Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1709 (2005) 181 – 190
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

The bacterial-like lactate shuttle components from heterotrophic *Euglena gracilis*

Ricardo Jasso-Chávez[a,*], Israel García-Cano[a], Álvaro Marín-Hernández[a], David Mendoza-Cózatl[a], Juan Luis Rendón[b], Rafael Moreno-Sánchez[a]

[a]*Departamento de Bioquímica, Instituto Nacional de Cardiología, Juan Badiano #1, Col. Sección XVI, Tlalpan, México, D. F. 14080, México*
[b]*Departamento de Bioquímica, Facultad de Medicina, UNAM, México*

Received 31 May 2005; received in revised form 22 July 2005; accepted 25 July 2005
Available online 9 August 2005

Abstract

The structural and kinetic analyses of the components of the lactate shuttle from heterotrophic *Euglena gracilis* were carried out. Mitochondrial membrane-bound, NAD^+-independent D-lactate dehydrogenase (D-iLDH) was purified by solubilization with CHAPS and heat treatment. The active enzyme was a 62-kDa monomer containing non-covalently bound FAD as cofactor. D-iLDH was specific for D-lactate and it was able to reduce quinones of different redox potential values. Oxalate and L-lactate were mixed-type inhibitors of D-iLDH. Mitochondrial L-iLDH also catalyzed the reduction of quinones, but it was inactivated during the extraction with detergents. Both L-iLDH and D-iLDH were inhibited by the specific flavoprotein-inhibitor diphenyleneiodonium, suggesting that L-iLDH was also a flavoprotein. Affinity chromatography revealed that the *E. gracilis* cytosolic fraction contained two types of NAD^+-dependent LDH specific for the generation of D- and L-lactate (D-nLDH and L-nLDH, respectively). These two enzymes were tetramers of 126–132 kDa and showed an ordered bi–bi kinetic mechanism. Kinetic properties were different in both enzymes. Pyruvate reduction by D-nLDH was inhibited by its two products; the D-lactate oxidation was 40-fold lower than forward reaction. L-lactate oxidation by L-nLDH was not detected, whereas pyruvate reduction was activated by fructose-1, 6-bisphosphate, K^+ or NH_4^+. Interestingly, membrane-bound L- and D-lactate dehydrogenases with quinone reductase activity have been only detected in bacteria, whereas the activity of soluble D-nLDH has been identified in bacteria and some yeast. Also, FBP-activated L-nLDH has been found solely in lactic bacteria. Based on their similar kinetic and structural characteristics, a possible common origin among bacterial and *E. gracilis* lactic dehydrogenase enzymes is discussed.

Keywords: Membrane-bound lactate dehydrogenase; NAD^+-dependent lactate dehydrogenase; Mitochondrion; Energy metabolism

1. Introduction

The NAD^+-independent lactate dehydrogenases (iLDH) are enzymes widely distributed among bacteria and yeast [1,2]. Lactate oxidation is coupled to the respiratory chain and hence to the generation of a transmembrane proton gradient and ATP synthesis. In bacteria, the quinone pool is the physiological electron acceptor of iLDH, while in yeast, cytochrome *c* is the main acceptor. In bacteria, D-iLDH and L-iLDH, both stereo-specific enzymes, are localized in the inner cytoplasmic membrane, while in *Saccharomyces cerevisiae*, they are located in the mitochondrial intermembranal space [3,4]. In both kinds of microorganisms, the iLDHs are flavoproteins, but in yeast, D- and L-iLDH also have a cytochrome b_2. Many of these enzymes have been purified and their molecular and kinetic properties have been determined [1,2,5]. In bacteria and yeast, active D-iLDH is a 60–66 kDa monomer which contains one non-covalently bound FAD molecule [1,2]; in yeast, D-iLDH seems to be mainly associated with the methylglyoxal pathway [6]. On the other hand, L-iLDH is a 45-kDa monomer in bacteria and a 240-kDa homotetramer in yeast

* Corresponding author. Tel.: +52 555 573 2911; fax: +52 555 573 0926.
E-mail address: rjassoch@aol.com (R. Jasso-Chávez).

0005-2728/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2005.07.007

and both enzymes possess non-covalently bound FMN as cofactor [2,4]. D-iLDH and L-iLDH are competitively or mixed-type inhibited by oxamate and/or oxalate, with K_i values ranging from 3 μM to 2 mM [1].

Bacteria constitutively express only one iLDH. Thus, while D-iLDH is present under many growth conditions in *Escherichia coli* [7], L-iLDH is induced only when cells are grown aerobically with L-lactate as carbon source. Since the main end product of glycolysis in *E. coli* is D-lactate, the expression of L-iLDH is repressed in the presence of glucose [8]. In yeast, iLDH expression depends on the availability of the corresponding lactate isomer, and oxygen, in the growth medium [9].

Amino acid alignment between iLDHs from bacteria and yeast reveals differences among sequences, suggesting a divergent origin for these enzymes [2]. In addition, the alignment between D-iLDH and other flavine-containing enzymes from diverse organisms shows that D-iLDH is a member of the new FAD-containing proteins super family [10].

Isolated mitochondria from the free living protist *Euglena gracilis,* cultured under aerobic conditions, have the capacity for coupling the oxidation of D- and L-lactate to oxidative phosphorylation by means of isomer-specific membrane-bound lactate dehydrogenases [11–13]. Like many bacterial systems, these enzymes in *E. gracilis* mitochondria are NAD^+-independent (D-iLDH and L-iLDH) and directly reduce the quinone pool [11,12]. Moreover, both mitochondrial iLDHs together with the cytosolic NAD^+-dependent-lactate dehydrogenase (nLDH) constitute a lactate shuttle in *E. gracili*s. In this shuttle, the pyruvate produced from paramylon (linear chain of β 1–3 glucan) catabolism by means of glycolysis is reduced by the cytosolic nLDH to generate lactate, which is oxidized by the membrane-bound iLDH to produce the reducing equivalents for oxidative phosphorylation [13]. Because *E. gracilis* mitochondria are unable to oxidize pyruvate for acetyl-CoA synthesis under aerobic conditions [13], it has been proposed that the lactate shuttle may be an essential component of the energy metabolism and hence of cellular duplication and other energy-dependent cellular events [13].

In *E. gracilis*, both D-iLDH and L-iLDH are constitutively expressed in all growth phases, independently of the carbon source used [13]. Oxygen availability and paramylon catabolism are strongly involved in the variation of the D- and L-iLDH activities. D-iLDH and L-iLDH have similar K_m values for their respective substrates, and are competitively and mixed-type inhibited by oxamate and oxalate, respectively. The D-iLDH is thermo-resistant up to 70 °C, while L-iLDH is inactivated from 50 °C [12].

Thus, both iLDHs from *E. gracilis* seem to be physiologically similar to the bacterial enzymes. In the present work, the partially purified components of the lactate shuttle were kinetically and structurally characterized. Their kinetic and molecular properties suggested that the lactate shuttle enzymes from *E. gracilis* share a common ancestor with the bacterial membrane-bound and cytosolic lactate dehydrogenases.

2. Materials and methods

2.1. Chemicals

Lubrol, D-lactate, and L-lactate were from ICN. Ubiquinone-1 (Q1), dichlorophenol-indophenol (DCPIP), cytochrome *c*, phenyl-methyl sulfonate fluoride (PMSF), CHAPS, pyruvate, fructose-1, 6-bisphosphate (FBP), NAD^+, NADH, diphenyleneiodonium (DPI), FAD, FMN and molecular weight markers for SDS-PAGE were from Sigma. The cyanogen-activated affinity resin was from BIO-RAD. The molecular exclusion Sephacryl S-200 and the blue-Sepharose resins, and molecular weight markers for molecular exclusion chromatography were from Pharmacia. Tetrachlorohidroquinone (TCHQ) was kindly provided by Dr. E. Escamilla (IFC-UNAM, México).

2.2. Cell culture and isolation of mitochondrial and cytosolic fractions

Euglena gracilis strain Z was cultured in the dark in presence of 33 mM glutamate and 17 mM malate as carbon source [12]. Isolation of the mitochondrial and cytosolic fractions was made as previously described [12–14] and kept at −70 °C until use.

2.3. Determination of metabolites

A 0.9-mL suspension containing approximately 1×10^8 washed cells, from different phases of growth, was mixed with perchloric acid (PCA, 3% final concentration). This mixture was vigorously mixed, centrifuged at 20,000×*g* for 5 min and the supernatant recovered and neutralized with KOH/Tris (3 M/0.1 M). NAD^+ and FBP were determined fluorometrically at 30 °C by using standard enzymatic methods [15].

2.4. Enzyme assays

D-iLDH activity was determined in 1 mL of a solution that contained 20 mM HEPES pH 7.5, 1 mM EGTA and 0.5 mM PMSF (HE buffer) supplemented with 0.2 (w/v) CHAPS, and 0.12% (w/v) Triton X-100. For the determination of K_m values for the electron acceptor, the rate of reduction of increasing concentrations of DCPIP from 5 up to 300 μM (λ 600 nm and $\varepsilon = 21.3\ mM^{-1}\ cm^{-1}$) [16], Q1 from 1 up to 75 μM (λ 273 nm and $\varepsilon = 18.1\ mM^{-1}\ cm^{-1}$) [12], or TCHQ from 5 up to 100 μM (λ 322 nm and $\varepsilon = 8.6\ mM^{-1}\ cm^{-1}$; data not shown) was followed in a spectrophotometer. The reaction was started by addition of 40 mM D-lactate. For determination of the K_m value for D-lactate, its concentration was varied between 0.1 and 30 mM, using

250 μM DCPIP as the electron acceptor. When TCHQ reduction was determined, Triton X-100 was omitted to avoid interference with the assay. For L-iLDH activity, both CHAPS and Triton X-100 were replaced by 0.2% (w/v) Lubrol. The activity of L-iLDH in Lubrol-solubilized mitochondria was measured in the presence of 250 μM DCPIP or 80 μM Q1, and the reaction was started by addition of 40 mM L-lactate.

The effect of DPI on D-iLDH and L-iLDH activities was determined as described elsewhere [17], with some modifications. Mitochondrial protein (0.1 mg) for the L-iLDH assay or 30 μg of isolated D-iLDH were incubated for 1 min with 0.5 mM L-lactate or D-lactate to maintain the enzyme reduced in 1 mL of HE buffer, which had been previously saturated with N_2 for 30 min. After the addition of different DPI concentrations, the mixture was further incubated for 5 min under N_2 atmosphere. The activity was determined by measuring the 0.3 mM DCPIP reduction at 600 nm, starting the reaction by adding 30 mM L- or D-lactate.

The activity of nLDH was measured in both directions of its reaction. The pyruvate reduction (forward) was determined in 1 mL of HE buffer at pH 7.0 plus 0.7 mM β-mercaptoethanol (HEB buffer) by measuring the rate of disappearance of 0.30 mM NADH at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}); the reaction was started by addition of freshly prepared pyruvate. The NAD^+-dependent lactate oxidation (reverse) was determined in 1 mL of 400 mM tricine and 500 mM glycine (pH 9) by measuring the reduction of 1.5 mM NAD^+ [15]; the reaction was started by the addition of either 100 mM D-lactate or L-lactate. To determine the D- and L-lactate production by the respective nLDH, 20–50 μg cytosolic protein was incubated with 150 μM NADH and 0.5 mM pyruvate in 1 mL of HEB buffer. After 1–3 min, the reaction was stopped with 3% (v/v) PCA, mixed and centrifuged and the supernatant neutralized as described above. The D- and L-lactate production was fluorometrically determined by using commercial specific NAD^+-dependent D- (Roche) and L-lactate dehydrogenases (Sigma Chem.), respectively [13].

2.5. D-iLDH purification

All steps were carried out at 4 °C. Mitochondria isolated from cells grown by 90–94 h [12] were diluted at 15–18 mg protein/mL in HE buffer. CHAPS was slowly added until a detergent/protein ratio of 0.7 was reached. The suspension was strongly mixed for 5 min and centrifuged at 40,000×*g* for 20 min. The solubilized D-iLDH in the supernatant was incubated in the presence of 10 mM oxalate at 60 °C for 45–50 min; the heated suspension was centrifuged at 40,000×*g* for 20 min. The supernatant was mixed with 50% (v/v) glycerol and stored at −70 °C until use. Under these conditions, enzyme activity was preserved by 70% after 1 month storage. The D-iLDH enrichment throughout the purification steps was monitored by SDS-PAGE and activity measurements.

2.6. Partial purification of nLDHs

The *E. gracilis* cytosolic fraction was precipitated with ammonium sulfate up to 30% (w/v) saturation and incubated for 30 min at 4 °C. This mixture was centrifuged at 20,000×*g* for 20 min. The supernatant was brought up to 75% saturation with ammonium sulfate and centrifuged. This second pellet was re-suspended in HEB buffer. This last solution was desalted in a Sephadex G-25 column (0.5 × 5 cm). The desalted solution was loaded on a Blue-Sepharose column previously equilibrated with HEB buffer. The nLDHs were eluted with a 0–1 M NaCl gradient in the same buffer, and concentrated in an Amicon ultrafiltration cell, using a 30-kDa cut-off membrane (YM30, Millipore). The concentrated protein was then loaded onto an oxamate-Sepharose column. The oxamate-Sepharose resin was prepared according to [18]. The column (1 × 15 cm) was previously equilibrated with HEB buffer containing 0.05 mM NADH and 1 mM FBP (HEBNF buffer). Protein eluted with 7.5 washing volumes of HEBNF buffer contained the D-nLDH activity, while L-nLDH eluted when HEBNF buffer was replaced by HEB buffer.

2.7. Molecular weight determination

The molecular weight of the *E. gracilis* lactate shuttle components, D-iLDH, D-nLDH, and L-nLDH, was determined by using molecular exclusion chromatography. The enzymes (2.5–5 mg protein) were loaded onto a Sephacryl S-200 column (56 × 1.7 cm) previously equilibrated with HE buffer supplemented with 0.2% (w/v) CHAPS. The column was calibrated with the following molecular standards (Pharmacia): blue dextran (2×10^6 Da), horse spleen ferritin (440,000 Da), bovine liver catalase (232,000 Da), rabbit muscle aldolase (158,000 Da), bovine serum albumin (66,000 Da), bovine pancreas chimotrypsinogen A (25,000 Da), bovine pancreas ribonuclease A (13,700 Da), and vitamin B12 (1,700 Da). The distribution coefficient (K_{av}) was determined with the equation.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = sample elution volume; V_o = column void volume = elution volume for Blue-dextran (59 mL); and V_t = total column volume (127 mL). The elution volume for each enzyme was taken to be the volume at which there was an absorbance peak at 280 nm, accompanied with the highest activity.

2.8. Structural characterization of D-iLDH

The enzyme (5–7.5 μg protein) was separated by 12% polyacrylamide SDS-PAGE under denaturing conditions. The extraction of flavine was made with a solution containing 0.4 mg enzyme protein and 10% trichloroacetic acid (TCA) [5]. After 15 min of incubation at 4 °C, the

solution was centrifuged at 20,000×*g* for 4 min. An aliquot of the supernatant was poured into a spectrofluorometer cuvette with 2 mL 50 mM citrate (pH 3). The fluorescent signal of flavine was measured at 520 nm, with the excitation wavelength set at 450 nm. Identification of flavine was determined by HPLC using a reverse phase (RP) C18 column (4.6 × 250 mm, Waters) equilibrated with 0.1% trifluoroacetic acid at a flux of 1 mL/min. Bound compounds were eluted with a linear gradient of 0–20% acetonitrile for 60 min. Absorbance was recorded at 450 and 360 nm, wavelengths at which the isoalloxazine ring of flavines shows two maximum peaks. FAD ($\varepsilon_{450\ nm}$=11.3 mM^{-1} cm^{-1}) [19–21] and FMN ($\varepsilon_{450\ nm}$=12.2 mM^{-1} cm^{-1}) [22] were used as standards.

3. Results

3.1. Purification and structural characterization of D-iLDH

Usual methodologies reported for bacterial D-iLDH purification are based on enzyme extraction with diverse non-ionic detergents, followed by ionic exchange and molecular exclusion chromatographies [5,20,23]. Our initial attempts to purify the D-iLDH from *E. gracilis* mitochondria were based on a similar strategy: pre-heating of the CHAPS-solubilized mitochondria at 50 °C for 20 min, followed by affinity chromatography (oxamate-Sepharose). An increase in specific activity after each purification step was achieved (for instance, from 50 nmol DCPIP reduced min^{-1} mg $protein^{-1}$ in homogenate up to 2000 nmol DCPIP reduced min^{-1} mg $protein^{-1}$, after affinity chromatography). However, poor enrichment of a 60-kDa protein was achieved (data not shown), which is a molecular weight similar to that reported for bacterial D-iLDH [2,5,20].

Taking advantage of the high thermo-stability of the D-iLDH [12], the CHAPS-solubilized mitochondrial solution was incubated at 60 °C for 45–50 min in the presence of 10 mM oxalate, as protecting ligand, to denature heat-sensitive proteins. With this rapid and easy procedure, a highly enriched fraction of a 62-kDa protein was obtained (Fig. 1A) with 80–85% of activity recovery and 29-fold enriched D-iLDH activity (Table 1). In addition, the 62-kDa protein showed D-iLDH activity in 10% polyacrylamide native gel electrophoresis (Fig. 1B).

The visible absorption spectrum of the purified enzyme showed a broad peak around 450 nm and a shoulder at around 488 nm, which suggested the presence of a flavoprotein [5,20,21]. Treatment of the isolated D-iLDH with TCA yielded a supernatant that showed fluorescence with two excitation peaks at 366 and 450 nm, which are typical of flavines (data not shown) [5,20,23]. All bacterial and yeast D-iLDH have a FAD molecule as cofactor [1,2,5,20]. In agreement with this notion, a compound with an identical retention time to FAD was identified by RP-HPLC (Fig. 1C), indicating that the purified *E. gracilis* D-iLDH contains a FAD molecule as cofactor. Similarly to bacterial and yeast enzymes [3,5,6,20], D-iLDH from *E.*

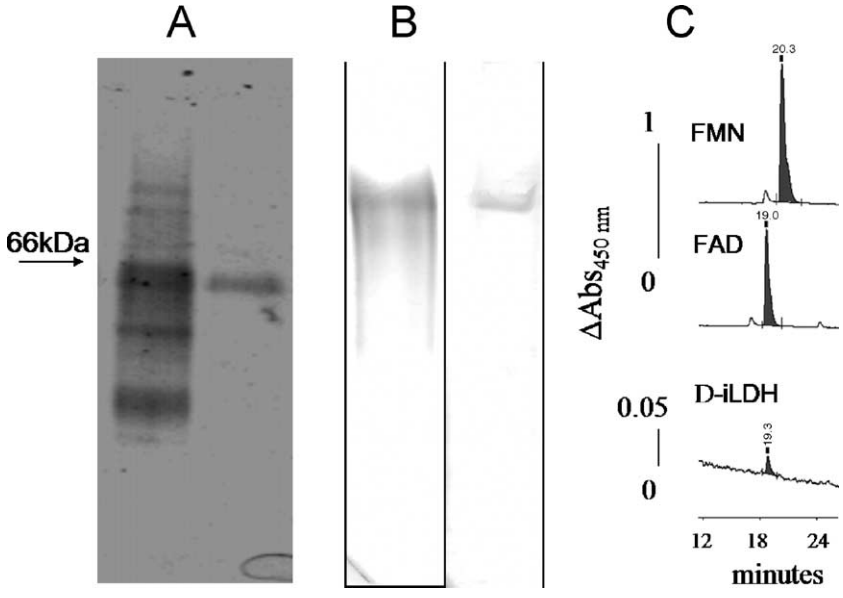

Fig. 1. Purification and flavine identification of mitochondrial D-iLDH from *E. gracilis*. (A) D-iLDH purification by heat treatment. Five μg protein were loaded onto a 12% acrylamide gel and stained with Coomassie blue. The molecular weight markers used ranged from 6.5 up to 205 kDa. Left lane, CHAPS-solubilized mitochondria and right lane, heat treatment supernatant. The last fraction showed 85% D-iLDH purity as judged by densitometric analysis (data not shown). (B) This last fraction of purified D-iLDH (5 μg protein) was also electrophorased in a native 10% acrylamide gel. Activity (left lane) was revealed by incubating at room temperature with 0.001% PMS, 0.001% NBT and 5 mM D-lactate for 5–10 min, whereas protein (right lane) was detected by staining with Coomassie blue. Omission of D-lactate or addition of 10 mM oxalate produced no color development (data not shown). (C) The flavine nature of isolated D-iLDH was determined by HPLC on reverse phase C18 column and at 450 nm. 4.2 nmol FMN (upper), 2.5 nmol FAD (middle), and 0.4 mg of D-iLDH acid extract (bottom) were eluted with a linear gradient of acetonitrile for 60 min (see methods section). Retention times are shown in the figure.

Table 1
Purification of D-iLDH from *E. gracilis*

	Total protein (mg)	Specific activity nmol/min × mg protein	Total activity nmol/min	Times of purification	Yield (%)
Mitochondria	51±8	210±82	10,700±2190	1	100
Solubilized mitochondria	21±2	510±100	10,710±180	2.4	100
60 °C, 45′ supernatant	1.57±0.25	6000±1400	9450±1500	29	88±6

Activity was measured in presence of 0.12% (w/v) Triton X-100 as described under Materials and methods. Values shown represent the mean±S.E.M. of at least 4 different independent preparations.

gracilis showed a molecular weight of 68 kDa by molecular exclusion chromatography, suggesting that active D-iLDH is a monomer (data not shown). Differences in molecular weight values found by exclusion chromatography (68 kDa) and by SDS-PAGE (62 kDa) might be related to the additional weight brought about by the presence of detergent micelles in the former case.

3.2. Kinetic characterization of D-iLDH and L-iLDH

The K_m values of the purified D-iLDH for D-lactate, DCPIP, and Q1, as well as the K_i value for oxalate, were very similar to those previously determined in intact *E. gracilis* mitochondria (Table 2) [12]. Similar to the *Neisseria meningitidis* enzyme [23], *E. gracilis* D-iLDH did not oxidize L-lactate (1.5% of D-lactate oxidation); instead, L-lactate inhibited D-lactate oxidation (Table 2). These data indicated that the enzyme from *E. gracilis* is specific for D-lactate, whereas L-lactate may physiologically modulate D-iLDH activity [13]. Pyruvate did not inhibit the D-iLDH activity at the concentration found into the cell (0.5 mM), whereas at 20 mM, 22% inhibition was observed.

D-iLDH was able to reduce Q1 and TCHQ, quinones with high (+100 mV) and low (−8 mV) redox potential [24], respectively (Fig. 2A, Table 2). The ability of D-iLDH to reduce quinones of high and low redox potential implied that both, ubiquinone-9 (+100 mV) and rhodoquinone-9 (−65 mV) [25] which are present in the mitochondrial quinone pool of *E. gracilis* [26], may be physiological substrates of D-iLDH. Similar to the *E. coli* enzyme, *E.*

Table 2
Kinetic parameters of the purified D-iLDH from *E. gracilis*

	E. gracilis D-iLDH	*E. gracilis* mitochondria	*E. coli* D-iLDH[a]
K_m D-lactate (mM)	2.0±0.8	2.7±0.3[b]	1.0
K_m DCPIP (μM)	10	35±5[b]	–
V_m (nmol DCPIP/min × mg)	6000±1400	480±40[b]	15,000
K_m Q1 (μM)	8.1	18.5±5[b]	8.5
V_m (nmol Q1/min × mg)	7000	620±70[b]	–
K_m TCHQ (μM)	21	30	–
V_m (nmol TCHQ/min × mg)	1480	310	–
K_i oxalate (mM)	0.14 (Mixed type)	0.09[b] (Mixed type)[c]	0.001 (Competitive)
K_i L-lactate (mM)[d]	10 (Mixed type)	19 (Competitive)	*Km* = 16 (Substrate)
$K_{0.5}$ Triton X-100 (%)	0.005	No effect	0.003

[a] Data reported by Futai [16].
[b] Data obtained with mitochondria were taken from [10] or determined in the present work.
[c] The global linear regression analysis of the oxalate inhibition data best fitted to a mixed-type inhibition [36]: $v = S \times V_m / [1+(I/(\alpha \times K_i))] / [K_m \times ((1+(I/K_i))/(1+(I/(\alpha \times K_i))) \times S]$, where v is the velocity; S is the substrate concentration; V_m is the maximal velocity, I is the inhibitor concentration, *Km* is the Michaelis–Menten constant, K_i is the inhibition constant, and α is the factor by which the substrate and inhibitor dissociation constants are affected when enzyme is in the presence of the other ligand. The factor α was 7 and 4 for oxalate and L-lactate, respectively.
[d] This parameter was determined in CHAPS-solubilized mitochondria.

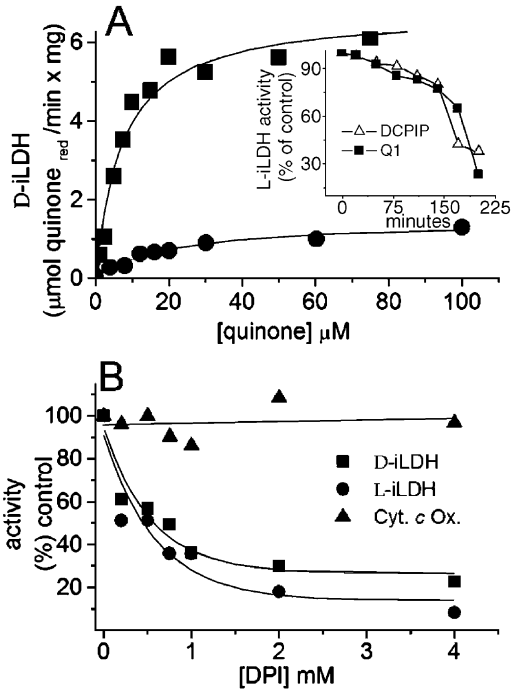

Fig. 2. Kinetics of quinone reduction and effect of DPI on D-iLDH and L-iLDH from *E. gracilis*. (A) 10 μg of Isolated D-iLDH was incubated at room temperature (27 °C) in 1 mL of HE buffer supplemented with 0.2 (w/v) CHAPS, and 0.12% (w/v) Triton X-100 pH 8 as described under Materials and methods. The reaction was started with 40 mM D-lactate in presence of different concentrations of Q1 (■) or TCHQ (●) as electron acceptors. Solubilized L-iLDH was also able to reduce Q1, but it was rapidly inactivated after solubilization (inset). (B) Isolated enzyme (D-iLDH) or isolated mitochondria (L-iLDH) were incubated in presence of different concentrations of DPI as described under Materials and methods. 100% of activity of isolated D-iLDH was 1566 nmol × (min × mg protein)$^{-1}$, 177 nmol × (min × mg protein)$^{-1}$ for L-iLDH, and 350 nano atoms oxygen × (min × mg protein)$^{-1}$ for cytochrome *c* oxidase. Experimental data are the mean of two experiments.

gracilis D-iLDH was activated by 0.12% (w/v) Triton X-100. Remarkably, addition of 0.1 mM FAD or FMN, or 0.03 mM cytochrome *c* did not increase the D-iLDH activity (data not shown).

Several works have determined that bacterial L-iLDH is inactivated in detergent-solubilized membranes [2,20,23]. We previously described that, for the *E. gracilis* enzyme, 11 mM β-octyl glucopyranoside solubilized up to 40–45% of total L-iLDH activity; however, such activity was lost after few hours [12]. Solubilization of mitochondria with β-octyl glucopyranoside at its critical micellar concentration (22 mM, indicated by the supplier Calbiochem™) brought about full inactivation of L-iLDH, suggesting a key role of membrane lipids for the enzyme functionality. However, in a fraction solubilized with Lubrol (0.98% w/v), the L-iLDH activity was stable at pH values of 7.6–9 for at least 60 min; such preparation was able to reduce Q1 in an oxalate-sensitive reaction (Fig. 2A, inset).

Diphenyleneiodonium chloride (DPI) has been used as a specific flavoenzyme inhibitor as it reacts with flavines to form irreversible adducts [17]. The activities of both, mitochondrial L-iLDH and isolated D-iLDH, were 93 and 75% inhibited by 4 mM DPI (Fig. 2B), with IC_{50} values of 0.44 and 0.56 mM, respectively.

3.3. Metabolic role of nLDH

Smillie showed in crude extracts of *E. gracilis* that D-lactate oxidation by nLDH was 3 times faster than L-lactate oxidation [27]. In our hands, the nLDH activity in an enriched cytosolic fraction of *E. gracilis* was 6 and 2 nmol × (min × mg protein)$^{-1}$, for D- and L-lactate oxidation, respectively [13], although these activities were 50–100 times lower than pyruvate reduction activity. Similar to L-nLDH from some hetero-fermentative bacteria [18,28–33], nLDH from *E. gracilis* was activated by FBP following a hyperbolic kinetic pattern. The $K_{0.5}$ value of L-nLDH for FBP was 0.22 ± 0.03 mM (n = 4, mean ± S.E.M.). FBP was a non-essential activator whose effect was to diminish the K_m values for NADH and pyruvate, from 0.082 ± 0.009 and 1.1 ± 0.1 mM, to 0.024 ± 0.003 and 0.47 ± 0.03 mM (n = 4; mean ± S.E.M.), respectively. The K_m value for pyruvate in the presence of FBP is identical to the pyruvate intracellular concentration (0.5 mM) [13]. In contrast to the cooperative FBP-dependent L-nLDH from *Bifidobacterium longum* [30,31], the enzyme from *E. gracilis* was inhibited by oxamate, a typical inhibitor of mammalian nLDH, with a K_i value of 1.2 mM; oxalate (20 mM) did not affect *E. gracilis* nLDH activity (data not shown).

In an attempt to elucidate whether the FBP activation on nLDH might have physiological relevance, the intracellular concentration of FBP in cells cultured in glutamate/malate or lactate as carbon source was determined. In general, the intracellular concentration of FBP was similar in both cultures, although it changed with the progress of the growth curve, from 0.26 ± 0.04 mM in the first 20 h of culture, to 0.51 ± 0.02 mM in the logarithmic phase, and up to 0.75 ± 0.04 mM in the stationary phase (n = 4; mean ± S.E.M.). This suggested that in *E. gracilis*, the L-nLDH activation by FBP may be relevant throughout the different growth phases.

3.4. Biochemical characterization of nLDH

In some homo-fermentative bacteria, FBP-activated L-nLDH may show either hyperbolic or sigmoidal saturation kinetics with respect to pyruvate. Co^{2+} and Mn^{2+} may also activate the enzyme [28,29,32,33], depending on the organism and pH of the reaction. Thus, the pH dependence of *E. gracilis* nLDH was analyzed in the cytosolic fraction with and without FBP or metals. The pH optimum for nLDH activity was 6.5–7.0. Activity in presence of 2 mM Mn^{2+} was approximately 33% higher than basal activity only at pH values of 7.7–8.5. FBP (0.8 mM) displaced the optimum pH value to 8.0 and induced a 40% increase in the basal nLDH activity. Co^{2+} did not affect nLDH activity.

Steps of nLDH purification are shown in Table 3. The nLDH activity was measured in the pyruvate reduction direction. The nLDH profile after passing the cytosolic fraction through the blue-Sepharose column showed 2 peaks of activity, the first eluting with the washing buffer (blue$^-$) (and which was not further examined), whereas the second was eluted with a 0–1 M NaCl gradient (blue$^+$) (Fig. 3A). The concentrated blue$^+$ fractions were pooled and loaded

Table 3
Separation of the cytosolic D-nLDH and L-nLDH from *E. gracilis*

	Total Protein (mg)	Specific activity (nmol $NADH_{ox}$ /min × mg protein)	Total activity (nmol /min)	Times of purification	Yield (%)
nLDH					
Homogenate	22,472	15	337,080	1	100
Cytosol	4240	75	318,000	5	94
70% $(NH_4)_2SO_4$ saturation	512	276	141,400	18.5	42
L-*nLDH*[a]					
Blue-seph	5.2	9025	46,930	601	14
Oxamato-seph	2.0	11,401	22,802	760	6.8
D-*nLDH*[b]					
Oxamato-seph$^-$	16.4	4380	70,550	292	21

[a] Enzymatic activity was measured in HE buffer plus 0.15 M ammonium sulfate. Concentrations of NADH and pyruvate were 0.3 and 5 mM, respectively. Oxamate-Sepharose pooled fractions yielded an activity of 3350 nmol L-lactate × (min × mg protein)$^{-1}$ in the absence of added NH_4^+ (which was an *E. gracilis* L-nLDH activator; see text), and did not generate D-lactate.

[b] Oxamate-seph$^-$ pooled fractions (enzyme activity not bound to the resin) produced 4350 nmol D-lactate × (min × mg protein)$^{-1}$, but did not generate L-lactate (see text). Times of purification were calculated by using the ratio of the increment of specific activity in each step/specific activity in homogenate.

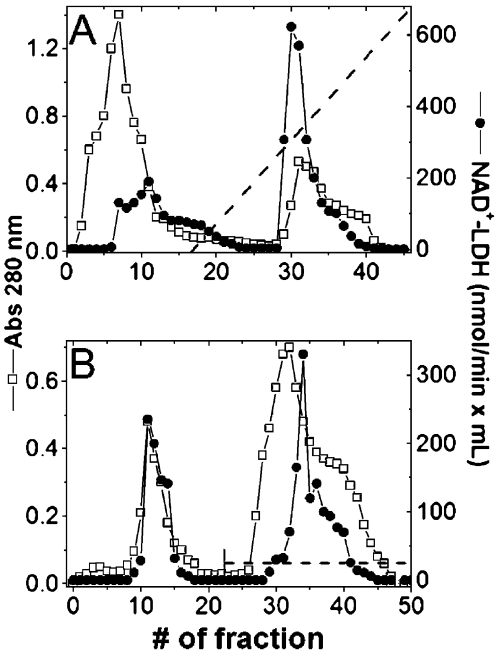

Fig. 3. Elution profiles of nLDH from affinity chromatography. (A) Blue-Sepharose chromatography. Buffer flux was 1.5 mL/min. The dashed line shows the 0–1 M NaCl gradient. (B) Oxamate-Sepharose chromatography. The HEBNF buffer flux was 1 mL/min. The dashed line shows the range in which HEBNF buffer was replaced by HEB buffer (see Materials and methods). The volume of the fractions was 6 mL. Absorbance at 280 nm (□), pyruvate reductase activity (●).

onto an oxamate-Sepharose column previously equilibrated with HEBNF buffer. In agreement with the reported ordered kinetic mechanism for bacterial L-nLDHs [18,29], the presence of NADH and FBP to increase the affinity for oxamate [18,28] was required for column enzyme binding.

Like the elution profile of a cell extract from *Lactobacillus plantarum*, which contains variable proportions of both D- and L-specific nLDHs [18], elution of the blue$^+$ fractions through the oxamate-Sepharose resin showed two peaks of activity (Fig. 3B). These corresponded to D-nLDH, which eluted by washing with HEBNF buffer, and L-nLDH, which remained strongly adsorbed to the resin, but eluted when HEBNF buffer was replaced by HEB buffer (Fig. 3B). These two nLDH activities were determined in the direction of pyruvate consumption (Table 3). SDS-PAGE of both D- and L-nLDH enriched fractions showed a 35-kDa protein, which was accompanied by significant amounts of contaminating proteins, resulting in a purity lower than 40% for both enzymes (data not shown). Exclusion molecular chromatography showed molecular weights of 126–132 kDa, indicating a tetrameric structure for both enzymes (data not shown). This was in agreement with the data reported for bacterial L-nLDH but not for bacterial D-nLDH which is a dimer [28,33]. Storage of D- and L-nLDH in 50% glycerol at −70 °C resulted in total loss of activity in the first 24 h, whereas there was 35–40% loss of both activities after 72 h when stored in saturated ammonium sulfate at 4 °C.

Table 4
Kinetic parameters of the semi purified D-nLDH and L-nLDHfrom *E. gracilis*

	D-NAD$^+$-LDH	L-NAD$^+$-LDH (+1 mM FBP)
$K_{m\ Pyruvate}$ (mM)	0.33±0.09 (3)	0.44±0.1 (3)
$K_{m\ NADH}$ (μM)	41 (2)	8±0.001 (3)
V_m Forward (μmol $NADH_{ox}$/min × mg protein)	20.5 (2)	19.3 (2)
V_m Reverse (μmol $NADH_{prod}$ /min × mg protein)	0.02 (2)	Not detected
$K_{i\ NAD}$ (μM)	0.13 (2) C	0.65 (2) C
$K_{i\ D\text{-lactate}}$ (mM)	15, α=4.5 (2) M	–

The values shown are the mean±S.E.M., with the number of different preparations assayed between parentheses. The determination of inhibition patterns was made at saturated concentrations of the other substrate. C: Competitive inhibition; M: mixed-type inhibition.

The optimum pH values of the enriched D- and L-nLDH activities were 8.5 and 9.2, respectively. Their kinetic parameters (Table 4) were similar to those of the respective bacterial nLDH [28,29,32–35]. D-nLDH showed substrate inhibition by pyruvate above 3 mM (Fig. 4), but did not by NADH (data not shown). In these enriched preparations, oxidation of D-lactate was lower than the rate of the forward reaction, whereas L-lactate oxidation was not detected (Table 4). The intersecting pattern for both pyruvate and NADH in a double reciprocal plot for *E.*

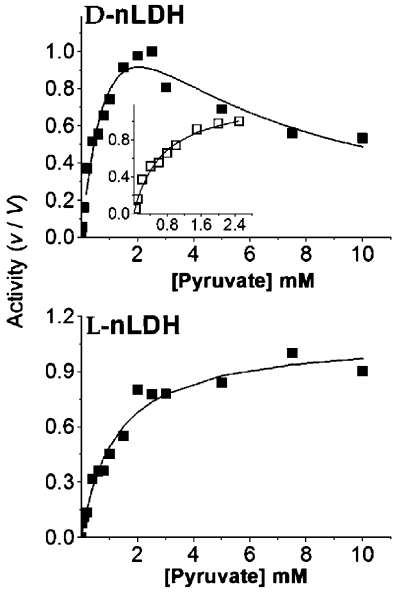

Fig. 4. Reduction of pyruvate by D-nLDH and L-nLDH. Activities were measured in the presence of 0.3 mM NADH, and in the absence of both FBP and ammonium sulfate. D-nLDH kinetic pattern was fitted to a substrate inhibition equation, whereas that of L-nLDH was fitted to the Michaelis–Menten equation (solid lines) [36]. In these representative experiments, V_m values were 30 and 19.3 μmol × (min × mg protein)$^{-1}$ for D-nLDH and L-nLDH, respectively. K_i (inhibition constant) value for pyruvate was 3.1 mM. Inset shows the Michaelis–Menten kinetic pattern of D-nLDH at low pyruvate concentrations.

gracilis L-nLDH suggests either a steady-state bi–bi ordered mechanism or a rapid equilibrium bi–bi random mechanism (data not shown) [36]. However, L-nLDH was adsorbed to the oxamate-Sepharose resin only in the presence of NADH; this is an observation consistent with an ordered mechanism, in agreement with observations in other bacterial FBP-activated L-nLDHs [18,29]. Contrary to bacterial L-nLDH, the *E. gracilis* enzyme showed 2- and 3-fold activation by 30 mM K^+ and 150 mM NH_4^+, respectively.

D-lactate exerted a mixed-type inhibition on D-nLDH versus pyruvate (at saturating NADH concentration), while NAD^+ showed a competitive inhibition versus NADH at saturated pyruvate concentration (Table 4). The inhibition pattern by the products of D-nLDH fitted well with a bi–bi ordered kinetic mechanism [36]. In this regard, the intracellular NAD^+ concentration determined in *E. gracilis* grown with glutamate/malate did not change throughout the progress of the growth curve (0.8 ± 0.08 mM, $n = 11$, mean ± S.E.M.). Similar to D-nLDH from bacteria, *E. gracilis* D-nLDH did not show activation by either FBP, NH_4^+ or divalent cations such as Mn^{2+} or Co^{2+} (data not shown).

4. Discussion

The aerobic energy metabolism in *E. gracilis* seems to be closely linked to the mitochondrial lactate oxidation through the functioning of a lactate shuttle constituted by mitochondrial membrane D- and L-iLDH, and by cytosolic nLDHs. In the present work, D-iLDH was purified and characterized; and two nLDHs were identified, one specific for the production of D-lactate and an unusual FBP-activated L-nLDH, specific for L-lactate formation.

The molecular weight, cofactor nature, kinetic parameters, and substrate specificity determined for the *E. gracilis* D-iLDH were almost identical to those reported for bacterial and yeast enzymes [1,5,6]. Remarkably, the ability to reduce quinones (*cf.* Fig. 2A) and the inability to reduce cytochrome *c* (see Results) suggests a common origin for *E. gracilis* and bacterial D-iLDH [5,20,21,23]. Indeed, *E. gracilis* L-iLDH was strongly inhibited by diphenyleneiodonium in contrast to the non-flavin enzyme cytochrome *c* oxidase (Fig. 2B), suggesting that L-iLDH from *E. gracilis* is also a flavoenzyme.

Moreover, yeast L-iLDH is a soluble tetramer [2,4] while bacterial and *E. gracilis* L-iLDHs are membrane-bound monomers [2,7,12], which are inactivated by detergent solubilization [20,23] (see also Fig. 2A, inset); this last observation suggests that both bacterial and protist L-iLDHs require lipids for their functional structure. Then, the data of the present work suggest that mitochondrial iLDHs from *E. gracilis* might share a common ancestor with the bacterial enzymes. It should be emphasized that sequence similarity between enzymes from different organisms does not suffice to accurately predict function similarity [37], thus making essential to carry out structural and kinetic studies.

In virtually all organisms, cytosolic L-lactate is formed by L-nLDH from pyruvate and NADH. In *E. coli* and other bacteria, D-lactate is the final product of both glycolysis and the methylglyoxal pathway [34,35,38]. In most of the studied eukaryotic organisms, D-lactate is synthesized exclusively by enzymes located in the methylglyoxal pathway (glyoxylases I and II). *Trypanosoma* sp., the most closely phylogenetic relative of *Euglena* [39] does not have D-nLDH; then, this parasite produces D-lactate by the methylglyoxal pathway [40]. Methylglyoxal is a toxic metabolite generated under conditions of highly active glycolysis [40]. In *E. gracili*s, glycolysis accelerates when paramylon catabolism is started [13]. Then, it is likely that in *E. gracilis* D-lactate may also be produced in high quantities by the methylglyoxal pathway, although this possibility remains to be examined.

We have previously showed that lactate formation in the cytosol of *E. gracilis* strongly depends on paramylon catabolism, and that lactate oxidation mediated by the mitochondrial lactate shuttle is essential in this protist bioenergetics when external carbon sources are scarce [13]. Thus, activation of the glycolytic L-nLDH by FBP ensures a constant flux towards lactate production for ATP synthesis from both glycolysis and oxidative phosphorylation. Likewise, to stimulate glycolysis and lactate production in the hetero-fermentative bacteria *Lactobacillus casei*, the culture medium is supplemented with substrates that lead to the formation of high FBP concentrations and hence to L-nLDH activation [28]. Like in *Streptococcus faecalis* [29], the *E. gracilis* L-nLDH showed hyperbolic saturation kinetics, and it was activated by FBP in a non-essential manner, such that the K_m values for both pyruvate and NADH were decreased.

FBP- and divalent cation-activated L-nLDHs are widespread in homo- and hetero-fermentative bacterial systems [18,28–32]. Regardless of the high identity percentage among the amino acid sequences of bacterial L-nLDH (up to 66%) [28], striking differences in the enzyme kinetics of the FBP-activated L-nLDH have been described. For instance, FBP activation can be essential [28,30,31,41,42] or non-essential [18,28,29,32,33,43], and the enzyme may show hyperbolic or sigmoidal kinetic respect to pyruvate [29–31]. These observations suggest that these enzymes probably diverged recently [42]. Moreover, the FBP-activated L-nLDHs are not present in all bacteria, suggesting that the enzyme in *E. gracilis* was the result of a lateral gene transfer event, which appears to be a common event in this protist [44–46] and in its parasitic relative *Leishmania major* [47].

Kinetic data showed that the D-nLDH K_i value determined for D-lactate was lower than its intracellular concentration [13]. This physiological inhibition by its own product ensures an efficient reducing equivalent transfer

from cytosol to mitochondria. On the other hand, the K_i value for NAD^+ was 6 times smaller than the NAD^+ concentration in *Euglena* (Table 4, see text). This high sensitivity for NAD^+ suggests that this enzyme may be fully active even if the glycolysis flux, and hence the NADH/NAD^+ ratio, are high. Hence, the activity of D-nLDH may be regulated by its products, depending on the cell growth conditions, while L-nLDH activity may be modulated also by changes in FBP intracellular concentration.

Assuming similar activities for the four lactate shuttle enzymes in crude extract ([13], see Table 3), and considering the K_m values for pyruvate (D- and L-nLDH, see Results section) and lactate (D- and L-iLDH [12]), the catalytic efficiencies (V_m/K_m) were 2–3 times higher for both nLDHs than those for iLDHs. This implies that D- and L-lactate (cytosolic) production is faster than their (mitochondrial) consumption, which is in agreement with the high intracellular concentrations of the two-lactate isomers previously described [13]. These data suggest that the flux control of the L- and D-lactate production may be mainly located in the consuming block (mitochondrial iLDHs).

Acknowledgements

The present work was partially supported by grants 203313 from PAEP, Facultad de Química, UNAM, México; and 43811-Q from CONACyT, México.

References

[1] E.I. Garvie, Bacterial lactate dehydrogenases, Microbiol. Rev. 44 (1980) 106–139.
[2] C.A. Fewson, D.P. Baker, R.M. Chalmers, J.N. Keen, I.D. Hamilton, A.J. Scott, M. Yasin, Relationships amongst some bacterial and yeast lactate and mandelate dehydrogenases, J. Gen. Microbiol. 139 (1993) 1345–1352.
[3] W.J. Ingledew, R.K. Poole, The respiratory chains of *Escherichia coli*, Microbiol. Rev. 48 (1984) 222–271.
[4] G. Daum, P.C. Ovni, G. Schatz, Import of proteins into mitochondria. Cytochrome b_2 and cytochrome *c* peroxidase are located in the intermembrane space of mitochondria, J. Biol. Chem. 257 (1982) 13028–13033.
[5] M. Futai, Membrane D-lactate dehydrogenase from *Escherichia coli*. Purification and properties, Biochemistry 12 (1973) 2468–2474.
[6] T. Lodi, Y. Ferrero, Isolation of the DLD gene of *Saccharomyces cerevisiae* encoding the mitochondrial enzyme D-lactate ferricytochrome *c* oxidoreductase, Mol. Gen. Genet. 238 (1993) 315–324.
[7] N. Haugaard, D- and L-lactic acid oxidase of *E. coli*, Biochim. Biophys. Acta 31 (1968) 66–72.
[8] S. Iuchi, E.C.C. Lin, *arc* A (dye), a global regulatory gene in *Escherichia coli* mediating repression of enzymes in aerobic pathways, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 1888–1892.
[9] M. Somlo, Induction des lactico-cytochrome *c* reductase (D- et L-) de la leuvre aerobie par les (D- et L-), Biochim. Biophys. Acta 97 (1965) 183–201.
[10] O. Dym, D. Eisenberg, Sequence–structure analysis of FAD-containing enzymes, Protein Sci. 10 (2001) 1712–1728.
[11] R. Moreno-Sánchez, R. Covián, R. Jasso-Chávez, S. Rodríguez-Enríquez, F. Pacheco-Moisés, M.E. Torres-Márquez, Oxidative phosphorylation supported by an alternative respiratory pathway in mitochondria from *Euglena*, Biochim. Biophys. Acta 1457 (2000) 200–210.
[12] R. Jasso-Chávez, M.E. Torres-Márquez, R. Moreno-Sánchez, The membrane-bound L- and D-lactate dehydrogenase activities in mitochondria from *Euglena gracilis*, Arch. Biochem. Biophys. 390 (2001) 295–303.
[13] R. Jasso-Chávez, R. Moreno-Sánchez, Cytosol-mitochondria transfer of reducing equivalents by a lactate shuttle in heterotrophic *Euglena*, Eur. J. Biochem. 270 (2003) 4942–4951.
[14] R. Moreno-Sánchez, J.C. Raya, Preparation of coupled mitochondria from *Euglena* by sonication, Plant Sci. 48 (1987) 151–157.
[15] H.U. Bergmeyer, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Vol. 3–9, Weinheim Verlag Chemie, Germany, 1983.
[16] J.M. Armstrong, The molar extinction coefficient of 2, 6-dichlorophenol indophenol, Biochim. Biophys. Acta 86 (1964) 194–197.
[17] S. Chakraborty, V. Massey, Reaction of reduced flavins and flavoproteins with diphenyliodonium chloride, J. Biol. Chem. 277 (2002) 41507–41516.
[18] N. Kelly, M. Delaney, P. OCarra, Affinity chromatography of bacterial lactate dehydrogenases, Biochem. J. 171 (1978) 543–547.
[19] C.A. Sheppard, E.E. Trimmer, R.G. Matthews, Purification and properties of NADH-dependent 5,10-Methylenetetrahydrofolate reductase (MetF) from *Escherichia coli*, J. Bacteriol. 181 (1999) 718–725.
[20] N. Allison, M.J. ODonell, M.E. Hoey, C.A. Fewson, Membrane-bound lactate dehydrogenase and mandelate dehydrogenase of *Acinetobacter calcoaceticus*, Biochem. J. 231 (1985) 407–416.
[21] E.A. Pratt, L.W.-M. Fung, J.A. Flowers, C. Ho, Membrane-bound D-lactate dehydrogenase from *Escherichia coli*: purification and properties, Biochemistry 18 (1979) 312–316.
[22] M.V. Fonseca, J.C. Escalante-Semerena, Reduction of Cob (III)alamin to Cob (II)alamin in *Salmonella enterica* Serovar Typhimurium LT2, J. Bacteriol. 182 (2000) 4304–4309.
[23] A.L. Erwin, E.C. Gotschlich, Oxidation of D-lactate and L-lactate by *Neisseria meningitidis*: purification and cloning of meningococcal D-lactate dehydrogenase, J. Bacteriol. 175 (1993) 6382–6391.
[24] J. Kim, T.D. Chung, H. Kim, Determination of biologically active acid based on the electrochemical reduction of quinone in acetonitrile+water mixed solvent, J. Electroanal. Chem. 499 (2001) 78–84.
[25] D.E. Buetow, The mitochondrion, in: D.E. Buetow (Ed.), The Biology of *Euglena*, vol. IV, Academic Press, New York, 1989, pp. 247–314.
[26] N.A. Castro-Guerrero, K. Krab, R. Moreno-Sánchez, The alternative respiratory pathway in *Euglena* mitochondria, J. Bioenerg. Biomembr. 36 (2004) 459–469.
[27] R.M. Smillie, Enzymology of *Euglena*, in: D.E. Buetow (Ed.), The Biology of *Euglena*, Vol. II, Academic Press, New York, 1968, pp. 2–54.
[28] R. Hensel, U. Mayr, K.O. Stetter, O. Kandler, Comparative studies of lactate dehydrogenases in lactic acid bacteria: amino-acid composition of an active-site region and chemical properties of the L-lactate dehydrogenase of *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, and *Lactobacillus acidophilus*, Arch. Microbiol. 112 (1977) 81–93.
[29] M.J. Hardman, G.G. Pritchard, Kinetics of activation of L-lactate dehydrogenase from *Streptococcus faecalis* by fructose 1, 6-bisphosphate and by metal ions, Biochim. Biophys. Acta 912 (1987) 185–190.
[30] S. Iwata, T. Ohta, Molecular basis of allosteric activation of bacterial L-lactate dehydrogenase, J. Mol. Biol. 230 (1993) 21–27.
[31] S. Fushinobu, K. Kamata, S. Iwata, H. Sakai, T. Ohta, H. Matsuzawa, Homotropic activation via the subunit interaction and allosteric symmetry revealed on analysis of hybrid enzymes of L-lactate dehydrogenase, J. Biol. Chem. 273 (1996) 2971–2976.
[32] H.A. Wyckoff, J. Chow, T.R. Whitehead, M.A. Cotta, Cloning,

sequence, and expression of L-(+) lactate dehydrogenase of *Streptococcus bovis*, Curr. Microbiol. 34 (1997) 367–373.
[33] H.W. Doelle, Nicotinamide adenine dinucleotide-dependent and nicotinamide adenine dinucleotide-independent lactate dehydrogenases in homofermentative and heterofermentative lactic acid bacteria, J. Bacteriol. 108 (1971) 1284–1289.
[34] M. Ogata, K. Arihata, T. Yagi, D-lactate dehydrogenase of *Desulfovibrio vulgaris*, J. Biochem. 89 (1981) 1423–1431.
[35] S. Kochhar, H. Hottinger, N. Chuard, P.G. Taylor, T. Atkinson, M.D. Scawen, D.J. Nicholls, Cloning and overexpression of *Lactobacillus helveticus* D-lactate dehydrogenase in *Escherichia coli*, J. Biochem. 208 (1992) 799–805.
[36] Y.H. Segel, Enzyme Kinetics, John Wiley and Sons, New York, 1975.
[37] M.L. Cárdenas, A. Cornish-Bowden, T. Ureta, Evolution and regulatory role of the hexokinases, Biochim. Biophys. Acta 1401 (1998) 242–264.
[38] I.R. Booth, G.P. Ferguson, S. Miller, C.L.B. Gunasekera, S. Kinghorn, Bacterial production of methylglyoxal: a survival strategy or death by misadventure? Biochem. Soc. Trans. 31 (2003) 1406–1408.
[39] A.H. Knoll, The early evolution of eukaryotes: a geological perspective, Science 256 (1992) 622–627.
[40] J.J. Cazzullo, Aerobic fermentation of glucose by trypanosomatids, FASEB J. 6 (1992) 3153–3161.
[41] K. Arai, T. Kamata, Y. Uchikoba, S. Fushinobu, H. Matsuzawa, H. Taguchi, Some *Lactobacillus* L-lactate dehydrogenases exhibit comparable catalytic activities for pyruvate and oxaloacetate, J. Bacteriol. 183 (2001) 397–400.
[42] K. Arai, A. Hishida, M. Ishiyama, T. Kamata, H. Uchikoba, S. Fushinobu, H. Matsuzawa, H. Taguchi, An absolute requirement of fructose 1, 6-bisphosphate for the *Lactobacillus casei* L-lactate dehydrogenase activity induced by a single amino acid substitution, Protein Eng. 15 (2002) 35–41.
[43] H. Taguchi, T. Ohta, Unusual amino acid substitution in the anion-binding site *Lactobacillus plantarum* non-allosteric L-lactate dehydrogenase, Eur. J. Biochem. 209 (1992) 993–998.
[44] J.D. Palmer, C.F. Delwiche, Second-hand chloroplasts and the case of the disappearing nucleus, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7432–7435.
[45] R.M. Figge, M. Schubert, H. Brinkmann, R. Cerff, Glyceraldehyde-3-phosphate dehydrogenase gene diversity in eubacteria and eukaryotes: evidence for intra- and inter-kingdom gene transfer, Mol. Biol. Evol. 16 (1999) 429–444.
[46] K. Matsui, N. Ishii, Z. Kawabata, Release of extracellular transformable plasmid DNA from *Escherichia coli* co cultivated with algae, Appl. Environ. Microbiol. 69 (2003) 2399–2404.
[47] T.J. Vickers, N. Greig, A.H. Fairlamb, A trypanothione-dependent glyoxalase I with a prokaryotic ancestry in *Leishmania major*, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 13186–13191.