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

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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 bc1 complex activity was fully inhibited by antimycin, but it was resistant to myxothiazol. Stigmatellin inhibited both L-lactate-dependent cytochrome c reductase and cytochrome bc1 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. © 2000 Elsevier Science B.V. All rights reserved.

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 Ca2+ [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,
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 bc1 and aa3), 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 bc1 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 32P and 3H-tetr phenylphosphonium (3H–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 MgCl2, of pH 7.25. For ATP synthesis, mitochondria were incubated at 30°C in 1 ml of standard medium which also contained 32P (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 32P
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-free aqueous phase was used for determination of \(^{32}\)P, 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 \(O_2\) 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 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 \(\mu\)M oxidized horse heart cytochrome c. The reaction was started by addition of either 10 mM l-lactate, 10 mM succinate, or 60 \(\mu\)M decylbenzoquinol (DBQ), which was reduced according to Rieske [19]. 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.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Rate of ATP synthesis (nmol/min/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>l-lactate</td>
</tr>
<tr>
<td>None</td>
<td>205.5 ± 11 (12)</td>
</tr>
<tr>
<td>+1 mM NaCN</td>
<td>4.2 ± 1 (8)</td>
</tr>
<tr>
<td>+0.5–1 (\mu)M Antimycin</td>
<td>91 ± 13 (9)</td>
</tr>
<tr>
<td>+12.5 (\mu)M Oligomycin</td>
<td>2.5 ± 0.1 (5)</td>
</tr>
<tr>
<td>+2.5 (\mu)M CCCP</td>
<td>2.1 ± 0.5 (7)</td>
</tr>
</tbody>
</table>

*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 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.

2.6. Membrane potential

The distribution of \(^{3}\)H-TPP\(^{+}\) (0.06–0.07 \(\mu\)Ci/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
bc1 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/107 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 (data not shown). Determination of the antimycin concentration, cytochrome c reductase activity was also extensively inhibited by antimycin (Table 2), indicating that electron transfer from succinate flowed preferentially through the cytochrome bc1 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 bc1 complex.

Myxothiazol, another specific inhibitor of mammalian cytochrome bc1 complexes [11], exerted a weak effect on Euglena cytochrome bc1 activity using DBQ or succinate as electron donors. Cytochrome bc1 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 bc1 complex (antimycin-sensitive

### Table 2

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

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).

To explore the existence of a cytochrome bc1 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 bc1 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 bc1 complex activity.

Thus, with DBQ as artificial electron donor, the activity of cytochrome bc1 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 bc1 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 bc1 complex.

Myxothiazol, another specific inhibitor of mammalian cytochrome bc1 complexes [11], exerted a weak effect on Euglena cytochrome bc1 activity using DBQ or succinate as electron donors. Cytochrome bc1 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 bc1 complex (antimycin-sen-
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.

The larger inhibition of t-lactate oxidation (Fig. 1), complete suppression of t-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 t-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 t-lactate and succinate, respectively. The respiratory rates with the two substrates were diminished in 85–
90% by 100 μM myxothiazol; likewise, with both substrates the sensitivity of state 3 respiration to myxothiazol (Fig. 3) was similar (i.e., similar IC50 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 IC50 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

<table>
<thead>
<tr>
<th>State</th>
<th>ΔψH+ (mV)</th>
<th>L-Lactate</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>146.4±10 (8)</td>
<td>139.5±6 (8)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>117±7 (16)</td>
<td>104.6±12 (16)</td>
<td></td>
</tr>
<tr>
<td>3+0.4-0.6 nmol</td>
<td>94±7 (14)</td>
<td>87±7 (7)</td>
<td></td>
</tr>
<tr>
<td>3+2 mM CN&quot;</td>
<td>42±3 (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3+0.3-0.6 nmol stigmatellin/mg protein</td>
<td>48±8 (6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mitochondria (2.5–3.5 mg protein/ml) were incubated at 30°C in 0.5 ml of standard medium with 0.8 μM [3H]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 [3H]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 bc1 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 bc1 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 1.8–1.53 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 mM antimycin, respectively. The activities of succinate and l-lactate oxidases, cytochrome c reductase, and cytochrome bc1 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.

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 myxothia-

### Table 4

Antimycin-resistant quinol oxidases

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (ng atoms oxygen/mg/min)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>+ Cyanide</td>
</tr>
<tr>
<td>DBQ</td>
<td>13.7±1.3 (8) 6.7±1.7 (7)</td>
</tr>
<tr>
<td>Duroquinol</td>
<td>23.7±2.8 (8) 15.8±1.7 (7)</td>
</tr>
<tr>
<td>l-lactate</td>
<td>109±13 (8) 33±3.9 (8)</td>
</tr>
</tbody>
</table>

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).
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$_2$O$_2$. 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-

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>0.5 mM DPA</th>
<th>12.5 mM nPG</th>
<th>5 mM SHAM</th>
<th>0.05 mM dithiouram</th>
<th>10 mM azide</th>
<th>50 μM myxothiazol</th>
</tr>
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<tbody>
<tr>
<td>Activity (% of control)</td>
<td>20 ± 3 (5)</td>
<td>17 ± 4 (4)</td>
<td>37 ± 4 (3)</td>
<td>21 (2)</td>
<td>59 (2)</td>
<td>56.5 (2)</td>
</tr>
</tbody>
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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 O$_2$ 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).
finity, identified as the cytochrome $bc_1$ complex, in which myxothiazol exerted a partial, mixed-type inhibition (R. Covia‡n 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.](image)

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 $\delta$-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 $\mu$M) has been identified as cytochrome $c$ oxidase, while the low affinity component ($K_i$ of $40–100$ $\mu$M) 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 $0.65 \pm 0.06$ (3) mM and $V_{max}$ of 604 $\pm$ 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 ($>10$ $\mu$M), 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 $\mu$M 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.

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**References**


