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Novel mitochondrial alcohol metabolizing enzymes of *Euglena gracilis*

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Abstract Ethanol is one of the most efficient carbon sources for *Euglena gracilis*. Thus, an in-depth investigation of the distribution of ethanol metabolizing enzymes in this organism was conducted. Cellular fractionation indicated localization of the ethanol metabolizing enzymes in both cytosol and mitochondria. Isolated mitochondria were able to generate a transmembrane electrical gradient ($\Delta\psi$) after the addition of ethanol. However, upon the addition of acetaldehyde no $\Delta\psi$ was formed. Furthermore, acetaldehyde collapsed $\Delta\psi$ generated by ethanol or malate but not by D-lactate. Pyrazole, a specific inhibitor of alcohol dehydrogenase (ADH), abolished the effect of acetaldehyde on $\Delta\psi$, suggesting that the mitochondrial ADH, by actively consuming NADH to reduce acetaldehyde to ethanol, was able to collapse $\Delta\psi$. When mitochondria were fractionated, 27% and 60% of ADH and aldehyde dehydrogenase (ALDH) activities were found in the inner membrane fraction. ADH activity showed two kinetic components, suggesting the presence of two isozymes in the membrane fraction, while ALDH kinetics was monotonic. The ADH *Km* values were 0.64–6.5 mM for ethanol, and 0.16–0.88 mM for NAD^+, while the ALDH *Km* values were 1.7–5.3 μM for acetaldehyde and 33–47 μM for NAD^+. These novel enzymes were also able to use aliphatic substrates of different chain length and could be involved in the metabolism of fatty alcohol and aldehydes released from wax esters stored by this microorganism.

Keywords ALDH · ADH · Ethanol · Acetaldehyde · Mitochondria · Transmembrane electrical potential · *Euglena gracilis*

Abbreviations

ALD	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
SMPs	Submitochondrial particles
mtxALDH	Mitochondrial matrix aldehyde dehydrogenase
mmALDH	Inner mitochondrial membrane aldehyde dehydrogenase
mtxADH	Mitochondrial matrix alcohol dehydrogenase
mmADH	Inner mitochondrial membrane alcohol dehydrogenase
NAD^+-LDH	Pyridine nucleotides dependent lactate dehydrogenase
D-iLDH	Pyridine nucleotides independent lactate dehydrogenase
SSADH	Succinate semialdehyde dehydrogenase
SSA	Succinate semialdehyde
($\Delta\psi$)	Transmembrane electrical gradient

Introduction

Euglena gracilis is a fresh water living protist with peculiar metabolism (Bégin-Heick 1973; Inui et al. 1982; Rotte et al. 2001; Shigeoka et al. 1986a; Tucci et al. 2010) and remarkable ability to resist and accumulate heavy metals

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(Avilés et al. 2003; Cervantes et al. 2006; Devars et al. 1998, 2011; García-García et al. 2009; Navarro et al. 1997; Mendoza-Cózatl and Moreno-Sánchez 2005; Rodríguez-Zavala et al. 2007). Furthermore, this organism has the ability to produce significant amounts of different metabolites of potential biotechnological interest such as paramylon, a glucose polymer with immune-stimulant and immune-potentiator properties (Fruehauf et al. 1983; Kataoka et al. 2002; Sugawara and Ishizaka 1984); carotenoids, vitamin A tetraterpenoid precursors (Schimmer and Krinsky 1966; Takeyama et al. 1997); and vitamin E (Fujita et al. 2008; Rodríguez-Zavala et al. 2010), an antioxidant used as a food complement, food preservative and in the cosmetic industry. Vitamin E produced by *E. gracilis* is composed by 97% alpha isomer (Shigeoka et al. 1986b), the isomer with highest biological activity (Kamal-Eldin and Appelqvist 1996), which represents an advantage with respect to other natural sources of vitamin E, composed of a mixture of several isomers. The use of carotenoids and vitamin E has become popular due to their protective role against oxidative stress (Demmig-Adams and Adams 2002; Ju et al. 2010; Park et al. 2009; SanGiovanni et al. 2007; Vardi et al. 2010;).

Our group recently reported that in the presence of ethanol *E. gracilis* produced high amounts of tyrosine (Rodríguez-Zavala et al. 2010), a vitamin E precursor, which reinforces the proposal that the *Euglena* whole cell might be used as a source of high quality protein (Takeyama et al. 1997).

This micro-organism can use a wide variety of substrates as carbon source for growth and survival (Barsanti et al. 2001; Fujita et al. 2008; Jasso-Chávez et al. 2005; Rodríguez-Zavala et al. 2006, 2010). This metabolic flexibility allows *Euglena* to populate different environments. Ethanol is one of the most efficient carbon sources for this micro-organism reaching maximal growth at an ethanol concentration of 177 mM. This is remarkable for an organism that, presumably, does not produce ethanol (Ono et al. 1995). Recently, it was shown that the combination of ethanol + glutamate + malate enhanced cell growth; the biomass yield reached was at least 5-fold higher that than obtained in the absence of ethanol (Rodríguez-Zavala et al. 2010). Increments in cell yield of this magnitude are of interest for the use of *Euglena* for biotechnological purposes. Thus, it appears relevant to understand the metabolism of ethanol in this micro-organism. Many alcohol dehydrogenases (ADHs) have been described in *Euglena*, under different culture conditions (Mego and Farb 1974; Munir et al. 2002) and recently, one of these ADHs has been cloned and characterized (Palma-Gutiérrez et al. 2008). Furthermore, mitochondrial alcohol (Ono et al. 1995) and aldehyde (Rodríguez-Zavala et al. 2006) dehydrogenases from this organism have been purified to homogeneity and characterized.

In the present work a bleached mutant of *Euglena gracilis* Z grown on glutamate/malate *plus* ethanol, was used to perform an in-depth analysis of the subcellular distribution and kinetics of alcohol and aldehyde dehydrogenases in this microorganism.

Materials and methods

Chemicals

Acetaldehyde, ADP, β-mercaptoethanol, butyraldehyde, butanol, CCCP (carbonyl cyanide m-chloro phenyl hydrazone), cysteine, 2,6-dichlorophenolindophenol (DCPIP), DL-dithiothreitol, dodecyl aldehyde, ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis(2-aminoethyl ether)-N,N,N′ N′-tetraacetic acid (EGTA), hexanal, D-lactate, MOPS (3-(N-morpholino) propanesulfonic acid), NAD^+, $NADP^+$, NADH, nonyl aldehyde, octanol, 2-oxoglutarate, phenazine methosulfate (PMS), phenylmethanesulfonyl fluoride (PMSF), propionaldehyde, pyrazole, rodamine 123, rotenone, succinate-semialdehyde tetradecanol and trypsin were from Sigma-Aldrich (St. Louis, MO, USA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was from Research Organics (Cleveland, OH, USA). Ethanol was from MERCK (Whitehouse Station, NJ, USA). 2-Amino-2-hidroximetil-propano-1,3-diol (Tris) was from GibcoBRL (Grand Island, NY, USA). 9-amino-6-chloro-2-methoxyacridine (ACMA) was from Molecular Probes (Eugene, OR, USA).

Culture conditions

Euglena gracilis strain Z was grown for 6 day in 1 l of the medium described by Schiff et al. (1971), supplemented with 1% absolute ethanol (Rodríguez-Zavala et al. 2010), at 25 °C and pH 3.5. To determine the cell growth, 100 µl aliquots of culture were diluted 1:50 with double-distilled water (ddH_2O) and the cells were immobilized by adding 2% HCl and counted in a Neubauer chamber.

Cell fractionation

Cells were harvested by centrifugation at 3,000 g for 5 min at 4 °C, washed twice with isotonic saline, and re-suspended in 15 ml of 100 mM Tris, pH 8.5 with 0.025% (*v/v*) β-mercaptoethanol. Cells (450×10^6/ml) were incubated in fresh 100 mM Tris, pH 8.5 with 0.025% (*v/v*) β-mercaptoethanol, in the presence of 15 mg trypsin (14,900 U/mg) at 35 °C for approximately 40 min, until about 25% of the cells were disrupted (which was determined by the uptake of 0.4% trypan blue). After incubation, the cells were diluted 10 times in ice-cold 100 mM Tris, pH 8.5 with 0.025% (*v/v*) β-mercaptoethanol, supplemented with 1 mM

EDTA and 1 mM PMSF to stop proteolysis, and centrifuged at 3,000 g and 4 °C for 5 min. The pellet was washed by re-suspending in the same buffer and centrifuging at 3,000 g for 5 min. Then, the pellet was re-suspended in 15 ml of 250 mM sucrose, 10 mM HEPES, 1 mM EGTA (SHE buffer), 0.025% β-mercaptoethanol, 1 mM PMSF, and disrupted by homogenization with a glass/teflon Potter-Elvehjem tissue grinder. Cell extracts prepared this way were centrifuged at 270 g and 4 °C for 10 min. The resulting supernatant was called fraction **S1**, and contained cytosol, mitochondria, and microsomes. The fraction **S1** was further centrifuged at 12,000 g and 4 °C for 10 min, to separate the mitochondrial fraction from the cytosol and microsomes (this second supernatant was fraction **S2**). The pellet (mitochondrial fraction) was re-suspended in 1 ml SHE buffer and supplemented with 1 mM ADP and 0.2% fatty acid-free bovine serum albumin, and incubated for 5 min at 4 °C. Then, the sample was diluted to 30 ml in SHE buffer and centrifuged again at 12,000 g at 4 °C for 10 min. The pellet was re-suspended in a small volume of SHE buffer and used for mitochondrial function and enzyme analysis. Fraction **S2** was centrifuged at 100,000 g for 45 min to separate the cytosol from the microsomal fraction. The pellet from this last centrifugation containing the microsomal fraction was re-suspended in a small amount of SHE buffer. Rat liver and kidney mitochondria were isolated as previously described (Rodríguez-Zavala et al. 1997).

Protein determination

Aliquots of the samples were poured into glass tubes, mixed with 1% sodium deoxycholate, vortexed, diluted with ddH_2O and mixed with Biuret reagent for protein determination (Gornal et al. 1949). Tubes were incubated for 15 min at room temperature and then centrifuged at 3,000 g for 20 min, to eliminate the turbidity caused by paramylon before reading the absorbance at 540 nm. Bovine serum albumin was used as standard.

Mitochondrial fractionation

Mitochondria were suspended in 10 ml of SHE buffer and disrupted by sonicating on ice, 10 cycles (10 s sonication/1 min rest), at 15% of the maximal output of the instrument (Branson sonifier model 450), using a tip probe of 1 cm diameter. The sonicated suspension was centrifuged at 100,000 g for 45 min at 4 °C. The supernatant was recovered and the pellet was re-suspended in 1 ml SHE buffer.

Enzyme activities

All the enzymatic determinations were carried out at 25 °C. Activities of all enzymes, except for D-iLDH, were determined following the increase in the absorbance at 340 nm owing to the formation of NAD(P)H. ALDH activity was measured by mixing an aliquot of each fraction with 100 mM Na_2HPO_4, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM pyrazole and 1 mM NAD^+; the reaction was started by adding the corresponding aldehyde. ADH activity was determined by mixing an aliquot of the supernatant from the mitochondrial fractionation with 100 mM HEPES, pH 8.5, 1 mM dithiothreitol, 2 mM NAD^+, 30 mM cysteine, and 2 mM Mg^{2+}; the reaction was started by adding 20 mM ethanol. The mmALDH activity was measured in a fluorometer (Aminco Bowman Series 2), mixing an aliquot of the mitochondrial membrane fraction with 100 mM Na_2HPO_4, pH 7.4, 100 mM NaCl, 1 mM dithiotreitol, 0.1 mM pyrazole, 1 mM NAD^+ and starting the reaction by adding the corresponding aldehyde. Excitation and emission wavelengths were 340 and 460 nm, respectively. Activity of mmADH was determined following the fluorescence of the NADH generated as described above. An aliquot of the membrane fraction was mixed with 100 mM HEPES, pH 8.5, 7 mM NAD^+, 30 mM cysteine, and 2 mM Mg^{2+}; the reaction was started by adding 2 to 30 mM of the corresponding alcohol.

Marker enzymes were used to determine cross-contamination of the cell fractions. NAD^+-LDH was used as cytosolic marker (Bergmeyer 1983). Membrane-bound D-iLDH was used as mitochondrial membrane marker (Jasso-Chávez and Moreno-Sánchez 2003) whereas SSADH (Tokunaga et al. 1976) and 2-oxoglutarate decarboxylase were used as mitochondrial matrix markers. NAD^+-LDH was assayed by mixing an aliquot of the indicated fraction with 100 mM MOPS, pH 7.5, and 0.1 mM NADH; the reaction was started by adding 5 mM pyruvate. SSADH was measured by adding an aliquot of a fraction to 100 mM HEPES, pH 8.0, and 1 mM $NADP^+$; the reaction was started by adding 2 mM succinate-semialdehyde. 2-oxoglutarate decarboxylase activity was determined by coupling the reaction with the $NADP^+$-dependent SSADH activity present in the sample (which was always in excess), adding 1 mM $NADP^+$ to saturate the coupling enzyme; the reaction was started by the addition of 1 mM 2-oxoglutarate. D-iLDH activity was determined by incubating a sample aliquot with 100 mM HEPES, pH 8.5, and 0.2 mM DCPIP, and following the increase in the absorbance at 600 nm; the reaction was initiated by the addition of 30 mM D-lactate.

Mitochondrial transmembrane electrical potential

Mitochondria (0.1–0.2 mg protein/ml) were added to 20 mM MOPS, pH 7.3, 0.5 mM EGTA and 120 mM KCl (KME buffer), in the presence of 2 mM phosphate, 2 mM MgCl and 50–100 nM Rhodamine 123, at 25 °C. The

excitation wavelength was 494 nm and the fluorescence recorded at 523 nm (Scaduto and Grotyohann 1999).

Synthesis of ethanol in isolated mitochondria

An aliquot of 30–50 μl of mitochondria (1–2 mg protein) was mixed with 50 μl of SHE buffer pH 7.2 *plus* 10 mM $MgCl_2$ and 1 mM malate. Then, the mixture was incubated 10 min in the presence or absence of 5 mM acetaldehyde and 0.1 mM pyrazole. Ethanol produced was determined using a gas chromatography equipment (Shimadzu GC2010), equipped with a capillary column HP-PLOT/U of 30 m length, 0.32 mm I.D. and 10 μm film (Agilent, Santa Clara, CA, USA) and a flame ionization detector (Shimadzu, Columbia, MD, USA).

Sub-mitochondrial particles isolation

Submitochondrial particles (SMPs) were prepared by sonicating mitochondrial suspensions as described by Jasso-Chávez et al. (2001). Briefly, 25 ml of mitochondrial suspension (18–25 mg protein/ml) were sonicated three times for 13 s at 15% of maximal output with 1 min rest, in a Branson sonicator with a probe tip of 0.5 cm diameter in an ice bath. The sonicated mitochondrial suspension was centrifuged at 17,370 g for 10 min to remove unbroken mitochondria. Hence, the supernatant was centrifuged at 105,000 g for 45 min and 4 °C. Then, the pellet was re-suspended in 20 ml SHE buffer and centrifuged again at 105,000 g for 30 min and 4 °C to obtain submitochondrial particles.

Measurement of the ΔpH generation by submitochondrial particles (SMPs)

The generation of ΔpH was assayed in SMPs (0.2–0.4 mg protein/ml) incubated in KME medium in the presence of 0.2 μM ACMA, 2 mM Mg^{2+} and 1 mM NAD^+. The generation of ΔpH was started by the addition of 5 mM succinate/1 μM rotenone or 0.2 mM acetaldehyde/0.1 mM pyrazole. ACMA was excited at 412 nm and the fluorescence collected at 476 nm (Rottenberg and Moreno-Sánchez 1993).

Results

Distribution of ADH and ALDH activities in *Euglena gracilis*

To evaluate the subcellular distribution of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activities in *Euglena* grown in the presence of glutamate, malate and ethanol, cellular fractionation was carried out. Most of the ADH and ALDH activities were localized in mitochondria (68 and 67% respectively; see Table 1), although still significant activity (26% for ADH, 24% for ALDH) was found in the cytosolic fraction. Robust validation of the subcellular enzyme distribution was provided by the low cross contamination of cytosol by the mitochondrial fraction as indicated by the marker enzymes (<4%; Table 1).

Effect of acetaldehyde on generation of transmembrane electric potential (Δψ)

Considering that acetaldehyde might be generated in the cytosol as some of the ADH activity was found in this compartment, it was explored whether acetaldehyde could permeate into the mitochondria to be further metabolized. For this goal Δψ generated by several oxidizable substrates was determined in coupled *Euglena* mitochondria. Addition of either ethanol, succinate semialdehyde or malate, substrates that generate NADH and feed the respiratory chain site I promoted Δψ generation (Fig. 1a, b and c); D-lactate a substrate of an enzyme that feeds electrons to the respiratory chain at the ubiquinone pool level, in the presence of rotenone (Jasso-Chávez and Moreno-Sánchez

Table 1 Activities of ADH and ALDH in different fractions of *Euglena gracilis* grown in the presence of glutamate, malate *plus* ethanol. Enzyme activities were assayed as indicated under the Materials and Methods section. The data shown are the mean ± SD of three independent preparations. Total activities in the first supernatant (nmol/min) were: 2,300–4,000 for NAD^+-LDH; 1,750–2,900 for D-iLDH; 2,500–4,380 for SSADH; 1,500–2,200 for ADH and 400–650 for ALDH

	% NAD^+- LDH	% D- iLDH	% 2-OGDC	% SSADH	% ADH	% ALDH
Fraction						
S1	100	100	100	100	100	100
Cyto + micro	<1	9±6	<1	6±2	29±6	32±10
Cytosol	95±6	<1	<1	4±2	26±4	24±4
Microsomes	<1	7±4	<1	<1	2±0.8	<1
Mitochondria	<1	85±6	98±10	93±8	68±11	67±6

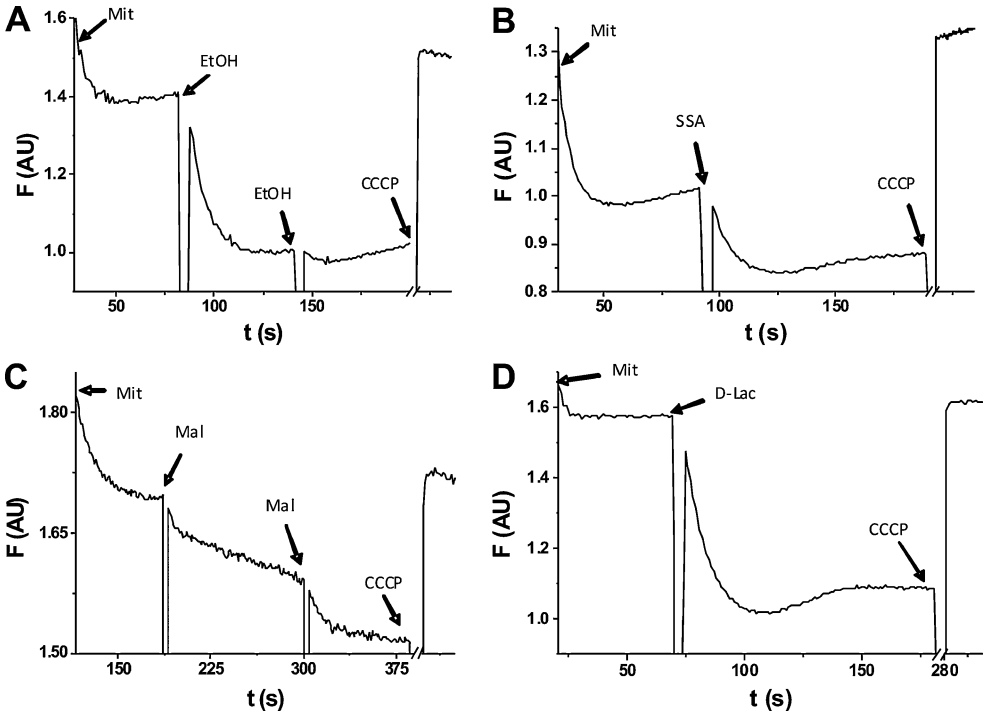


Fig. 1 Generation of transmembrane electrical potential by *Euglena* mitochondria. Membrane potential was measured with Rhodamine 123 as detailed under Materials and Methods. Figures are representative of at least 4–5 experiments with independent mitochondria preparations. Mit, mitochondria; EtOH, 1 mM ethanol; CCCP, 2 μM carbonylcyanide-3-chlorophenylhydrazone; SSA, 0.25 mM succinate semialdehyde; Mal, 2 mM malate; D-Lac, 0.1 mM D-Lactate

2003), also promoted generation of a high Δψ (Fig. 1d). On the other hand, acetaldehyde did not generate Δψ and in fact collapsed that driven by endogenous substrates (Fig. 2a) and by added ethanol, 2-oxoglutarate or malate, but not that driven by SSA (Fig. S1). In contrast, acetaldehyde did promote Δψ generation in rat liver and kidney mitochondria (Fig. 2b and c). When ethanol, 2-oxoglutarate or malate were added to *Euglena* mitochondria after the addition of acetaldehyde, no Δψ generation occurred (Fig. 2d), whereas D-lactate was still able to induce significant Δψ (Fig. 2a and d). These observations suggested that acetaldehyde inhibited the respective

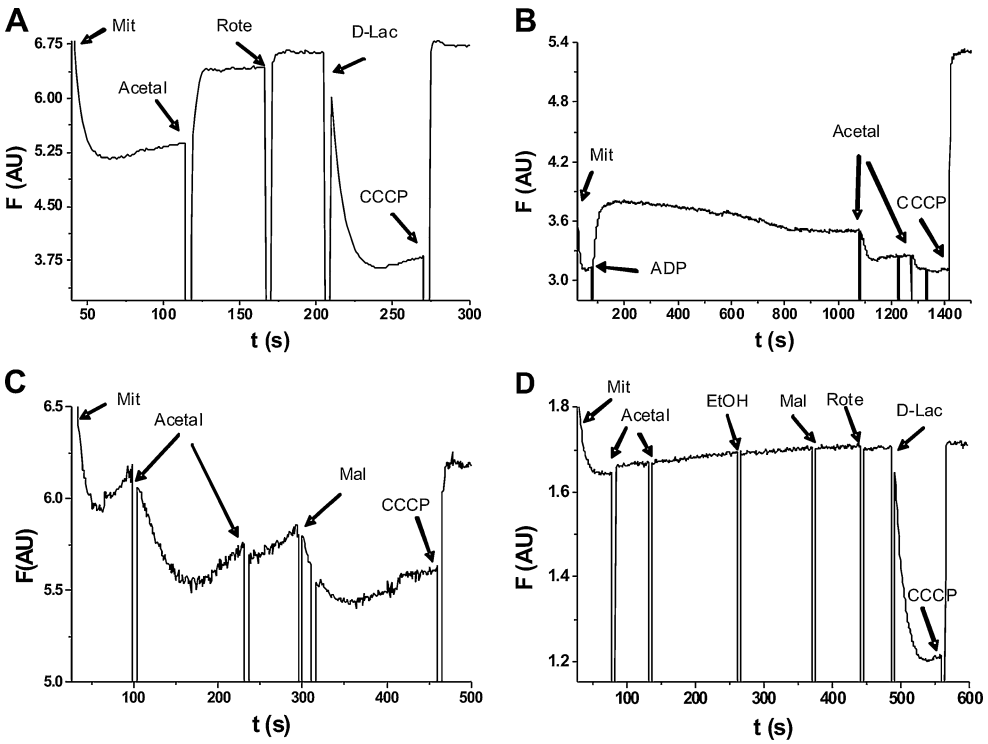


Fig. 2 Effect of acetaldehyde on the generation of transmembrane potential (Δψ) by mitochondria from different sources. Mitochondria were isolated as indicated under Materials and Methods. **a** *Euglena* mitochondria; **b** Rat liver mitochondria; **c** Rat kidney mitochondria. Mit, mitochondria; Acetal, 0.2 mM acetaldehyde; EtOH, 1 mM ethanol; Rote, 2 μM rotenone; CCCP, 2 μM carbonylcyanide-3-chlorophenylhydrazone; ADP, 25 μM adenosine diphosphate; SSA, 0.25 mM succinate semialdehyde; Mal, 2 mM malate; D-Lac, 0.1 mM D-Lactate. Results are representative of experiments with at least 3–5 independent mitochondria preparations

dehydrogenases and/or site I of the respiratory chain in *Euglena* mitochondria.

Effect of acetaldehyde on the activity of mitochondrial NAD^+-dependent enzymes

To establish whether acetaldehyde was indeed a dehydrogenase inhibitor, the activities of some dehydrogenases were evaluated in detergent-permeabilized mitochondria. Acetaldehyde inhibited the NAD^+-dependent SSADH (Fig. 3a), and malate dehydrogenase activities (data not shown), whereas $NADP^+$-dependent SSADH was insensitive (Fig. 3b). The addition of pyrazole, a specific inhibitor of ADH (Li and Theorell 1969), promoted the recovery of the NAD^+-dependent SSADH (Fig. 3a) and malate dehydrogenase activities (data not shown). The ADH activity was also inhibited by acetaldehyde (Fig. 3c). Remarkably, pyrazole revealed a pronounced mitochondrial ALDH activity (Fig. 3d). Furthermore, generation of $\Delta\psi$ in isolated mitochondria by acetaldehyde was achieved in the presence of pyrazole (Fig. 4).

Ethanol synthesis by isolated *Euglena* mitochondria

Euglena mitochondria synthesized 37±15 nmol of ethanol (n=3) after 10 min following the addition of 5 mM acetaldehyde (and in the presence of malate for the production of NADH). Ethanol synthesis was almost completely inhibited by 0.1 mM pyrazole (residual production = 0.5±0.2 nmol ethanol/mg protein; n=3). In the absence of acetaldehyde no ethanol synthesis was observed.

ADH and ALDH activities in sub-mitochondrial fractions

Isolated *Euglena* mitochondria were disrupted by sonication and the ADH and ALDH activities were evaluated in the sub-mitochondrial fractions. Most of the ADH activity (60%) was found in the mitochondrial matrix, but surprisingly, a significant fraction (27%) was found in the inner mitochondrial membrane (Table 2). For ALDH activity, 20% was localized in the mitochondrial matrix and 60% in the membrane fraction (Table 3). Again, robust validation of the sub-mitochondrial ADH and ALDH distribution was provided by the low cross contamination of the membrane fraction with matrix proteins.

Kinetics of mitochondrial ALDH and ADH

Both membrane (mmALDH) and matrix (mtxALDH) aldehyde dehydrogenases showed Michaelis-Menten kinetics with similar *Km* values for acetaldehyde and NAD^+ (Table 3). Both ALDH isoforms also showed significant activity with aliphatic aldehydes of different chain length (Table 3), but not with aromatic aldehydes. In turn, the

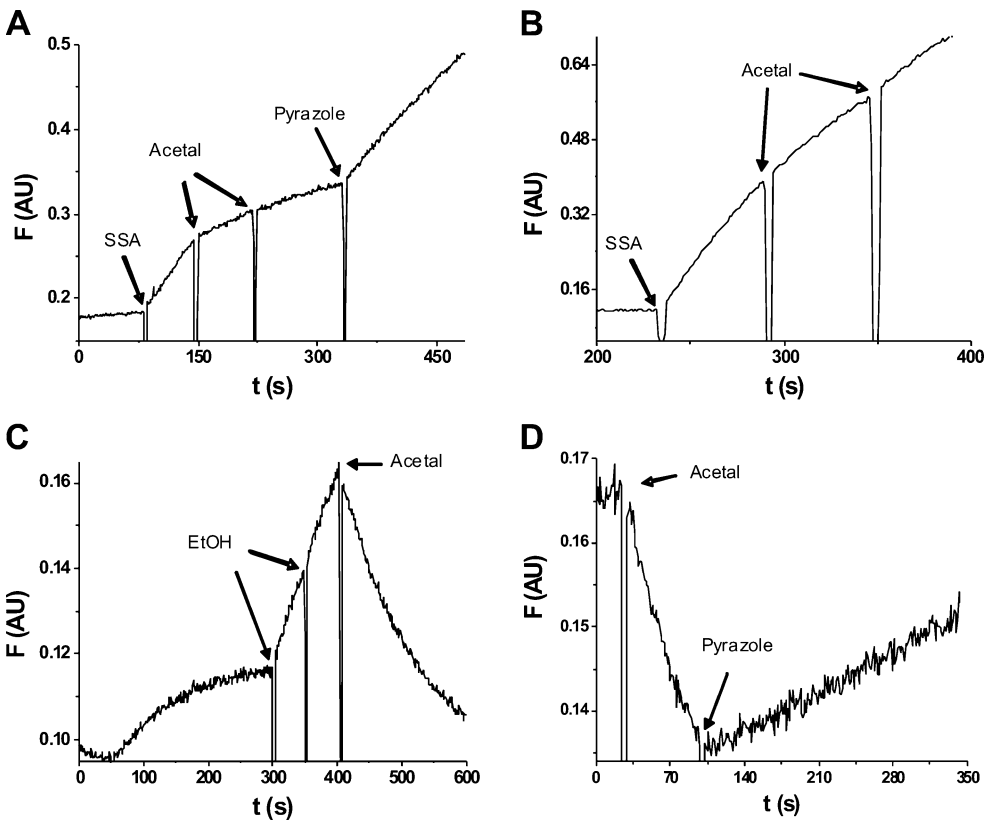


Fig. 3 Effect of acetaldehyde on the mitochondrial SSADH activities. Enzyme activities were determined with 0.5 mM NAD^+ (**a**) or 0.5 mM $NADP^+$ (**b**) as indicated under Material and Methods. Acetal, 0.2 mM acetaldehyde; Pyrazole, 0.1 mM pyrazole; EtOH, 1 mM ethanol; SSA, 0.25 mM succinate semialdehyde. Results are representative of experiments with at least 3 independent mitochondrial extracts

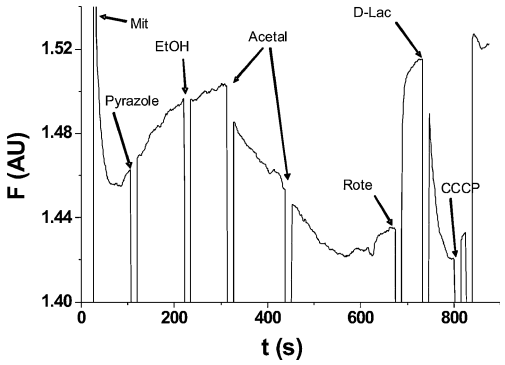


Fig. 4 Restoration of acetaldehyde-inhibited Δψ by pyrazole. Acetal, 0.2 mM acetaldehyde; Pyrazole, 0.1 mM pyrazole; Rote, 2 μM rotenone; D-Lac, 0.1 mM D-Lactate; CCCP, 2 μM carbonylcyanide-3-chlorophenylhydrazone. Results are representative of experiments with at least 3 independent mitochondria preparations

activity of mmADH exhibited two well-defined kinetic components (Fig. S2), showing high and low affinities for both substrates, whereas mtxADH kinetics was similar to that of the membrane high affinity component (Table 3). The three ADH isoforms also showed significant activity with alcohols of different chain length but not with aromatic alcohols. These ADHs were fully sensitive to the inhibitor pyrazole (data not shown).

Generation of ΔpH by submitochondrial particles

Submitochondial sealed particles were prepared to further assess the presence of ALDH in the inner mitochondrial membrane. Generation of ΔpH by succinate in the presence of rotenone was used as control (Fig. S3A). Acetaldehyde in the presence of NAD^+ and pyrazole, prompted the generation of ΔpH, which was collapsed by the uncoupler CCCP (Fig. S3B).

Orientation of the active sites of mmALDH and mmADH

Sealed mitochondria were incubated in the presence of 1 mM NAD^+ *plus* 1 mM acetaldehyde, or 10 mM ethanol, to elucidate whether the active sites of mmALDH or mmADH were orientated towards the cytosolic side of the membrane. No activity was observed in these assays until 0.01% of the detergent triton x-100 was used to permeabilize the membranes, indicating that the active sites of these enzymes were not exposed to the cytosolic compartment.

Discussion

Effect of acetaldehyde on Δψ generation

Several ADHs have been reported for *Euglena*, some localized in the cytosol (Mego and Farb 1974; Munir et al. 2002; Palma-Gutiérrez et al. 2008) and one in the mitochondria (Ono et al. 1995), while only a mitochondrial ALDH has been described (Rodríguez-Zavala et al. 2006) for this microorganism. When culturing *Euglena* in the presence of ethanol, this substrate can be oxidized by cytosolic ADH to form acetaldehyde. The toxicity of acetaldehyde makes necessary its fast in situ oxidation and/or rapid transport into the mitochondria to be detoxified to avoid cellular damage. In this regard, significant ALDH activity was found in the cytosol as it also occurs in other organisms (Crow et al. 1974; Henehan et al. 1985; Kirch et al. 2004; Tottmar et al. 1973; Wang et al. 1998; Wood and Duff 2009), which supports the requirement for this specific cellular defense mechanism.

However, some of the acetaldehyde produced in the cytosol might permeate into the mitochondria. Acetaldehyde collapsed the mitochondrial transmembrane electrical potential. Initially it was thought that acetaldehyde was altering the integrity of the membrane or that it was inhibiting site I of respiratory chain. The generation of Δψ induced by D-lactate in the presence of acetaldehyde, discarded the first possibility, whereas the pyrazole restoring effect on Δψ and on ADH and NAD^+-SSADH activities indicated that site I was active. The explanation of the acetaldehyde effect was that the ADH activity, in the presence of added acetaldehyde, was responsible for the diminution of Δψ, by sequestering the NADH produced by the other enzymes and thus masking their activities. This explanation was supported by the restoring pyrazole effect on Δψ and on the activity of the mitochondrial matrix NAD^+-dependent enzymes, both inhibited by acetaldehyde.

Table 2 Activities of ADH and ALDH in the different mitochondrial fractions. Enzyme activities were assayed as indicated under Materials and Methods. Results are the mean ± SD of three independent mitochondrial preparations. Total activities in mitochondrial extracts (nmol/min) were: 2,600–4,600 for D-iLDH; 2,600–3,800 for $NADP^+$-SSADH; 600–1,200 for ADH and 300–480 for ALDH

Fraction	% SSADH	% D-iLDH	% ADH	% ALDH
Intact mitochondria	100	100	100	100
Mitochondrial extract	95±4	93±3	81±10	87±7
Mitochondrial matrix	91±6	<1	52±6	21±3
Mitochondrial membrane	5±2	89±8	27±8	61±11

Table 3 Kinetic parameters of mitochondrial ALDHs and ADHs

	mtxALDH		mmALDH			
Substrate	*Km* (μM)	*Vm* (% of *Vm* Acetaldehyde)	*Km* (μM)	*Vm* (% of *Vm* acetaldehyde)		
NAD^+	47±9 (*n*=3)	100	33±11 (*n*=3)	100		
Acetaldehyde	5.3±1.4 (*n*=3)	100	1.7±1.1 (*n*=3)	100		
Butanaldehyde	1.8±0.8 (*n*=4)	139±19	2.2±0.2 (*n*=3)	173±35		
Hexanaldehyde	5.3±4.5 (*n*=3)	77±8	2.14±0.53 (*n*=3)	80±9		
Nonyl aldehyde	3.1±1.1 (*n*=3)	132±7.8	6.4±1.1 (*n*=3)	105±15		
Dodecyl aldehyde	5.6±2 (*n*=3)	45.5±10	6±1.8 (*n*=3)	45.3±8.5		
	mtxADH		mmADH			
	Km (mM)	*Vm*	*Km*1 (mM)	*Vm*1 (% of *Vm*2 EtOH)	*Km*2 (mM)	*Vm*2 (% of *Vm*2 EtOH)
NAD^+	0.88±0.71 (*n*=3)	100	0.16±0.08 (*n*=3)	67±8.5	0.66±0.25 (*n*=3)	100
Ethanol	0.64±0.32 (*n*=3)	100	1.75±0.83 (*n*=3)	74±16	6.5±1.5 (*n*=3)	100
Butanol	0.27 (*n*=2)	97	0.9 (*n*=2)	77	4.7 (*n*=2)	103
Octanol	1.18±0.13 (*n*=3)	89±14	0.16 (*n*=2)	90	0.6 (*n*=2)	116
Myristic alcohol	0.93 (*n*=2)	13	3.1 (*n*=2)	120	10 (*n*=2)	144

Vm mtxALDH = 12–20 nmol/min*mg; *Vm* mmALDH = 60–90 nmol/min*mg. *Vm* mtxADH =10–27 nmol/min*mg; *Vm* mmADH = 19–30 nmol/min*mg. *mtx* mitochondrial matrix; *mm* inner mitochondrial membrane. Where indicated, results are the mean ± SD of the number of experiments shown in parenthesis

Acetaldehyde did not affect the generation of Δψ by SSA, or the activity of $NADP^+$-dependent SSADH, but it did inhibit NAD^+-SSADH. As site I of the respiratory chain cannot oxidize NADPH, one explanation of the last results was that an efficient transhydrogenase catalyzed a fast NADPH/NADH exchange. A second possibility was that the synthesis of succinate by $NADP^+$-SSADH, a substrate that feeds the pool of quinones of the respiratory chain, bypassed acetaldehyde inhibition. However, generation of Δψ by SSA in the presence of 12 mM malonate (this concentration of malonate completely inhibited the activity of *Euglena* succinate dehydrogenase; data not shown) was only 15% inhibited.

Like in yeast, metabolism of acetaldehyde is displaced to ethanol synthesis in *Euglena* mitochondria (see Figs. 2 and 3). However, in yeast ethanol synthesis takes place in the cytosol under anaerobic conditions. In higher organisms, acetaldehyde metabolism is shifted to oxidation, which occurs mainly in the mitochondrial matrix (Svanas and Weiner 1985).

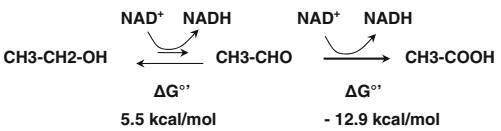
Thermodynamics of ethanol oxidation

The standard ΔG of the reaction for acetaldehyde reduction at pH 7.0 is −5.5 kcal/mol and hence the *Keq* value at 25 °C is 1×10^4, indicating that acetaldehyde reduction is thermodynamically favored (scheme 1). However, at ethanol/acetaldehyde and NAD^+/NADH ratios values higher than 100, the direction of the reaction can be changed. On the other hand, ΔG°' for acetaldehyde oxidation is −12.9 kcal/mol and *Keq* 2.6×10^9, indicating that the reaction of acetaldehyde oxidation is practically irreversible under near-physiological conditions.

Possible role of membrane-bound ethanol metabolizing enzymes

Different membrane-bound ADHs and ALDHs have been reported for several Gram-negative bacteria (Fukaya et al. 1989; Gómez-Manzo et al. 2010; Goodwin and Anthony 1998; Kay et al. 2004; Wu et al. 2011), which use pyrroloquinoline quinone (PQQ) as the redox prosthetic group. A $NAD(P)^+$-dependent ALDH has also been found in the mouse liver microsomal membrane (Masaki et al. 1989; Vasiliou et al. 1996) and this enzyme has been related to the detoxification of medium chain aldehydes produced during lipid peroxidation (Lindahl and Petersen 1991; Mitchell and Petersen 1989).

Under anaerobic conditions, *Euglena* produces and accumulates wax esters (Kolattukudy 1970), synthesized at the expense of paramylon storage (Inui et al. 1982,



Scheme 1 Reactions of ethanol and acetaldehyde oxidation

1992). Wax esters form water insoluble aggregates of high molecular weight that are accumulated in the cytosol (Inui et al. 1982). Under aerobic conditions, aliphatic alcohols and aldehydes are produced from the degradation of wax esters in the cytosol (Inui et al. 1986). Thus, it is possible that the different ADHs and ALDHs expressed by *Euglena* might be involved in the metabolism of the aliphatic alcohols produced during wax esters degradation. Fatty alcohols and aldehydes are poorly soluble in water and therefore preferentially interact with the membrane. The presence of ADH and ALDH in the inner mitochondrial membrane may favor the utilization of ethanol and acetaldehyde in the vicinity of the internal mitochondrial membrane, thus preventing the toxic effects of these compounds on respiratory chain and mitochondrial matrix enzymes.

Euglena does not possess an active pyruvate dehydrogenase complex (Inui et al. 1984; Shigeoka et al. 1986a). Thus, pyruvate can only be used to transfer redox equivalents to the mitochondria through the lactate shuttle (Jasso-Chávez and Moreno-Sánchez 2003). However, carbon equivalents from pyruvate have to be used by other pathways to avoid its accumulation. The results shown in the present work permit to visualize a model (Fig. 5) in which acetaldehyde can be synthesized in the cytosol from pyruvate by pyruvate descarboxylase (Hurlbert and Rittenberg 1962); in the cytosol, acetaldehyde could be used by cytosolic alcohol dehydrogenase (ADHc) to produce ethanol. Alternatively, acetaldehyde can also be produced by ADHc when cells are cultivated in the presence of ethanol. Then, acetaldehyde can be oxidized to acetate by cytosolic aldehyde dehydrogenase (ALDHc). Acetate can enter into the mitochondria to be metabolized by acetate thiokinase that produces acetyl CoA to feed the Krebs cycle. A fraction of the cytosolic acetaldehyde might permeate the mitochondrial membrane to be oxidized by ALDHm or reduced by ADHm. Ethanol can also permeate into the mitochondria where it is oxidized to acetaldehyde by mtxADH (Ono et al. 1995) or by the mmADHs with production of NADH. In turn, acetaldehyde is oxidized to acetate by mtxALDH (Ju et al. 2010) or mmALDH, also with the generation of NADH. Acetate can then be used by acetate thiokinase to synthesize acetyl CoA to feed the Krebs cycle (Fig. 5).

The presence of different ADHs and ALDHs in cytosol and mitochondria of *Euglena gracilis* raises the possibility that in this microorganism an ethanol-acetaldehyde shuttle such as that described for yeast (Bakker et al. 2000;

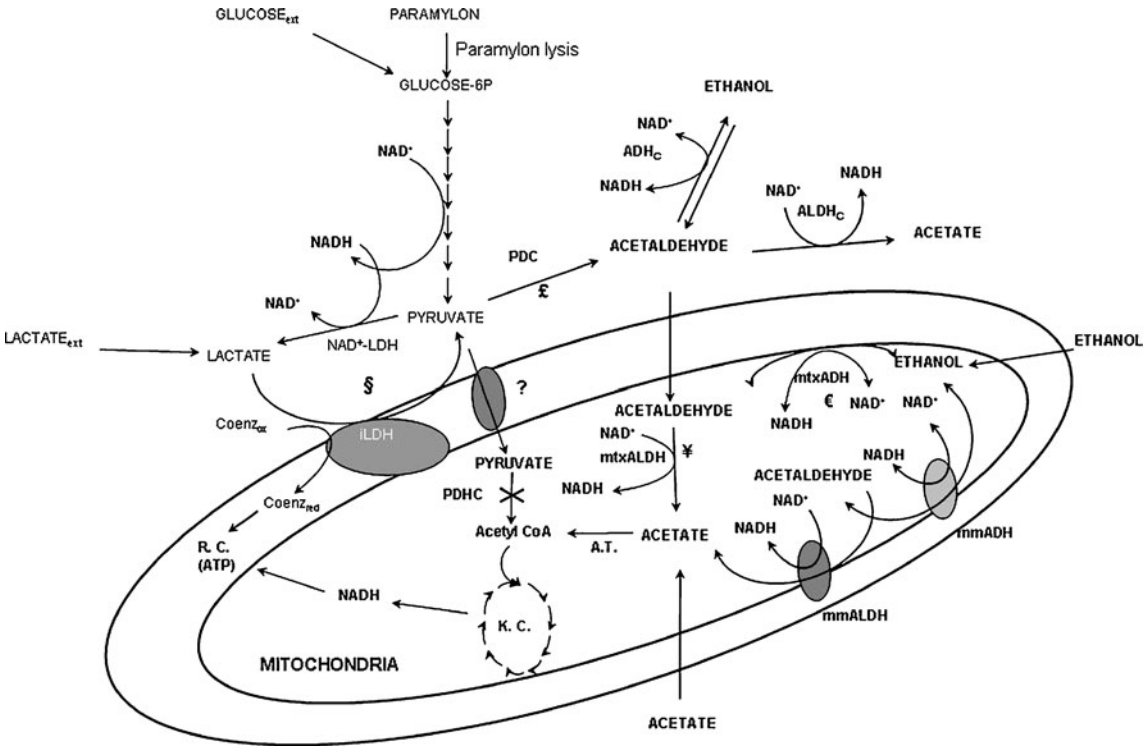


Fig. 5 Scheme of the ethanol metabolism in *Euglena gracilis.* PDC, pyruvate decarboxylase; ALDc, cytosolic alcohol dehydrogenase; nLDH, NAD^+-dependent lactate dehydrogenase; iLDH, NAD^+-independent lactate dehydrogenase; ALDHc, cytosolic aldehyde dehydrogenase; mtxADH, mitochondrial matrix aldehyde dehydrogenase; mtxALDH, mitochondrial matrix aldehyde dehydrogenase; mmADH, mitochondrial membrane alcohol dehydrogenase; mmALDH, mitochondrial membrane aldehyde dehydrogenase; PDHC, pyruvate dehydrogenase complex; A.T., Acetate thiokinase; K.C., Krebs cycle; R.C., respiratory chain. **£** Reported by Hurlbert and Rittenberg 1962. **§** Described by Jasso-Chávez and Moreno-Sánchez 2003. **¥** Characterized by Rodríguez-Zavala et al. 2006. **€** Reported by Ono et al. 1995

Lertwattanasakul et al. 2009) might be operating. It has been suggested that this shuttle is important for the transference of reduced equivalents from mitochondria to cytosol under anaerobic or microaerophilic conditions, in which generation of NADH still occurs (Bakker et al. 2000). It is worth noting that *Euglena* is a facultative organism and can switch its metabolism from aerobic to anaerobic and vice versa depending on the environmental conditions (Coleman et al. 1988; Inui et al. 1984; Tucci et al. 2010). In order to clarify this possibility, the purification and in-depth characterization of these novel membrane bound ADH and ALDH of *Euglena gracilis* is necessary and it is currently under progress in our laboratory.

In conclusion, *Euglena gracilis* possesses several alcohol metabolizing enzymes distributed in cytosol and mitochondria; three new isozymes of this pathway were found bound to the inner mitochondrial membrane. These isozymes can use aliphatic aldehydes and alcohols of different chain size. mmALDH and mmADHs could be part of the antioxidant defense mechanism and could also participate in the metabolism of complex alcohols and aldehydes, produced as part of the metabolism of wax esters, accumulated by this organism in anaerobic conditions.

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