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The Alternative Respiratory Pathway of *Euglena* Mitochondria

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Mitochondria, isolated from heterotrophic *Euglena gracilis*, have cyanide-resistant alternative oxidase (AOX) in their respiratory chain. Cells cultured under a variety of oxidative stress conditions (exposure to cyanide, cold, or H_2O_2) increased the AOX capacity in mitochondria and cells, although it was significant only under cold stress; AOX sensitivity to inhibitors was also increased by cold and cyanide stress. The value of AOX maximal activity reached 50% of total respiration below 20°C, whereas AOX full activity was only 10–30% of total respiration above 20°C. The optimum pH for AOX activity was 6.5 and for the cytochrome pathway was 7.3. GMP, AMP, pyruvate, or DTT did not alter AOX activity. The reduction level of the quinone pool was higher in mitochondria from cold-stressed than from control cells; furthermore, the content of reduced glutathione was lower in cold-stressed cells. Growth in the presence of an AOX inhibitor was not affected in control cells, whereas in cold-stressed cells, growth was diminished by 50%. Cyanide diminished growth in control cells by 50%, but in cold-stressed cells this inhibitor was ineffective. The data suggest that AOX activity is part of the cellular response to oxidative stress in *Euglena*.

KEY WORDS: Alternative oxidase inhibitors; cold stress; oxidative stress.

INTRODUCTION

Respiratory electron transfer in mitochondria from higher plants, fungi, and several protists is mediated by the phosphorylating cytochrome pathway and also by an alternative nonphosphorylating no-cytochrome pathway. The branching point between these two pathways is located at the ubiquinone pool level (Ito *et al*., 1997; Siedow and Umbach, 2000). The alternative pathway is resistant to antimycin and cyanide and, apparently, consists of only one enzyme, the alternative oxidase (AOX).

AOX is widespread in plants, protists, and fungi (Jarmunszkiewics *et al*., 1997; Joseph-Horne *et al*., 1998) as shown by the comparison of amino acid sequences (Day *et al*., 1995), and the cross-reactivity with an antibody raised against *Sauromatum guttatum* AOX protein (Elthon *et al*., 1989). The dimeric plant AOX is regulated by α-keto acids and by the redox-state of essential SH-groups. In contrast, fungi and protists have the active AOX as a monomeric protein regulated by purine mononucleotides (AMP or GMP) (Milani *et al*., 2001; Vanderleyden *et al*., 1980). Inhibitors of both dimeric and monomeric AOX are diphenylamine, salicylhydroxamic acid (SHAM), alkyl gallates, and disulfiram (Murphy *et al*., 1997; Siedow and Girvin, 1980; Schonbaum *et al*., 1971).

A respiratory activity resistant to antimycin and cyanide has also been described in mitochondria of the free-living protist *Euglena gracilis* (Devars *et al*., 1992; Sharpless and Butow, 1970a,b). A complex respiratory chain has been proposed for *E. gracilis*, in which not one but two additional alternative components participate in electron transfer (Moreno-Sánchez *et al*., 2000). Diphenylamine (Sharpless and Butow, 1970a), SHAM, *n*-propyl gallate (*n*PG) and disulfiram (Moreno-Sánchez *et al*., 2000) block one of these components, AOX; the other component is an antimycin-resistant quinol-cytochrome

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c oxidoreductase (Moreno-Sánchez *et al*., 2000). In regard to the entry of reduced equivalents, stereo-specific NAD^+-independent lactate dehydrogenases that donate electrons directly to the quinone pool have been described (Jasso-Chávez *et al*., 2001).

Stimulation of AOX activity by AMP is attained in mitochondria from *E. gracilis* cells cultured with succinate (Sharpless and Butow, 1970a), but not from cells cultured with glutamate plus malate as carbon source (Moreno-Sánchez *et al*., 2000; Sharpless and Butow, 1970a,b). An increase in AOX activity is achieved when cells are grown in the presence of antimycin (Sharpless and Butow, 1970a) or with ethanol as carbon source (Devars *et al*., 1998). These observations indicate that AOX in *E. gracilis* might be modulated by changing the culture conditions, i.e., by subjecting cells to a variety of stress conditions. In this regard, it is well documented that AOX activity in plants increases with cold (Stewart *et al*., 1990) or oxidative stress (Wagner, 1995).

The role of *Euglena* AOX in the physiology of the cell is unknown. Moreover, a full description of its biochemical features remains to be made. Therefore, in the present work we explored different stress culture conditions that might induce a higher AOX capacity; glutamate plus malate was used as carbon source since a higher biomass is obtained. In addition, to advance in the understanding of the AOX functioning in *E. gracilis* mitochondria, substrate dependence and inhibitor, oxygen, pH, and temperature sensitivity were also examined. The role of the second alternative component, the antimycin-resistant quinol-cytochrome *c* oxidoreductase was not explored; its participation in electron flux was merged with that of the cytochrome pathway.

MATERIAL AND METHODS

Chemicals

Salicylhydroxamic acid (SHAM), D- and L-lactic acid, *N*,*N*,*N*,*N*,tetramethyl-*p*-phenylendiamine (TMPD), disulfiram, 1,6-dichlorophenol indophenol (DCPIP), fatty acid-free bovine serum albumin, and decylbenzoquinone (DBQ) were purchased from Sigma. Sodium cyanide and sodium dithionite were from J. T. Baker; sodium azide, *n*-propyl gallate (*n*PG), and horse heart cytochrome *c* were from ICN, and diphenylamine (DPA) from Aldrich.

Cell Culture and Preparation of Mitochondria

Euglena gracilis Z, kept in the dark in liquid medium for several months, was reactivated and axenically grown in the dark with glutamate + malate as carbon source at 25 ± 2°C under orbital shaking (125 rpm) as previously described (Moreno-Sánchez and Raya, 1987). Additionally, cells were cultured for 72 h at 25°C and then either transferred at 4°C for 15 h or the medium was supplemented with H_2O_2. Cells were also cultured for 96 h at 25°C in the presence of 0.3 mM sodium cyanide added at the beginning of the culture. Cells were harvested by centrifuging at $1000 \times g$ for 10 min at 4°C and washing once in SHE medium [250 mM sucrose, 10 mM HEPES (4-(-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*,*N*-tetraacetic acid), pH 7.3].

A cellular suspension of approximately 2×10^9 cells in 25 mL was sonicated as previously reported (Moreno-Sánchez and Raya, 1987) with one modification. The sonication time with a probe tip of 0.5 cm diameter was 7 s four times, with 1 min rest at 50–60% of maximal output in a Branson sonicator (Danbury, CT, USA). This modification yielded mitochondrial preparations with higher respiratory, including AOX, and dehydrogenase activities (30%, approximately). Mitochondrial protein was determined by the Biuret method as described previously (Uribe and Moreno-Sánchez, 1992).

Oxygen Uptake

The respiration of *E. gracilis* mitochondria was measured at 15–30°C, with a Clark-type oxygen electrode, in an air-saturated standard medium that contained 120 mM KCl, 20 mM MOPS (3-[*N*-morpholino]propanesulfonic acid), 1 mM EGTA (KME buffer), 5 mM phosphate at pH 7.3. The oxygen solubility was determined to be 533 and 400 ng atoms/mL at 15 and 30°C, respectively.

To determine the O_2 affinity of the TMPD (cytochrome *c*) oxidase, the dissolved O_2 in the KME buffer was diminished by titrating with a freshly prepared solution of dithionite. For AOX O_2 affinity, N_2-bubbled buffer was used to adjust the O_2 concentration; dithionite reaction products, particularly sulfite strongly inhibited lactate oxidation.

Dehydrogenase Activities

These were determined as previously described (Jasso-Chávez *et al*., 2001) at room temperature in 1 mL of KME buffer, 0.3 mM DCPIP, and 0.05–0.1 mg of mitochondrial protein. Succinate dehydrogenase (SDH) activity was not further increased by adding phenazine

methasulfate (0.4 mM) or Triton X-100 (0.05%), which by-pass the membrane barrier for electron transfer from the external phase.

Cytochrome *c* Reductase Activity

Mitochondria (0.1 mg protein) were incubated in 1 mL of SHE medium with 1 mM ADP and 30 μM oxidized horse heart cytochrome *c*. Adding D-lactate, L-lactate, succinate, or DBQH started the reaction. Reduction of DBQ with sodium borohydride and isolation of reduced DBQ (DBQH) with cyclopentane was made as described elsewhere (Trumpower and Edwards, 1979). The rate of cytochrome *c* reduction at 25°C was followed by the increase in the absorbance difference at 550 *minus* 540 nm in a dual wavelength SLM-Aminco DW-2000 spectrophotometer (Urbana, IL, USA); an extinction coefficient of 19.1 mM^{-1} cm^{-1} was used in the calculations (Degli Esposti and Lenaz, 1991).

Quinone Content

Extraction of quinones with isooctane from lyophilized mitochondria was made according to the procedure described by Ding *et al*. (1992). The quinones were separated and identified by reverse-phase HPLC according to Wagner and Wagner (1995). A Waters C18 Spherisorb S5 ODS2 analytical column (4.6 × 150 mm, Waters PSS 831913) was equilibrated with nitrogen-purged ethanol/methanol (3:2 v/v), and this mixture was used as the mobile phase. The flow rate was 0.5 mL/min. Detection of the quinones was performed at 275 nm. The chromatograms showed mainly the UQ-9 and RQ-9 peaks, indicating the almost complete oxidation of the quinones by this procedure. To determine the redox-state of the mitochondrial quinones under steady-state conditions, rapid extraction with petroleum ether was used (Wagner and Wagner, 1995). Quantification of quinones was estimated from the areas under the curve of the corresponding peaks using commercial Q_9 as standard. For rhodoquinone-9 identification, thin layer chromatography (TLC) and infrared spectroscopy were also used.

Cytochrome Content

Mitochondria were firstly incubated in 50% (v/v) glycerol, 60 mM KCl, 10 mM MOPS, and 0.25 mM EGTA, pH 7.3 for 30 min at 25°C under orbital shaking to induce full oxidation of endogenous substrates. The baseline (oxidized *minus* oxidized) was recorded after the mitochondrial samples were further agitated and kept under 100% O_2 gassing during the acquisition of the spectrum. The content of $c + c_1$ cytochromes was determined from ascorbate-reduced *minus* oxidized spectra; *b*-type cytochrome was estimated from the dithionite-reduced *minus* ascorbate-reduced spectra; and cytochrome $a + a_3$ from dithionite-reduced *minus* oxidized spectra. Spectra were taken in an SLM-Aminco DW2000 spectrophotometer with the bandwidth set at 2 nm and a scanning speed of 2 nm s^{-1}. The content of cytochromes $a + a_3$, b, and $c + c_1$ was estimated by using the extinction coefficients of 16 mM^{-1} cm^{-1}, 25.6 mM^{-1} cm^{-1}, and 17.5 mM^{-1} cm^{-1}, respectively (Degli Esposti and Lenaz, 1991; Gray *et al*., 1994; Priest and Hajduk, 1992).

Fatty Acids and Glutathione Content

Extraction, trans-esterification of phospholipids and derivatization of total fatty acids to methyl-esters was made as described elsewhere (Jasso-Chávez *et al*., 2002). The content of reduced glutathione in cellular extracts was determined by HPLC as previously described (Mendoza-Cózatl *et al*., 2002).

RESULTS

Growth Under Stress Conditions

Culture of *E. gracilis* in the presence of 0.3 mM cyanide diminished the rate of cell growth and lowered the cell density reached in the stationary phase (Fig. 1). The change in the incubation temperature from 25 to 4°C after 72 h of culture also lowered the growth rate and the cell density reached in the stationary phase (Fig. 1). Cells exposed to 4°C from the beginning of the culture were unable to reach the stationary phase after 7 days (data not shown). The exposure to 2 mM H_2O_2 after 72 h of incubation stopped cell growth; addition of 10 mM H_2O_2 induced cell death within the next 2 h (data not shown).

Oxidation and Dehydrogenation Rates in Isolated Mitochondria

Mitochondria isolated from cells grown in the presence of cyanide (cyanide mitochondria) showed diminished rates of L-lactate oxidation (Table I) and dehydrogenation (Table II), whereas the respective succinate-dependent reactions were unaltered. Respiratory control

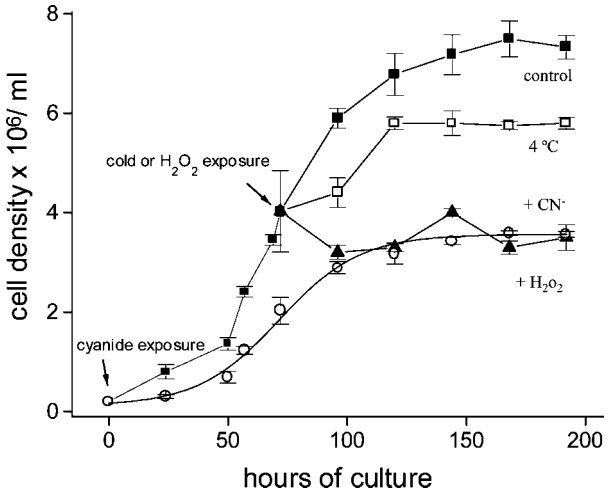

Fig. 1. Effect of stress conditions on growth of *Euglena*. The cultures were started by addition of an inoculum of 2×10^5 cells/mL in a control medium without cyanide (■) or with 0.3 mM cyanide (○). Cold (□) and 2 mM H_2O_2 (▲) stress were undertaken on control cells after 72 h of incubation. Viability was higher than 95% in all cultures.

values (rate of ADP-stimulated respiration/rate of basal respiration) were similar (1.6–1.8) in all types of mitochondrial preparations with L-lactate as substrate. With D-lactate, the rate of dehydrogenation also diminished in cyanide mitochondria, although the rate of oxidation was not affected. In mitochondria from cold-exposed cells (cold mitochondria) only L-lactate oxidation diminished, whereas succinate and TMPD oxidases, and D-iLDH and SDH activities increased. Mitochondria from H_2O_2-exposed cells (peroxide mitochondria) showed lower rates of L- and D-lactate oxidation, but similar rates of dehydrogenation, to those attained in control mitochondria; TMPD oxidase and SDH activities also decreased in peroxide mitochondria. The activity of cytochrome bc_1 complex measured directly with an artificial quinone (DBQH) (Table II) or associated with substrate dehydrogenation (not shown) also diminished in the three types of stressed-mitochondria. It should be noted that both SDH and cytochrome bc_1 activities were lower than the corresponding oxidases, indicating a suboptimal estimate of these enzyme activities. In turn, the iLDH activities fully accounted for the measured oxidase activities.

The rate of cyanide-resistant respiration in control mitochondria amounted to only 13% of total respiration with L-lactate (Table I) and was similar to previously reported data (Moreno-Sánchez *et al*., 2000). In percentage values the cyanide-resistant respiration was higher with succinate. However, it should be noted that the absolute values of cyanide-resistant respiration with either substrate were rather similar. Cyanide-resistant respiration in the other types of mitochondria followed a similar pattern to that observed in control mitochondria, except for peroxide mitochondria in which D-lactate and succinate-supported cyanide-resistant respiration were severely diminished (Table I).

Interestingly, culturing under cyanide, peroxide, or cold stress induced an enhanced cyanide-resistant respiration in isolated mitochondria with L-lactate as substrate, reaching a value of 29–34% of total respiration (Table I). In absolute rates, a significantly higher AOX capacity was attained in cold mitochondria with L-lactate or succinate.

Table I. Substrate Oxidation in *Euglena gracilis* Mitochondria

Substrate	Control mitochondria	Cyanide mitochondria	Cold mitochondria	Peroxide mitochondria
		ng atoms oxygen min^{-1} (mg protein)$^{-1}$		
L-lactate	215 ± 15 (3)	134 ± 9 (3)[a]	174 ± 33 (8)[b]	109 ± 12 (3)[a]
D-lactate	260 ± 16 (3)	253 ± 6 (3) ns	297 ± 56 (8) ns	215 ± 24 (3)[b]
Succinate	63 ± 5 (3)	52 (2)	126 ± 30 (8)[a]	69 ± 7 (3) ns
TMPD(ascorbate	410 ± 73 (4)	264 ± 98 (3) ns	566 ± 85 (3)[b]	238 ± 22 (3)[c]
L-lactate + CN^-	28 ± 16 (3)	39 ± 10 (3) ns	50 ± 14 (8)[d]	38 ± 4 (3) ns
D-lactate + CN^-	43 ± 10 (3)	25 ± 6 (3)[b]	44 ± 15 (8) ns	19 ± 3 (3)[c]
Succinate + CN^-	34 ± 8 (3)	23 ± 8 (3) ns	58 ± 19 (6)[b]	11 ± 3 (3)[a]
TMPD-Asc + CN^-	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)	0 ± 0 (3)

Note. Values are mean ± SD. ns = not significantly different from control. Student *t* test for nonpaired samples. (1–2 mg protein -mL) isolated from control, 0.3 mM NaCN, 4°C or 2 mM H_2O_2 exposed were incubated in KME buffer +5 mM potassium phosphate of pH 7.3 at 30°C. L-lactate, D-lactate, and succinate were added at a final concentration of 10 mM, TMPD was 2 mM (+5 mM ascorbate, Asc). 1 mM NaCN was added after 4-min incubation.

[a] $P < 0.005$ versus control.
[b] $P < 0.05$ versus control.
[c] $P < 0.025$ versus control.
[d] $P < 0.01$ versus control.

Table II. Dehydrogenase and Cytochrome *c* Reductase Activities in *E. gracilis* Mitochondria

	Control mitochondria	Cyanide mitochondria	Cold mitochondria	Peroxide mitochondria
	nmol DCPIP red min^{-1} (mg protein)$^{-1}$			
L-$_i$LDH	258 ± 70 (5)	102 ± 40 (3)[a]	353 ± 86 (5) ns	236 ± 74 (3) ns
D-$_i$LDH	407 ± 117 (4)	209 ± 85 (3)[b]	705 ± 251 (5)[b]	385 ± 81 (3) ns
SDH	33 ± 14 (4)	27 ± 13 (3) ns	113 ± 18 (5)[c]	11 ± 3 (3)[b]
	nmol cyt *c* red min^{-1} (mg protein)$^{-1}$			
Cyt *c* reductase	96 ± 9 (5)	61 ± 7 (3)[c]	50 (2)	52 (2)

Note. Values are mean ± SD. ns = not significantly different from control. Mitochondria +0.1 mg protein /mL) were incubated at 25°C with KME buffer plus 0.3 mM DCPIP. The rates of L-$_i$LDH, D-$_i$LDH, and SDH were determined after addition of 10 mM L-lactate, 10 mM D-lactate, or 10 mM succinate, respectively. The rate of cytochrome *c* reductase was started by addition of 10 mM L-lactate in the presence of 30μM oxidized cytochrome *c*.
[a] $P < 0.025$ versus control.
[b] $P < 0.05$ versus control.
[c] $P < 0.005$ versus control.

It should be noted that inhibition of the cytochrome pathway with cyanide to fully reveal the AOX pathway does not allow for determination of the contribution to total respiration of either pathway, since inhibition of one pathway promotes the activity of the other (Day *et al*., 1996). Culturing for 40 h at 4°C yielded similar results to those achieved with 15 h of cold exposure.

In agreement with data from isolated mitochondria, AOX capacity was 26 (n = 2) and 62 ± 13 (n = 3) ng atoms oxygen $(10^7$ cells$)^{-1}$ min^{-1} in intact control and cold-stressed cells, respectively. After correcting for the O_2 uptake remaining in the presence of 3 mM *n*PG (and 1 mM cyanide), the AOX capacity in control and cold-stressed cells was 18.5 and 32 ± 3% of total cellular respiration, respectively. Moreover, the rate of cyanide-resistant quinol (50 μM Q_1) oxidase was 21.6 ± 2 (n = 5) and 33 ± 2 ng atoms oxygen (mg protein)$^{-1}$ min^{-1} (n = 5) in control and cold mitochondria at 30°C, respectively. At lower temperature (15°C), the cyanide-resistant Q_1 oxidation rates were 27 (2) and 46.6 (2) ng atoms oxygen (mg protein)$^{-1}$ min^{-1} in control and cold mitochondria, respectively. Thus, Q_1-dependent AOX capacity was 34 and 45% at 30°C, and 40 and 52% at 15°C of total quinol oxidase. The significant difference between these values ($P < 0.005$) suggested an increased AOX activity under cold stress.

Kinetic Properties of AOX

The temperature dependence of the respiratory activity showed that the AOX capacity remained constant throughout the range studied, whereas the cytochrome pathway diminished drastically with several substrates (Fig. 2(A)). In consequence, the contribution of each pathway to the total electron flux, in control mitochondria, is expected to vary in opposite directions with the lowering in the temperature; at 15°C both pathways showed identical maximal rates (Fig. 2(B)). In mitochondria from cold-stressed cells, the AOX pathway would appear to predominate from 10 to 20°C (Fig. 2(B)). The rate of total respiration decreased by 74, 71, and 27% at 15°C, in comparison to 30°C, in control, cyanide, and cold mitochondria, respectively (Table III). However, the rate of cyanide-resistant respiration at 15°C was similar to that attained at 30°C in control and cold mitochondria (Table III), suggesting an enhanced AOX contribution to total respiration at low temperatures.

The pH dependence of the respiratory activity at 30°C showed that the AOX capacity exhibited a maximum at a pH value of 6.55, whereas the cytochrome oxidase pathway maximum was 7.3 (Fig. 2(C)). Essentially identical pH optimal values were attained at 15°C for AOX and cytochrome pathway (not shown). These data are similar to those reported for *Acantamoeba castellani* mitochondria (Jarmuszkiewicz *et al*., 2002) in which the maximal respiratory activities were attained at pH values of 6.8 and 7.4 for the alternative and cytochrome pathway, respectively.

The TMPD oxidase exhibited a 20-fold higher affinity for oxygen than AOX in control mitochondria (Fig. 2(D); Table IV). Cold-stress slightly increased the AOX affinity for O_2 and induced a significant increase in the K_m value for oxygen of TMPD oxidase (Table IV). The K_m values for O_2 in control mitochondria were in the same range of values reported for both pathways in plant mitochondria (Ribas-Carbo *et al*., 1994; Sluse and Jarmuszkiewicz, 1998).

Titration with different AOX inhibitors showed full blockade of cyanide-resistant respiration at 15 and 30°C. The sensitivity of AOX activity towards SHAM and *n*PG was much lower in control *Euglena* mitochondria than in

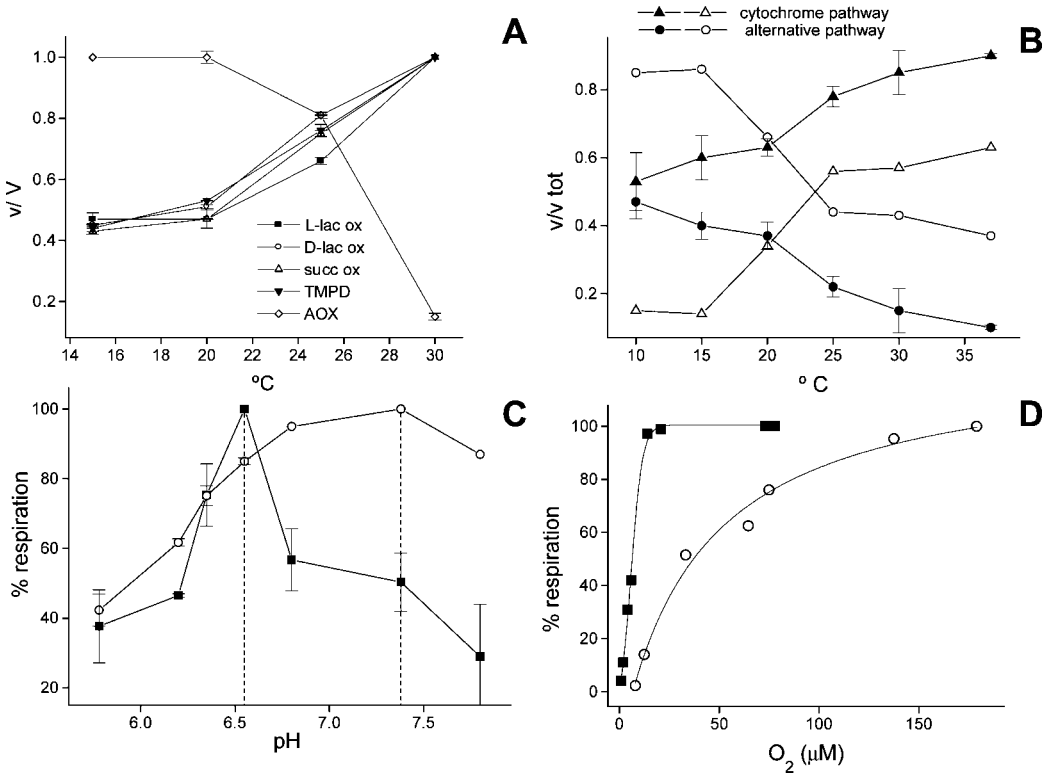

Fig. 2. Temperature, pH, and O_2 concentration dependence of the cytochrome and alternative respiratory pathways. Mitochondria (2 mg protein/mL) isolated from control (A, B) and cold-stressed cells (B) were incubated in KME buffer + 5 mM potassium phosphate, pH 7.3. After 2 min, 10 mM L-lactate (■), 10 mM D-lactate (○), 10 mM succinate (△), or 2 mM TMPD (+ 5 mM ascorbate) (▼) were added. NaCN (1 mM) was added 4 min later to mitochondria respiring with L-lactate (◇) to determine the AOX capacity. (B) The rates of respiration (v) of control (closed symbols) and cold mitochondria (open symbols) were normalized with respect to the maximal rates attained at 30°C (V, whose values are shown in Table I). (C) Control mitochondria were also incubated at 30°C at the indicated pH values. The incubation medium was as described above except that 20 mM MOPS was replaced by 10 mM MES + 10 mM HEPES + 10 mM MOPS + 10 mM Tris. The oxidizable substrate was 10 mM L-lactate and 1 mM NaCN was used to determine the AOX capacity (■). The rate of cyanide-resistant respiration was 41 ± 5 at pH 6.55. The rate of the cytochrome pathway (○) was estimated from the total rate of respiration *minus* the rate of cyanide-resistant respiration. (D) Incubation medium of pH 7.2 and thermo-stated at 30°C was either titrated with sodium dithionite (TMPD oxidase, ■), or flushed with a N_2 stream (AOX, ○), to diminish the concentration of soluble O_2. Mitochondria were then added to these media with different O_2 concentration which also contained 2 mM TMPD + 5 mM ascorbate or 10 mM L-lactate + 1 mM cyanide (AOX); the solid lines represent the best fit to the Michaelis–Menten equation.

Table III. L-Lactate Oxidation at 15°C

Substrate	Control mitochondria	Cyanide mitochondria	Cold mitochondria
	ng atoms oxygen min^{-1}(mg protein)$^{-1}$		
L-lactate	55.7 ± 8.1 (4)	38.5 ± 3.6 (3)[a]	127.4 ± 15.2 (3)[b]
L-lactate + CN^-	24.1 ± 13 (4)	20 ± 4.4 (3) ns	53 ± 11.2 (3)[a]

Note. Values are mean ± SD. ns = not significantly different from control. Student t test for nonpaired samples. Mitochondria (1-2 mg protein /mL) isolated from control, 0.3 mM NaCN or 4°C exposed were incubated in KME buffer +5 mM potassium phosphate at 15°C. L-lactate was added at a final concentration of 10 mM. 1 mM NaCN was added after 4-min incubation.
[a] $P < 0.025$ versus control.
[b] $P < 0.005$ versus control.

Table IV. AOX Affinity to O_2 and Sensitivity to Inhibitors

Inhibitor	Control mitochondria	Cyanide mitochondria	Cold mitochondria	Reference
		$K_{0.5}$ for oxygen (μM)		
TMPDox	6.1 ± 2.6 (3)	140 ± 37 (3)[a]	90 ± 50 (4)[a]	
AOX	92.5 ± 14.8 (3)	nd	38.7 ± 1.1 (3)	
		IC_{50} (mM)		
SHAM				
15°C	0.2 ± 0.05 (3)	0.27 ± 0.06 (3)	0.45 (1)	
30°C	2.1 ± 0.3	0.29 (2)	0.3 ± 0.06 (4)[a]	0.26[b]
*n*PG				
15°C	0.5 ± 0.1 (3)	0.2 (2)	0.56 (1)	
30°C	0.8 ± 0.1	0.23 ± 0.05 (3)[a]	0.05 ± 0.008 (3)[a]	0.005[c]

Note. Values are mean ± SD. ns = not significantly different from control. The K_m for oxygen of TMPD oxidase and AOX was estimated from plots such as that shown in Fig. 3(D). IC_{50}, inhibitor concentration required to reach 50% inhibition of cyanide-resistant respiration, was measured as indicated in Fig. 4. Rates of AOX respiration are in Table I.

[a] $P < 0.005$ versus control.

[b] Skunk cabbage mitochondria[12].

[c] Mung bean mitochondria[11].

plant (Schonbaum *et al*., 1971; Siedow and Girvin, 1980), *Plasmodium* (Murphy *et al*., 1997), or in cyanide- and cold *Euglena* mitochondria (Table IV).

Stimulation of AOX activity by AMP, ADP, or GMP has been reported for several organisms (Milani *et al*., 2001; Vanderleyden *et al*., 1980). In *Euglena* mitochondria isolated from cells grown with succinate as carbon source, stimulation of succinate oxidase by AMP has been described (Sharpless and Butow, 1970a). However, in mitochondria isolated from cells grown with glutamate-malate, the AMP stimulation is negligible (Sharpless and Butow, 1970a; Moreno-Sánchez *et al*., 2000). AMP, ADP, GMP (0.5– 5 mM), as well as pyruvate (5 mM), or pretreatment with DTT (1 mM) did not affect the AOX activity in control, cyanide- and cold-mitochondria (data not shown).

Quinone, Cytochrome, Fatty Acid, and AOX Content

The HPLC analysis of mitochondrial extracts (Fig. 3(A)) revealed the presence of ubiquinone-9 (UQ-9) and rhodoquinone-9 (RQ-9), in agreement with previous reports (Powls and Hemming, 1966; Threlfall, 1972). Growth under stress conditions prompted an increase in the content of both quinones, being higher under cyanide stress (Table V). In plant tissues stored at 4°C, an increase in the total mitochondrial ubiquinone content is also found (Wagner and Purvis, 1998). Although the RQ-9/UQ-9 ratio varied from 1.3 in control mitochondria to 1.92 and 0.75 in cyanide- and cold-mitochondria, respectively, a similar lack of correlation between the rate of AOX and the contents of either UQ-9 or RQ-9 was observed (Fig. 3(B)).

The percentage reduction of the UQ-9 pool was 24.5 and 37% for control- and cold-mitochondria respiring at 30°C with L-lactate, respectively. Variation in the redox-state of the RQ-9 pool was not explored. Addition of cyanide + *n*PG prompted full UQ-9 reduction (>90%) in both types of mitochondria. Addition of cyanide alone induced full reduction of the ubiquinone pool in control mitochondria, whereas in cold mitochondria reduction was only 60.5%. In contrast, addition of *n*PG promoted 43 and 90% ubiquinone reduction in control and cold mitochondria, respectively.

Evaluation of cytochrome content in the different mitochondrial preparations showed that *a*-type cytochrome in cyanide-exposed cells diminished from 122 in control to 84 pmol (mg protein)$^{-1}$. No differences were observed between control and cold mitochondria, which were in agreement with the TMPD oxidase activities (see Table I). There were no other differences between control and cyanide- or cold-exposed cells: *b*-type and $c + c1$ cytochrome contents were 42–58 and 86–100 pmol (mg protein)$^{-1}$ for control, cyanide, and cold mitochondria.

The analysis of the fatty acid composition of the mitochondrial membranes revealed that there was a significant increase in total fatty acids induced by the exposure to stress conditions (Table VI). All the fatty acid species increased, being the polyunsaturated fatty acid (PUFA) fraction the most abundant. The unusual fatty acid $\Delta^{7,10}$ hexadecenoic acid (C16:2n-6) found in a significant amount (3–10% of total) in the plasma membranes of *Euglena*

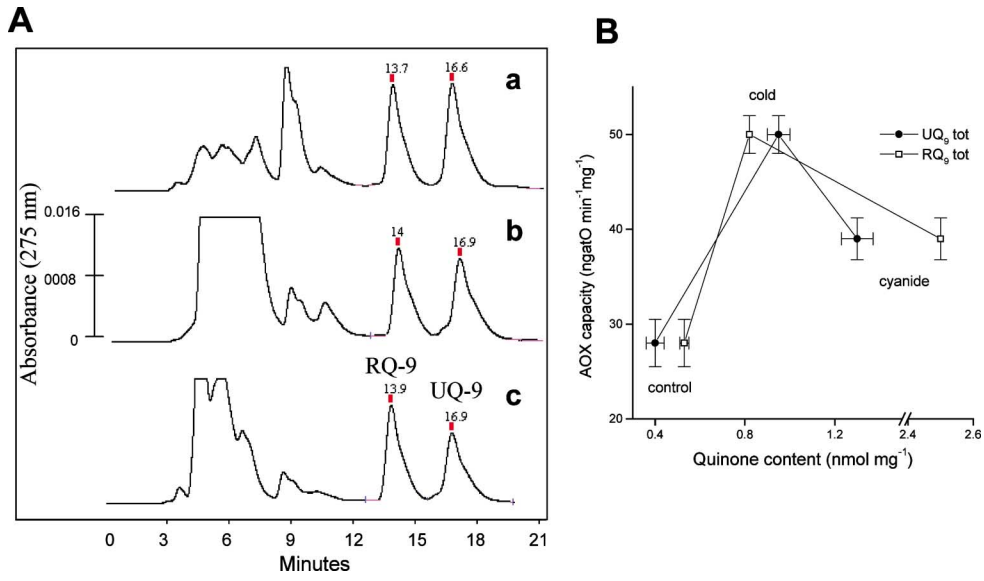

Fig. 3. (A) Quinone HPLC representative profile of *E. gracilis* mitochondria. Extraction of quinones from mitochondria (50 mg protein) was made as described in the Methods section. Mitochondria were isolated from control (a), subjected to cold stress (b), and cyanide exposed cells (c). The extract samples were 1:10, 1:20, and 1:50 diluted, respectively, and an aliquot of 50 μL was applied to the HPLC apparatus. (B) Relationship between ubiquinone and rhodoquinone content with AOX capacity in control, cyanide, and cold mitochondria. The oxidizable substrate was L-lactate. The data are presented as mean ± SD, except for the values in the *y*-axis, which represent mean ± SE for clarity purposes.

(Jasso-Chávez *et al*., 2002) was not detected in the mitochondrial membranes.

Western blot analysis of *Euglena* mitochondria with antibodies raised against AOX from either *Sauromatum guttatum* (Elthon *et al*., 1989) or *Chlamydomonas reinhardtii* (Nakamoto, 2001) revealed the presence of an increased content of a 38 kDa protein, the putative AOX, in cold mitochondria (not shown).

Physiological Role of AOX

To assess the physiological relevance of an enhanced AOX capacity, cells grown under stress conditions were further exposed to additional stimuli. Thus, cells exposed to cold stress (see Fig. 1) were harvested and further cultured in a medium that contained cyanide. The cellular density reached after 150–200 h (stationary phase) was significantly higher than that attained by control cells grown with cyanide (Fig. 4(A)). In another set of experiments, addition of *n*PG to the culture medium slightly modified the cellular density reached by control cells (approximately 15% inhibition between 120 and 200 h of culture). In contrast, *n*PG induced a marked (45%) diminution in the cellular density achieved by cold-stressed cells (Fig. 4(B)). The glutathione content in control and cold-exposed cells after 96 h culture was 4 ± 1 and 1.46 ± 0.2 nmol/(1×10^7 cells) (n = 3), respectively; the cysteine content was similar in both cellular types (0.3 nmol/(1×10^7cells).

Table V. Quinone Content in *Euglena* Mitochondria

	Control mitochondria	Cyanide mitochondria	Cold mitochondria
nmol (mg protein)$^{-1}$			
RQ_9	0.53 ± 0.02(3)	2.5 ± 0.1 (3)[a]	0.82 ± 0.2 (3)[b]
Q_9	0.4 ± 0.04 (3)	1.3 ± 0.07 (3)[a]	0.95 ± 0.05 (3)[a]

Note. Values are mean ± SD. Mitochondrial extracts (50 mg protein) were subjected to HPLC as shown in Fig. 2. Commercial Q_9 was used as standard.
[a] $P < 0.005$ versus control.
[b] $P < 0.05$ versus control.

DISCUSSION

Kinetic Properties of AOX

The *Euglena* AOX sensitivity towards three different specific inhibitors, which was similar to that observed in plant and *Plasmodium* AOX, suggests a close structural resemblance with AOX from taxonomically diverse species, at least in the binding site domain. However, the

Table VI. Fatty Acid Composition of *Euglena* Mitochondrial Membranes

	Content (μg/mg protein)		
Fatty acid	Control mitochondria	Cyanide mitochondria	Cold mitochondria
C16:0	4.3 ± 0.8	10.8 ± 3.8	14.7 ± 5.6
C16:1n-7	0.9 ± 0.3	1.6 ± 0.3	3 ± 1.5
C18:0	1.1 ± 0.6	3 ± 2.2	3.4 ± 0.6
C18:1n-9	1.5 ± 0.6	2 ± 1.7	5.7 ± 2.6
C18:2n-6	0.6 ± 0.4	1.1 ± 0.7	2.5 ± 1.7
C18:3n-6	0.4 ± 0.1(7)	0.93 ± 0.1	3 ± 1.2 (6)
C18:2n-3	0.4 ± 0.1	1.2 ± 0.6	1.6 ± 0.5
C20:0	0.3 ± 0.1 (6)	0.93 ± 0.06	1.6 ± 0.5 (6)
C20:1n-9	0.3 ± 0.1 (7)	0.6 ± 0.2	0.8 ± 0.2 (6)
C20:2n-6	1.3 ± 0.4	3.6 ± 1.6	3.6 ± 1.8
C20:3n-6	0.9 ± 0.2	3 ± 0.6	3.4 ± 1.3
C20:4n-6	7.1 ± 1.5	13.7 ± 2	17.8 ± 5.6
C20:5n-3	5.5 ± 2.1	15.7 ± 4	13.5 ± 7.6
C22:4n-6	4.5 ± 2	4.5 ± 0.8	10.3 ± 6.3
C22:5n-3	0.3 ± 0.2 (5)	1.5 ± 0.2	1 ± 0.5 (8)
C22:6n-3	5.3 ± 2	12.3 ± 3	13.2 ± 7.8
SFA	5.8 ± 1.3	14.5 ± 5.4	19 ± 6.6
MUFA	2.6 ± 0.5	4.3 ± 1.5	8.3 ± 3.5
PUFA	26.5 ± 5.5	57.5 ± 9.5	68 ± 29
TOTAL	35 ± 5.5	77.8 ± 15.7	96.5± 39

Note. Values in control, cyanide, and cold mitochondria are mean ± SD of 8, 4, and 11 independent determinations, respectively, except where indicated otherwise. SFA, MUFA, and PUFA are saturated, monounsaturated, and polyunsaturated fatty acids, respectively.

lack of effect of α-keto acids, DTT, and purine nucleotide monophosphates on *Euglena* AOX activity clearly indicates significant differences in the regulatory mechanisms with respect to other reported AOX.

The current structural model of AOX proposes an integral protein tightly bound to the inner membrane (Andersson and Nordlund, 1999), in which AOX would be sensing the matrix pH. There is no reason to expect a similar pH dependence of both cytochrome and alternative pathways. Thus, variation in the medium pH, as a tool to concomitantly vary the matrix pH, showed that optimum pH of AOX activity was slightly more acidic than that of the cytochrome pathway. A similar pattern was reported for *A. castellani*, *A. italicum*, and *S. guttatum* mitochondria (Elthon *et al*., 1986; Jarmuszkiewicz *et al*., 2002). To establish a regulatory role for the matrix pH on the AOX activity from 7.5 (matrix pH value reported for respiring mammalian mitochondria in the presence of 2 mM Pi) to 6.8, it would be required to document large physiological changes in cytosolic (and hence matrix) pH. A diminution in pH may be expected when the cytochrome pathway, which contains the H^+ pumps, becomes inactive (Jarmuszkiewicz *et al*., 2002), due to cold, oxidative or respiratory inhibitor stress.

Analysis of the steady-state UQ reduction correlated well with the respiratory rate data. The basal level of UQ reduction was lower and the respiration rate higher, in control than in cold-stressed mitochondria. Control mitochondria also showed full UQ reduction with cyanide but partial reduction with *n*PG. This UQ reduction pattern agreed with the apparent prominent contribution of the cytochrome pathway and low AOX capacity in these mitochondria. In cold mitochondria, the opposite pattern was observed in which the AOX capacity reached a value of 30% of total respiration. This correlation between UQ reduction and respiration suggests that the redox-state of the UQ pool be mainly controlled by the AOX activity in stressed mitochondria.

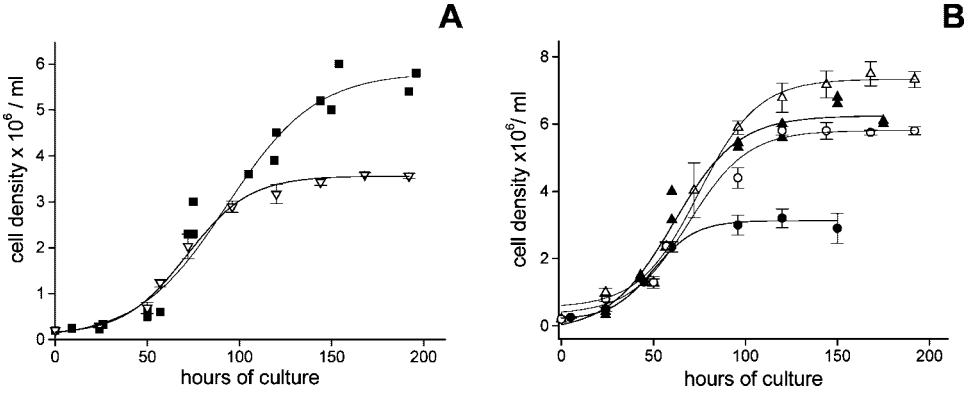

Fig. 4. AOX-dependent cellular growth. The cultures were started by addition of an inoculum of 2×10^5 cells/mL. (A) Control (▽) or cold-stressed (■) cells were cultured in the presence of 0.3 mM cyanide. (B) Control (△, ▲) or cold-stressed (○, ●) cells were cultured in the presence of 3 mM *n*PG (▲, ●).

Rhodoquinone Pool

In *Euglena* mitochondria, RQ-9 is present in equimolar quantities to UQ-9 (Powls and Hemming, 1966; Threlfall, 1972). In this work, an increase in RQ-9 content was observed when cells were exposed to different stress conditions (cf. Table V, Fig. 3(B)). However, a role for RQ-9 in oxygen uptake has not been described. Fumarate reductase uses rhodoquinol under anoxic conditions in some eukaryotic microorganisms (Van Hellemond *et al*., 1995), but this has not been evaluated in mitochondria from *E. gracilis* grown either under aerobic or anaerobic conditions. Then, the role of RQ-9 in *E. gracilis* mitochondria, particularly under stress conditions where its content increases, remains to be explored.

Physiological Role of AOX

Mitochondria, isolated from *Euglena* cells subjected to different types of stress during culturing, showed a diminished cytochrome pathway activity and an increased capacity of the alternative respiratory pathway. The increased AOX as induced by cold stress could result from the observed change in either the quinone pool, in the fatty acid composition, or in the protein content.

Several physiological roles have been assigned to the mitochondrial AOX: (a) in plant tissues with a high AOX activity, a thermogenic effect has been proposed. The elevation in temperature would bring about volatilization of aromatic compounds to attract pollination insects (Meeuse, 1975). (b) AOX functions as an overflow valve for high levels of oxidable substrates or when the cytochrome pathway has been blocked. The electron flow provided by AOX avoids Krebs cycle complete inhibition and allows anaplerotic reactions to maintain biosynthetic pathways (Palmer, 1976). (c) AOX activity is part of the defense cellular mechanism against oxidative stress by impeding the accumulation of reduced UQ, which in turn favors the formation of reactive oxygen species (Wagner and Moore, 1997). The cyanide-resistant oxidase of *Pseudomonas aeruginosa* seems involved in cell division, growth, temperature-sensitivity, antibiotic-resistance, and oxidative stress (Reza Tavankar *et al*., 2003).

It should be noted that the three types of stress conditions used in the present work may promote accumulation of reactive oxygen species (Popov *et al*., 2001). Thus, the relative increase in AOX activity in mitochondria isolated from stressed cells suggests a role for this enzyme in the mechanisms involved in the management of oxidative stress. Furthermore, the lowering in the incubation temperature of isolated mitochondria also increased the AOX capacity, which was accompanied by a lowering of the activity of the cytochome pathway (a condition that brings about oxidative stress).

Growth of control cells was insensitive to the addition of AOX inhibitors to the culture medium. On the other hand, growth of stressed cells was highly sensitive to AOX inhibitors and resistant to cyanide. Thus, these data suggest an AOX protective role for cellular growth under stress conditions.

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