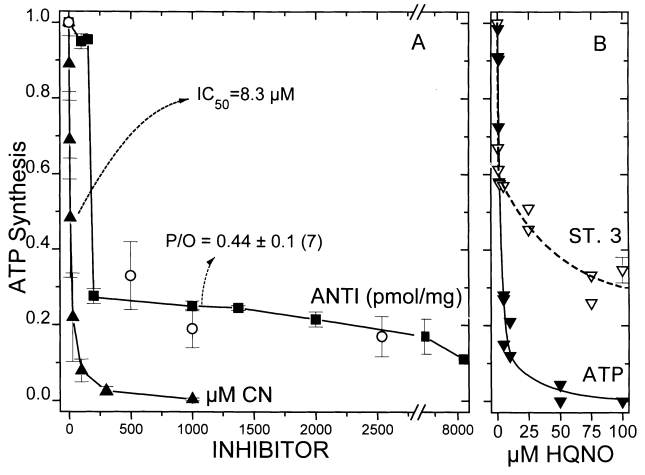


Fig. 2. Inhibition of ATP synthesis by antimycin, cyanide and HQNO. (A) *Euglena* mitochondria (0.35–0.5 mg protein/ml) were incubated with $^{32}P_i$ as described in Section 2 with 10 mM L-lactate (filled symbols) or 10 mM succinate (empty circles) and the indicated concentrations of inhibitors. After 2 min, 1 mM ADP was added and the reaction was stopped 2 min later with trichloroacetic acid. (B) Mitochondria were incubated with L-lactate and the indicated concentrations of HQNO. Respiration and the incorporation of $^{32}P_i$ into ATP were determined as detailed in Section 2. The values shown represent the mean ± S.E.M. of titrations with 3–12 different preparations. The rates of ATP synthesis in the absence of inhibitors were 252.4 ± 27 (14) and 112 ± 24 (5) nmol/mg protein/min for L-lactate and succinate, respectively.

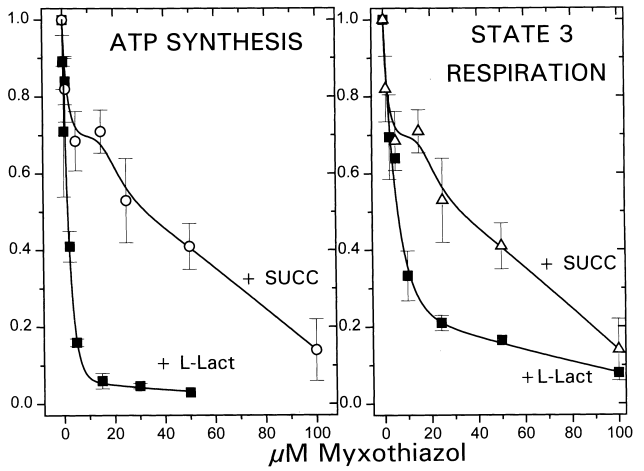


Fig. 3. Inhibition of ATP synthesis and respiration by myxothiazol. See legends to Figs. 1 and 2 for experimental details. The values shown are the mean ± S.E.M. of 3–10 different preparations.

90% by 100 μM myxothiazol; likewise, with both substrates the sensitivity of state 3 respiration to myxothiazol (Fig. 3) was similar (i.e., similar IC_{50} values) to that observed for ATP synthesis, after correction of the non-inhibited fluxes. The biphasic pattern of inhibition by myxothiazol on respiration and ATP synthesis supported by succinate (Fig. 3) was also observed for the L-lactate-cytochrome *c* reductase activity (data not shown). In the latter case, the IC_{50} values for myxothiazol were 0.2 and 24.7 μM ($n = 2$). The low affinity component disappeared when the titration of the reductase activity with myxothiazol was made in the presence of antimycin (not shown). Moreover, in the presence of both antimycin plus myxothiazol, the L-lactate-cytochrome *c* reductase activity was suppressed (Table 2).

With 20 μM myxothiazol, the concentration that inhibited ATP synthesis with L-lactate, but which slightly affected that supported by succinate (Fig. 3), the membrane potential diminished to 105 ± 2 (7) mV with L-lactate (i.e., 12 mV lower H^+ gradient), but it was not affected with succinate.

Stigmatellin, at a concentration of 0.3 nmol/mg protein, suppressed ATP synthesis and state 3 respiration supported by L-lactate or succinate (data not shown); it also collapsed the membrane potential (Table 3). The stigmatellin- and cyanide-resistant respiration was of the same extent; stigmatellin plus

Table 3
Steady-state H^+ gradient in *Euglena* mitochondria

	$\Delta\psi_{H^+}$ (mV)	
	L-Lactate	Succinate
State 4	146.4 ± 10 (8)	139.5 ± 6 (8)
State 3	117 ± 7 (16)	104.6 ± 12 (16)
State 3+0.4–1.0 nmol antimycin/mg protein	94 ± 7 (14)	87 ± 7 (7)
State 3+2 mM CN^-	42 ± 3 (6)	
State 3+0.3–0.6 nmol stigmatellin/mg protein	48 ± 8 (6)	

Mitochondria (2.5–3.5 mg protein/ml) were incubated at 30°C in 0.5 ml of standard medium with 0.8 μM [^{3}H]TPP^+ and 20 mM L-lactate or 20 mM succinate. After 2 min, 2 mM ADP was added, except for state 4 where no addition was made. The reaction was stopped by rapid centrifugation at 14 000 × *g* for 2 min at 4°C in an Eppendorf refrigerated microcentrifuge. Aliquots of the pellet and supernatant were used to calculate the distribution of [^{3}H]TPP^+ across the inner membrane following the corrections for unspecific binding to the Nernst equation by Rottenberg [21].

cyanide did not produce further inhibition of the rate of respiration supported by L-lactate. Since iLDH activity was not affected by these inhibitors (data not shown), the site of action of HQNO and stigmatellin on ATP synthesis and respiration supported by L-lactate is very likely located after the reduction of quinones; myxothiazol (50 μM) slightly inhibited (15.5 ± 6%; 5) iLDH activity, but its main site of action may also be after the formation of quinol, because of its more pronounced effect on respiration, cytochrome *c* reductase and ATP synthesis.

3.5. Antimycin-resistant pathway

To determine whether the antimycin-resistant respiratory component oxidizes quinol or receives electrons directly from iLDH, the activity of antimycin-resistant quinol oxidase was determined. Several artificial quinones were assayed, but the two that yielded the higher rates were DBQ and duroquinol (Table 4). For comparison, the activity of antimycin resistant L-lactate oxidase was also measured in the same mitochondrial preparations. The activities were partially inhibited by cyanide, indicating that a fraction of electron flow from the oxidation of quinol reached cytochrome *c* oxidase, notwithstanding the complete blockade of cytochrome bc_1 complex by antimycin. The existence of quinol and L-lactate oxidase activities, in the presence of both antimycin and cyanide, suggests that a second alternative pathway branches from quinone.

Irradiation of mitochondria with ultraviolet light for 100 min, to destroy the quinone pool [30], diminished by 79 and 61% the activities of succinate and L-lactate oxidases, respectively. UV light also affected, but to a lower extent, the activity of quinone independent TMPD oxidase (38% inhibition). The activities of cytochrome *c* reductase, using L-lactate or succinate as electron donors, were also decreased by 20 and 40%, respectively, after UV irradiation; the bc_1 and iLDH activities decreased by 20 and 50%, respectively (data not shown).

Extraction of quinones from mitochondria with iso-octane [18] also decreased the activities of succinate and L-lactate oxidases by 60–90%; these were restored to control values by addition of 0.2 mM DBQone: the L-lactate oxidase activity was enhanced by 4.3 ± 0.5 times (3) and that of succinate oxidase by 2.8 ± 0.3 times (3) by DBQone. The value of stimulation of L-lactate oxidase by DBQone, when corrected for respiration due to endogenous substrates, was 8.4–10.5 times higher for iso-octane extracted mitochondria, and 1.18–1.53 times for control, lyophilized mitochondria. These data indicated an extensive, although incomplete, extraction of quinones. Antimycin (2 μM) strongly inhibited (81 ± 7%; 3) the stimulation of L-lactate oxidase by DBQone, while 20 μM myxothiazol only diminished the activity by 43% (2). The cytochrome *c* reductase activity, with L-lactate or succinate as electron donor, was also restored by added DBQone; these activities were 76 and 50% inhibited by 0.4 μM antimycin, respectively. The activities of succinate and L-lactate oxidases, cytochrome *c* reductase, and cytochrome bc_1 complex were higher in iso-octane treated than in control mitochondria, probably due to a limitation of natural quinones in the latter and saturation by artificial quinones in the former conditions.

Table 4
Antimycin-resistant quinol oxidases

Substrate	Activity (ng atoms oxygen/mg/min)	
		+ Cyanide
DBQ	13.7 ± 1.3 (8)	6.7 ± 1.7 (7)
Duroquinol	23.7 ± 2.8 (8)	15.8 ± 1.7 (7)
L-lactate	109 ± 13 (8)	33 ± 3.9 (8)

Mitochondria (1–1.5 mg protein/ml) were incubated at 30°C in 2 ml of standard medium, which also contained 0.8 μM antimycin (540–810 pmol/mg protein) and 1.25–2.5 mM dithiothreitol. After approximately 5–7 min (to exhaustion of endogenous substrates), 0.22 mM DBQone, 0.25 mM duroquinone or 10 mM L-lactate were added. Where indicated, 1 mM NaCN was present from the beginning of the incubation. The data shown are mean ± S.E.M. (*n*).

3.6. Cyanide-resistant pathway

In all our preparations the rate of respiration with L-lactate as substrate was always partially inhibited (85.4 ± 1.7%; 18) by 1 mM cyanide (or 10 mM azide). One micromolar antimycin, 0.1 mM HQNO or 0.5 μM stigmatellin did not further decrease the rate of cyanide-resistant respiration with 10 mM L-lactate as substrate. On the other hand, inhibitors of plant alternative respiratory pathway strongly inhibited the cyanide-resistant pathway, whereas myxothi-

Table 5
Cyanide-resistant pathway

Inhibitor	0.5 mM DPA	12.5 mM nPG	5 mM SHAM	0.05 mM disulfiram	10 mM azide	50 μM myxothiazol
Activity (% of control)	20 ± 3 (5)	17 ± 4 (4)	37 ± 4 (3)	21 (2)	59 (2)	56.5 (2)

Three milligram protein of mitochondria were incubated at 30°C in 2 ml of standard medium with 1 mM sodium cyanide and the indicated concentrations of the inhibitors. After 5–7 min, 10 mM L-lactate was added and the steady-state rate of 0_2 uptake was measured. The cyanide-resistant respiration in the absence of other inhibitors was 51 ± 8 (9) ng atoms oxygen/mg protein/min, which in turn was 17 ± 2 (9)% of the rate reached in the absence of cyanide. The data shown are mean ± S.E.M. (*n*).

azol and azide inhibited, but to a lower extent (Table 5). Catalase exerted a very small inhibitory effect of the cyanide-resistant respiration, with or without DPA or nPG (data not shown), indicating that this activity was not associated to the production of H_2O_2. Cyanide-resistant respiration was stimulated by 34 ± 5% (7) by 5 mM AMP; oligomycin (2.5 μM) and oleic acid (15–20 nmol/mg protein) partially suppressed AMP stimulation, whereas pyruvate (5 mM) or DTT (1.25 mM) did not induce any effect [31].

4. Discussion

The values of the P/O ratio with L-lactate or succinate indicated the involvement of two energy conservation respiratory sites, i.e., the cytochrome bc_1 complex and the cytochrome *c* oxidase. This is in agreement with the early proposal of Sharpless and Butow [1]. Thus, similarly to succinate oxidation, dehydrogenation of L-lactate produces reduction of the quinone pool. This is further supported by the drastic diminution of L-lactate oxidation and L-lactate dependent cytochrome *c* reduction in iso-octane treated mitochondria, and their restoration by addition of artificial oxidized quinone. In addition, the direct reduction of added Q_1 by iLDH through a reaction inhibited by oxalate, oxamate or sulfite (R. Jasso-Chávez et al., unpublished data), also illustrated their connection to the quinone pool.

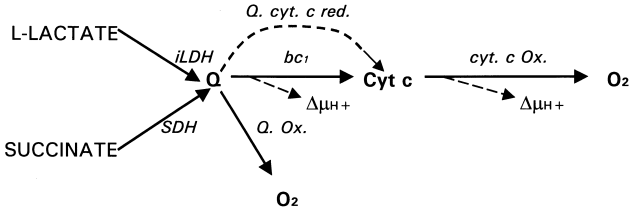
The partial inhibition of oxidative phosphorylation by antimycin indicated a branching for the oxidation of quinol, in which electrons flow through the cytochrome bc_1 complex, the reaction completely sensitive to antimycin, or through an alternative route, resistant to antimycin but still able to generate a H^+ gradient. The existence of an alternative respiratory component that catalyzes the same reaction like cytochrome bc_1 complex, by an antimycin-resistant process without the coupled pumping of H^+, was suggested by the following observations. (a) The diminished P/O ratio of 0.44 induced by the presence of antimycin (see also [1]); (b) the reduction of cytochrome *c* by dehydrogenation of L-lactate (or succinate) in the presence of antimycin; and (c) the total suppression of ATP synthesis by cyanide with similar IC_{50} values either in the presence or in the absence of antimycin. In consequence, L-lactate-dependent oxidative phosphorylation, in the presence of antimycin, would be supported by cytochrome *c* oxidase H^+ pumping activity. Cytochrome *c* reductase activity resistant to antimycin has also been reported in *Candida parapsilosis* mitochondria [32]. Antimycin-resistant electron transfer between NADH and cytochrome *c* was described for trypanosomatids [33,34] and *Euglena* mitochondria [1], although quinone involvement was not explored.

Myxothiazol was a weak inhibitor of the activity of cytochrome bc_1 complex as well as of the succinate-dependent activities of respiration, cytochrome *c* reductase and ATP synthesis. This pattern of inhibition by myxothiazol, together with the strong inhibition of the same activities by antimycin, further supported the notion that succinate was preferentially oxidized through the cytochrome bc_1 complex [4,10]. In contrast, the high sensitivity of L-lactate dependent activities to myxothiazol, and the lower inhibition by antimycin, indicated a preferential electron flow through the phosphorylating alternative branch, sensitive to myxothiazol. Stigmatellin was equally effective on both branches. The biphasic pattern of inhibition by myxothiazol (cf. Fig. 3; two slopes in a Dixon plot of [I] versus 1/L-lactate-cytochrome *c* oxidoreductase activity, not shown) suggested the presence of two components; one with high affinity, presumably the alternative quinol:cytochrome c oxidoreductase, and another with low af-

finity, identified as the cytochrome bc_1 complex, in which myxothiazol exerted a partial, mixed-type inhibition (R. Covián et al., unpublished data).

Likewise, the inhibition of the antimycin-resistant pathway from L-lactate to cytochrome *c*, by submicromolar concentrations of myxothiazol or stigmatellin, also indicated that there was a non-bc_1 quinol: cytochrome *c* oxidoreductase, since these two highly hydrophobic molecules interact with membrane-bound enzymes that use quinol or quinone as substrates, such as NADH dehydrogenase [35], bacterial quinol oxidases [36,37] and quinone reductases [38], in addition to cytochrome bc_1 complex [11].

On the basis of the results of the present study a model of the respiratory chain of *Euglena* mitochondria is proposed (Scheme 1). iLDH and succinate dehydrogenase catalyze the transfer of electrons from L-lactate or succinate to quinone; the cytochrome bc_1 complex transfers electrons from quinone to cytochrome *c* in an antimycin-sensitive reaction, which is coupled to H^+ pumping (and ATP synthesis). Cytochrome *c* oxidase (*cyt. c Ox.*) catalyzes the reduction of oxygen in a cyanide-sensitive reaction also coupled to H^+ pumping. There is also a cyanide-resistant quinol oxidase (*Q. Ox.*) that reduces oxygen in a reaction partially blocked by DPA, nPG, SHAM and disulfiram, and an alternative component with an antimycin resistant activity of quinol:cytochrome *c* oxidoreductase (*Q. cyt. c red.*), but sensitive to myxothiazol, stigmatellin and HQNO.



Scheme 1.

iLDHs from yeast mitochondria are soluble enzymes located in the intermembrane space that catalyze the electron transfer from lactate to cytochrome *c* [39], while bacterial iLDHs are membrane-bound enzymes of the respiratory chain that catalyze the electron transfer from lactate to the quinone pool [40,41]. Moreover, the respiratory chain of *Paracoccus denitrificans* has a D-lactate oxidase activity that is also resistant to antimycin [42], and is coupled to the generation of a H^+ gradient, apparently by the activity of a H^+ pumping terminal oxidase [43]. Hence, the involvement of quinones in the oxidation of L-lactate suggests that the *Euglena* iLDH is similar to that present in bacteria.

It is pointed out that some experimental results are not fully explained by the above described model. For instance, inhibition of TMPD oxidase by cyanide exhibits a biphasic pattern which has been interpreted as the presence of two oxidases with different sensitivity to cyanide. The component of high affinity (K_i for cyanide ranging from 1 to 10 μM) has been identified as cytochrome *c* oxidase, while the low affinity component (K_i of 40–100 μM) has been associated to an alternative oxidase [10,41]. However, a biphasic pattern of inhibition may also result from two states of the enzyme with different affinity for the inhibitor [44,45], as consequence of negative cooperativity in inhibitor binding, or from a partial or mixed-type inhibition [46]. Since the TMPD oxidase activity follows a Michaelis–Menten kinetics with respect to the TMPD concentration (i.e., only one component), with a K_m of 0.65 ± 0.06 (3) mM and V_{max} of 604 ± 20 (3) ng atoms oxygen/mg protein/min (data not shown), it is suggested that the biphasic kinetics of cyanide inhibition is the result of the presence of oxidized and reduced enzymes which have different cyanide affinities.

There was a low lactate-dependent ATP synthesis in the presence of high concentrations of myxothiazol (> 10 μM), although a H^+ gradient of a sufficient magnitude to support the process was available. However, at these concentrations, myxothiazol exerted inhibitory effects on other enzymes of the pathway like iLDH (15% inhibition) and ATP synthase ($14 \pm 2\%$; $n = 3$; activity measured as ATP hydrolysis in the presence of 1 μM CCCP).

We previously reported the ability of *Euglena* mitochondria to generate a H^+ gradient supported by L-lactate oxidation in the presence of 0.1 mM cyanide [4,5]. This observation is apparently at variance with the proposed model of the respiratory chain, in which there is only one cyanide-sensitive component (cytochrome *c* oxidase) and, hence, no H^+ gradient should be built up in the presence of cyanide. A low cyanide concentration was previously used, because

it was thought that such concentration could totally inhibit cytochrome *c* oxidase, while still allowing the observation of the low affinity component. However, 0.1 mM cyanide does not completely abolish TMPD oxidase and, therefore, nor respiration or ATP synthesis (see Figs. 1 and 2).

The lower activity of antimycin-resistant quinol oxidase, in comparison to that of antimycin-resistant L-lactate oxidase, could be due to a lower affinity for the artificial quinones used. The natural quinones found in the membranes of *Euglena* mitochondria are ubiquinone-9, traces of ubiquinone-8 and rhodoquinone-9 [47], a quinone with an amine group instead of methoxy in the position 2 of the aromatic ring [48]. This last quinone is abundant in organisms subjected to anoxic environments in which a fumarate reductase activity is increased [49]. Although its function in the respiratory chain of *Euglena* mitochondria is yet unknown, rhodoquinone might be a more specific substrate for the putative antimycin-resistant quinol/cytochrome *c* oxidoreductase, since DBQ and other artificial quinones prompted high rates of the cytochrome bc_1 complex, but they were unable to reconstitute fully the antimycin-resistant activity.

Acknowledgements

This work was partially supported by Grants 25274-M and 25465-M from CONACYT-México. The authors thank Dr. A. Gómez-Puyou for his suggestions and critical reading of the manuscript.

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