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Biochimica et Biophysica Acta 1708 (2005) 13 – 22
BIOCHIMICA ET BIOPHYSICA ACTA
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Transport and metabolism of D-lactate in Jerusalem artichoke mitochondria

Anna Atlante[a], Lidia de Bari[a,b], Daniela Valenti[a], Roberto Pizzuto[b], Gianluca Paventi[b], Salvatore Passarella[b,*]

[a]*Istituto di Biomembrane e Bioenergetica, CNR, Via G. Amendola 165/A 70126, Bari, Italy*
[b]*Dipartimento di Scienze Animali, Vegetali e dell'Ambiente, Università del Molise, Via De Sanctis, 86100 Campobasso Italy*

Received 21 May 2004; received in revised form 8 March 2005; accepted 9 March 2005
Available online 31 March 2005

Abstract

We report here initial studies on D-lactate metabolism in Jerusalem artichoke. It was found that: 1) D-lactate can be synthesized by Jerusalem artichoke by virtue of the presence of glyoxalase II, the activity of which was measured photometrically in both isolated Jerusalem artichoke mitochondria and cytosolic fraction after the addition of *S*-D-lactoyl-glutathione. 2) Externally added D-lactate caused oxygen consumption by mitochondria, mitochondrial membrane potential increase and proton release, in processes that were insensitive to rotenone, but inhibited by both antimycin A and cyanide. 3) D-lactate was metabolized inside mitochondria by a flavoprotein, a putative D-lactate dehydrogenase, the activity of which could be measured photometrically in mitochondria treated with Triton X-100. 4) Jerusalem artichoke mitochondria can take up externally added D-lactate by means of a D-lactate/H^+ symporter investigated by measuring the rate of reduction of endogenous flavins. The action of the D-lactate translocator and of the mitochondrial D-lactate dehydrogenase could be responsible for the subsequent metabolism of D-lactate formed from methylglyoxal in the cytosol of Jerusalem artichoke.

Keywords: D-lactate; Jerusalem artichoke mitochondria; D-lactate dehydrogenase; Glyoxalase II; Mitochondrial transport; Methylglyoxal pathway

1. Introduction

There is accumulating evidence for the importance of a methylglyoxal pathway in the physiology of plants. For example, the development of methylglyoxal toxicity in cultured plant cells has been established by [1] and more recently a glyoxalase pathway was reported to play a role in conferring salinity tolerance to plants [2].

The methylglyoxal pathway is responsible for the detoxification of α-oxoaldehydes to the corresponding aldonic acids [3]. In brief, glyoxalase I converts the adduct between methylglyoxal and reduced glutathione (GSH) to *S*-lactoylglutathione (Lact-GSH), from which D-lactate and glutathione are released by glyoxalase II [4]. These processes have been extensively investigated using animal systems [5] and yeasts [6].

As far as plants are concerned, it has been shown that glyoxalase I and II are present in spinach and *Aloe vera* leaves [7] and the existence of a D-lactate producing-glyoxalase II has been reported in *Zea mais* [8]. In addition, a mitochondrial glyoxalase II has been purified from spinach leaves [9] and *Arabidopsis thaliana* [10]. In spite of these advances, there is an important gap in the knowledge of the processes in plants, in that there is currently no information

Abbreviations: AA, antimycin A; ASC, ascorbate; β-NH_2-BUT, β-aminobutyrate; BUT, butyrate; CN^-, cyanide; α-CCN^-, α-cyano-4-hydroxycinnamate; DCIP, dichloroindophenol; D-LAC, D-lactate; D-LDH, D-lactate dehydrogenase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ΔΨ, electrical membrane potential; e.u., enzymatic unit; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; G3P, glycerol-3-phosphate; GSH, reduced glutathione; JAM, Jerusalem artichoke mitochondria; Lact-GSH, *S*-D-lactoylglutathione; L-LAC, L-lactate; L-LDH, L-lactate dehydrogenase; MERS, mersalyl; TNB, 5-thio-2-nitrobenzoic acid; OLIGO, oligomycin; PheSUCC, phenylsuccinate; PHT, phthalonate; PMS, phenazine methosulphate; PYR, pyruvate; RAC, rotenone+antimycin A+cyanide; ROT, rotenone; SUCC, succinate; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; TX-100, Triton X-100; VAL, valinomycin

* Corresponding author. Tel.: +39 080 5443364; fax: +39 080 5443317.
E-mail address: passarel@unimol.it (S. Passarella).

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doi:10.1016/j.bbabio.2005.03.003

about the metabolic fate of the D-lactate that is produced in the pathway, or about the possible role of mitochondria in its metabolism. There is reason to suspect that mitochondria may be involved in the light of our previous work in which it was shown that D-Lactate is transported into the organelles isolated both from rat liver [11] and from *Saccharomyces cerevisiae* [12] and metabolized there.

Here we show the presence of glyoxalase II activity both in the cytosol fraction and in isolated Jerusalem artichoke mitochondria (JAM). Moreover, externally added D-lactate was found to enter mitochondria via a novel carrier, namely the D-lactate/H^+ symporter. We also show that D-lactate can be oxidized by JAM via a putative flavin-dependent D-lactate dehydrogenase (D-LDH). The process involved consumption of oxygen, proton release from mitochondria, generation of membrane potential and synthesis of ATP.

2. Materials and methods

2.1. Materials

ADP, antimycin A, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone, dichloroindophenol, lactoyl-glutathione, mannitol, phenazine methosulphate, NADH, $NADP^+$, rotenone, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, β-amino-*N*-butyric, ascorbic, butyric, α-cyano-4-hydroxycinnamic, glycerol-3-phosphoric, D-lactic, L-lactic, pyruvic and succinic acids were obtained from SIGMA; phenylsuccinic acid was obtained from Fluka. Sucrose, Triton X-100, HEPES, Tris and sodium arsenite were from Baker. Phthalonic acid was prepared as described in Ref. [13].

All chemicals were of purest grade available and were used as Tris salts at pH 7.0–7.4 adjusted with Tris or HCl. ROT, AA, FCCP and valinomycin were dissolved in ethanol.

Jerusalem artichoke tubers were kindly supplied by Prof. A. De Santis (University of Ancona).

2.2. Isolation of JAM and peroxisomes and preparation of the cytosolic fraction

Jerusalem artichoke mitochondria (JAM) were isolated as in Ref. [14] and checked for their intactness [12,15].

Peroxisomes were isolated as in Ref. [16].

The cytosolic fraction was obtained by centrifuging (105,000 ×*g* for 60 min at 4 °C) the supernatant obtained during isolation of JAM. Glucose-6-phosphate dehydrogenase (G6PDH) (E.C. 1.1.1.49) was assayed as in Ref. [17].

2.3. Oxygen uptake studies

Oxygen uptake measurements were carried out at 25 °C in 1.5 ml of the respiratory medium consisting of 210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 20 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 5 mM KH_2PO_4/K_2HPO_4 using a Gilson 5/6 oxygraph with a Clark electrode.

2.4. Assay of glyoxalase II

Glyoxalase II assay was done photometrically at 412 nm as in Ref. [9] using a Perkin-Elmer Lambda-5 spectrophotometer. Briefly, either the cytosolic fraction or JAM treated with TX-100 (0.2%) was incubated at 25 °C in 2 ml of a standard medium consisting of 0.29 mM sucrose, 10 mM KCl, 20 mM HEPES–Tris pH 7.2, 1 mM $MgCl_2$. GSH formation was monitored following absorbance increase at 412 nm due to its reaction with 2 mM DTNB. The reaction was started by the addition of Lact-GSH at the concentrations reported in the legends of the relevant Figures. The specific activity was expressed as nmol NTB formed/min × mg protein. ε_{NTB}, as determined under our experimental conditions, was 13.9 $mM^{-1}cm^{-1}$ in a fairly good agreement with the value given by Ref. [8].

2.5. Assay of D-lactate dehydrogenase

D-LDH assay was done photometrically at 600 nm using a Perkin-Elmer Lambda-5 spectrophotometer, as in Ref. [11]. Briefly, the mitochondrial sample was incubated at 25 °C in 2 ml of the standard medium in the presence of 30 μM PMS and 50 μM DCIP. D-LDH activity was assayed by measuring the decrease in absorbance at 600 nm due to the reduction of DCIP that occurred when 15 mM D-lactate was added to the sample. The activity was expressed as nmol DCIP reduced/min × mg protein (ε_{DCIP}=21 mM^{-1} cm^{-1}).

2.6. Safranin O response assay

Safranin O response was monitored as in Ref. [11]. Measurements were carried out at 25 °C in 2 ml of standard medium containing 12.5 μM safranin O and 1 mg mitochondrial protein.

2.7. Measurements of proton movement

Proton movement across the mitochondrial membrane was followed, as previously reported [11], using a Gilson 5/6 Oxygraph, equipped with a Gilson pH 5 Servo Channel electrode. Mitochondria were added, at 25 °C, to 1.5 ml of the proton medium consisting of 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA–Tris, 2 mM Tris–HCl, pH 7.0. Changes in pH of the mitochondrial suspension were continuously monitored and directly recorded. The calibration was made by using HCl.

2.8. Fluorimetric and photometric assays

Changes in the redox state of either flavin or mitochondrial pyridine nucleotides were monitored fluorimetrically, using a Perkin Elmer luminometer LS-5 with excitation–emission wavelengths of 450–520 and 334–456 nm, respectively [18].

Uptake of D-lactate was monitored as in Ref. [11] by following the reduction of intramitochondrial FAD/FMN caused by externally adding the substrate to JAM (1 mg protein). The mitochondria were pre-incubated in 2 ml of standard medium and then ROT (2 μg), AA (1.5 μg) and cyanide (CN^-, 1 mM) were added 2 min later to prevent the oxidation of the newly synthesized $FADH_2/FMNH_2$ via the respiratory chain.

Uptake of pyruvate was monitored by following the reduction of intramitochondrial $NAD(P)^+$, as in Ref. [18], caused by externally adding the substrate to mitochondria previously energized with an increase of ΔpH. The latter was achieved by adding ascorbate (ASC, 5 mM) plus *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD, 0.5 mM) followed by valinomycin (0.5 μg) plus KCl (25 mM), and treatment 3–5 min later with 2 μg rotenone to prevent the oxidation of the newly synthesized NAD(P)H via the respiratory chain.

The rate of fluorescence change was obtained as tangent to the initial part of the progress curve and expressed as arbitrary units of either FAD/FMN or $NAD(P)^+$ reduced/mg mitochondrial protein.

3. Results

3.1. The occurrence of glyoxalase II activity in the cytosolic fraction and in solubilized mitochondria from Jerusalem artichoke tubers

To establish whether production of D-lactate takes place in Jerusalem artichoke tubers, the occurrence of glyoxalase II, which produces D-lactate from Lact-GSH, was examined. This was done photometrically by measuring the absorbance increase at 412 nm as in Ref. [8] both in isolated JAM and in the cytosol. Either JAM (0.5 mg protein) or cytosolic fraction (0.2 mg protein) was suspended in the presence of DTNB (0.2 mM) and, when the absorbance was constant, Lact-GSH (1 mM) was added. In the case of the cytosol fraction an increase of absorbance due to the reduction of DTNB occurred, thus showing the existence of glyoxalase II activity (not shown). No glyoxalase II activity was observed with intact mitochondria, but the addition of TX-100 (0.2%) led to reduction of DTNB, showing that glyoxalase II is present in the inner mitochondrial compartment (not shown). In a control experiment, we found about 95% intactness of the mitochondrial outer membrane, measured as in Ref. [12]. In addition, we found negligible fumarase activity [15] in suspensions of mitochondria thus further confirming the intactness of the inner membrane.

The kinetic characteristics of the glyoxalase II reactions were studied by determining the dependence of the rate of reduction of DTNB on increasing concentrations of externally added Lact-GSH both in JAM solubilized with TX-100 (Fig. 1A) and in the cytosol fraction (Fig. 1B). Saturation kinetics were found with K_m values of 200 and 60 μM, respectively; the V_{max} values were 74 and 13 nmol/min × mg of sample protein. In three experiments, carried out with different preparations, the mean K_m values for the mitochondrial and cytosolic glyoxalase II were found to be 200±10 and 55±5 μM, respectively; these were significantly different as judged by the Student *t*-test ($P<0.01$).

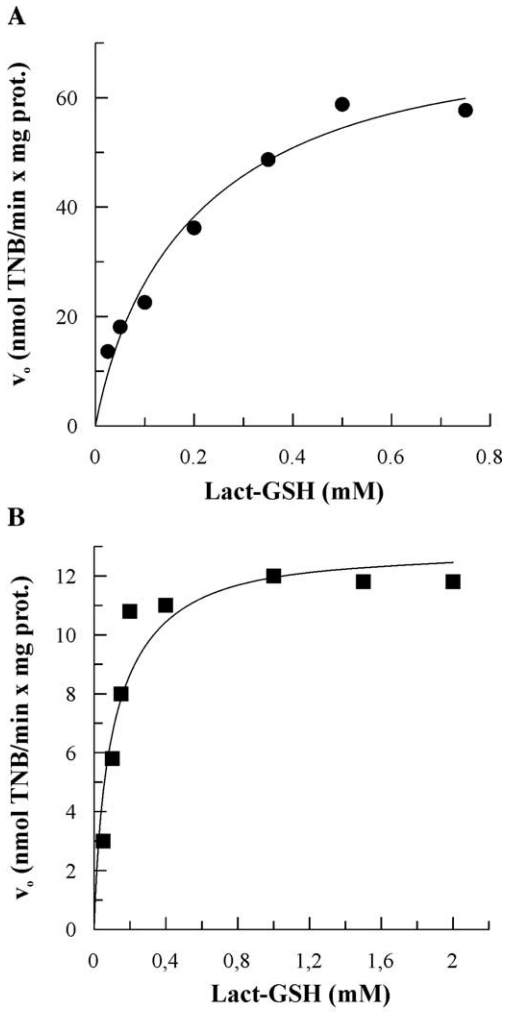


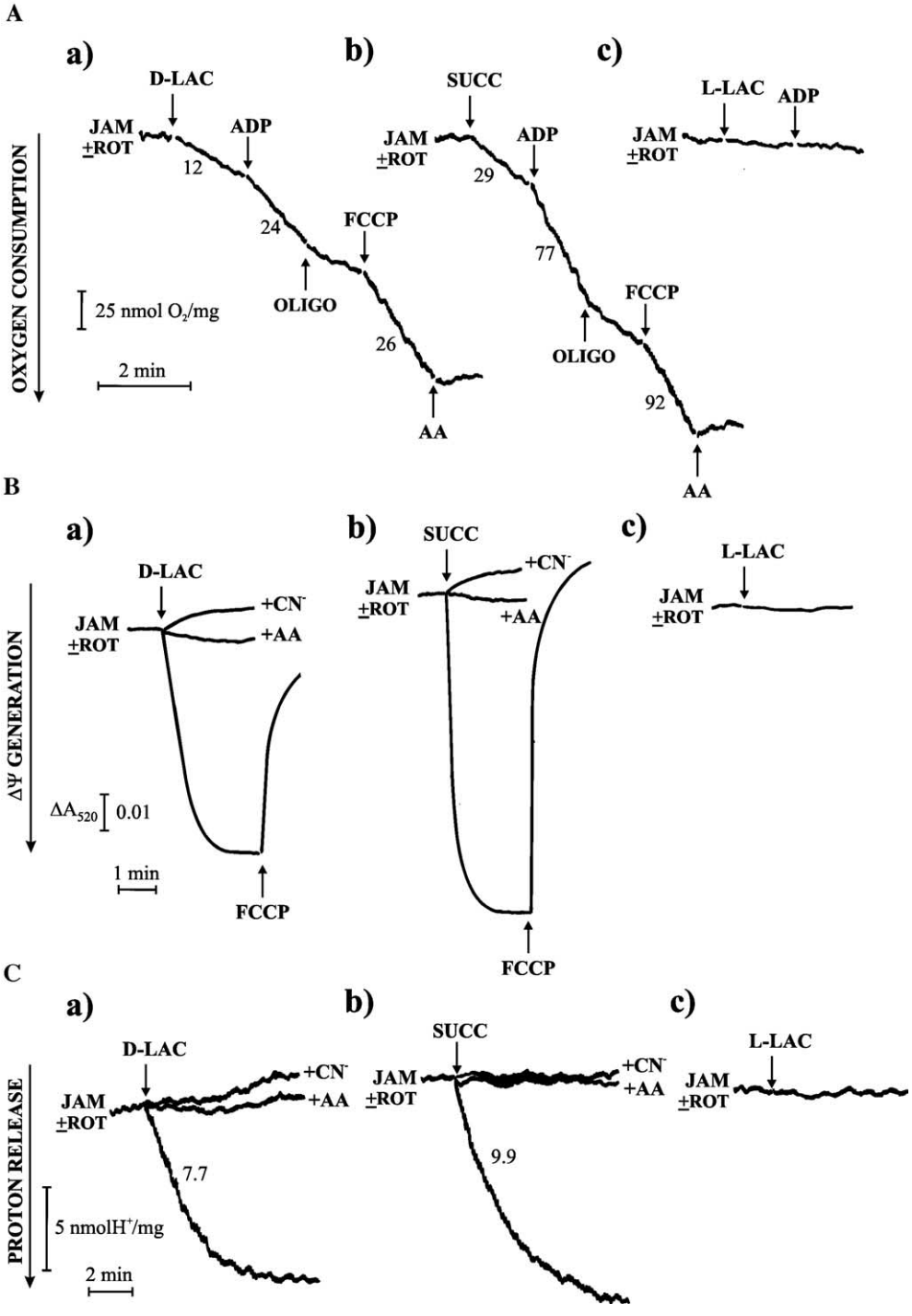
Fig. 1. Dependence of the rate of glyoxalase II on increasing concentrations of Lact-GSH in mitochondria and cytosol from Jerusalem artichoke. Either JAM (0.5 mg protein) solubilized with TX-100 (0.2%) (A) or Jerusalem artichoke cytosol (0.2 mg protein) (B) were suspended at 25 °C in 2 ml of standard medium in the presence of 0.2 mM DTNB. Glyoxalase II activity was monitored at 412 nm as described in the Methods. Lact-GSH was added at the indicated concentrations with the rate (v_o) of DTNB reduction calculated as the tangent to the initial part of the progress curve and expressed as nmol TNB formed/min × mg of sample protein.

3.2. Oxygen uptake, ΔΨ generation and proton release caused by the addition of D-lactate to JAM

To determine whether isolated JAM can oxidize D-lactate taken up from the cytosol, in addition to that synthesized in the matrix, JAM (1 mg protein) were incubated with D-lactate (5 mM) and measurements of oxygen consumption were made in both State 4 and State 3 in the presence of 0.5

mM ADP (Fig. 2A). L-lactate and succinate, also at 5 mM, were used as controls. No oxygen uptake was found as a result of the addition of L-lactate in the absence or presence of ADP. Oxygen uptake was found with a control respiratory index equal to 2 and 2.5 for D-lactate and succinate, respectively. In both cases, OLIGO (2.5 μg) was found to reduce the rate of oxygen consumption drastically, but the rate was restored by the addition of the uncoupler FCCP (1.25 μM). Both D-lactate and succinate-dependent oxygen consumption were completely inhibited by AA (2 μg), an inhibitor of complex III of the respiratory chain, but was totally insensitive to ROT (2 μg), an inhibitor of complex I.

The ability of either the lactate isomers or succinate to generate membrane potential ($\Delta\Psi$) was determined by using safranin O as a $\Delta\Psi$ probe. D-lactate and succinate (5 mM each), but not L-lactate, were found to cause $\Delta\Psi$ generation as shown by the decrease in safranin O absorbance at 520 nm (Fig. 2B). The effect was completely insensitive to ROT (2 μg), but was prevented by AA (2 μg) or cyanide (CN^-, 1 mM) and was abolished by FCCP (1.25 μM).



Proton movement across the mitochondrial membrane due to the addition of either D-lactate or succinate (10 mM each) was measured as a function of time (Fig. 2C) in the absence or presence of ROT (2 μg). In both cases, proton efflux from JAM was found at a rate decreasing with time. No proton ejection was found in the presence of either AA or CN^-, or when L-lactate (10 mM) was added.

3.3. Localization of a putative D-lactate dehydrogenase in JAM

To determine the localization of the enzyme responsible for the oxidation of D-lactate, intact JAM were assayed for D-lactate dehydrogenase activity as reported in the Methods. A negligible decrease in DCIP absorbance at 600 nm was found when D-lactate (15 mM) was added to JAM (Fig. 3A), indicative of the absence of D-LDH activity in outer membrane, in the intermembrane space or on the outer side of the mitochondrial inner membrane, or in any contamination, including peroxisomes, of the mitochondrial suspension. Externally added phenylsuccinate (PheSUCC, 10 mM) failed to inhibit D-LDH activity. In a control experiment, we confirmed that no oxidation of succinate by succinate dehydrogenase (which is located on the matrix side of the inner mitochondrial membrane) occurred with intact JAM. Oxidation of succinate did occur after the addition of TX-100 (0.2%) which solubilized the mitochondrial membranes and allowed the interaction between DCIP and the succinate dehydrogenase complex (see inset a to Fig. 3A). Consistently, the oxidation of D-lactate was similarly found after the addition of TX-100 (0.2%) to JAM showing that the localization of the putative D-lactate dehydrogenase is either on the inner side of the inner mitochondrial membrane or in the matrix (Fig. 3A).

Moreover, in order to confirm that PheSUCC itself cannot affect the DCIP based assay, we incubated JAM with glycerol-3-phosphate (G3P, 5 mM) in the absence or presence of PheSUCC (10 mM). A decrease of absorbance was found, possibly due to the reduction of external glycerol-phosphate dehydrogenase, in a manner completely insensitive to PheSUCC (see inset b to Fig. 3A).

The dependence of the rate of D-lactate oxidation on the concentration of externally added D-lactate was investigated in JAM treated with TX-100. Saturation kinetics were found with K_m and V_{max} values equal to 5 mM and 24 nmol/min × mg protein, respectively (Fig. 3B). In three experiments, carried out with different preparations, the mean K_m and V_{max} values for the mitochondrial D-LDH were found to be 5 ± 0.5 mM and 25 ± 2 nmol/min × mg protein, respectively.

3.4. D-lactate transport in JAM

The experiments reported above pose the question as to how D-lactate produced in the cytosol can cross the mitochondrial membrane. As an initial approach to this problem, swelling experiments were carried out as in Ref. [19]. JAM suspended in 0.18 M ammonium D-lactate showed spontaneous swelling with a rate and an extent significantly lower than those found with ammonium L-lactate, as judged by statistical analysis carried out on five swelling experiments, according to the Student *t*-test ($P < 0.02$). This shows that both D- and L-lactate can enter mitochondria, but that the uptake is stereospecific (not shown). D-lactate uptake was further investigated by monitoring changes in the pH of the mitochondrial suspension due to D-lactate addition to JAM incubated with a cocktail of the respiratory chain inhibitors ROT (2 μg), AA (2 μg) and CN^-(1 mM) (RAC). The addition of D-lactate (5 mM) resulted in proton uptake by the mitochondria (Fig. 4A). PheSUCC (10 mM) and mersalyl (MERS, 0.1 mM), both non-penetrant compounds, were found to inhibit the rate of D-lactate-dependent proton uptake, whereas α-cyano-4-hydroxycinnamate(α-CCN^-, 25 μM) and β-aminobutyrate(β-NH_2-BUT, 10 mM) had no effect on it. In a control experiment, when we energized JAM with an increase of ΔpH by adding ASC (5 mM) plus TMPD (0.5 mM) and valinomycin (VAL, 0.5 μg) in the presence of KCl (25 mM), we found proton uptake due to the addition of 1 mM pyruvate (PYR, 1 mM) which was completely insensitive to β-NH_2-BUT (10 mM) but prevented by 5 μM α-CCN^-(Fig. 4A).

Mitochondrial energization, strictly required for pyruvate-dependent proton uptake to occur, produced a significant increase in the proton uptake caused by D-lactate.

The results in Fig. 4A suggest that D-lactate/H^+ symport is carrier-mediated and that it does not occur via the pyruvate

Fig. 2. Oxygen consumption, ΔΨ generation and proton movement across the mitochondrial membrane accompanying D-lactate uptake into JAM. (A) JAM (1 mg protein) were suspended at 25 °C in 1.5 ml of the respiratory medium and the amount of residual oxygen was measured as a function of time. At the arrows the following additions were made: D-lactate (D-LAC, 5 mM) (a), succinate (SUCC, 5 mM) (b), L-lactate (L-LAC, 5 mM) (c), either in the presence or absence of ROT (2 μg), ADP (0.5 mM), OLIGO (2.5 μg), FCCP (1.25 μM), AA (2 μg). Numbers along the curves are rates of oxygen uptake expressed as nmol O_2/min × mg mitochondrial protein. (B) JAM (1 mg protein) were suspended at 25 °C in 2 ml of standard medium containing safranin O (12.5 μM). At the arrows the following additions were made: D-lactate (D-LAC, 5 mM) (a), succinate (SUCC, 5 mM) (b), L-lactate (L-LAC, 5 mM) (c), either in the presence or absence of ROT (2 μg), FCCP (1.25 μM). Where indicated, AA (2 μg) or CN^- (1 mM) was added 2 min before addition of the substrate. The safranin O response was monitored at 520 nm as described in the Methods. (C) JAM (1 mg protein) were suspended at 25 °C in 1.5 ml of proton medium consisting of 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA–TRIS, 2 mM TRIS–HCl, pH 7.00. At the arrows the following additions were made: D-lactate (D-LAC, 10 mM) (a), succinate (SUCC, 10 mM) (b), L-lactate (L-LAC, 10 mM) (c), either in the presence or absence of ROT (2 μg). Where indicated, AA (2 μg) or CN^- (1 mM) was added 2 min before addition of the substrate. Number along the curve is the rate of proton efflux calculated as the tangent to the initial part of the progress curve and expressed as nmol H^+/min × mg protein.

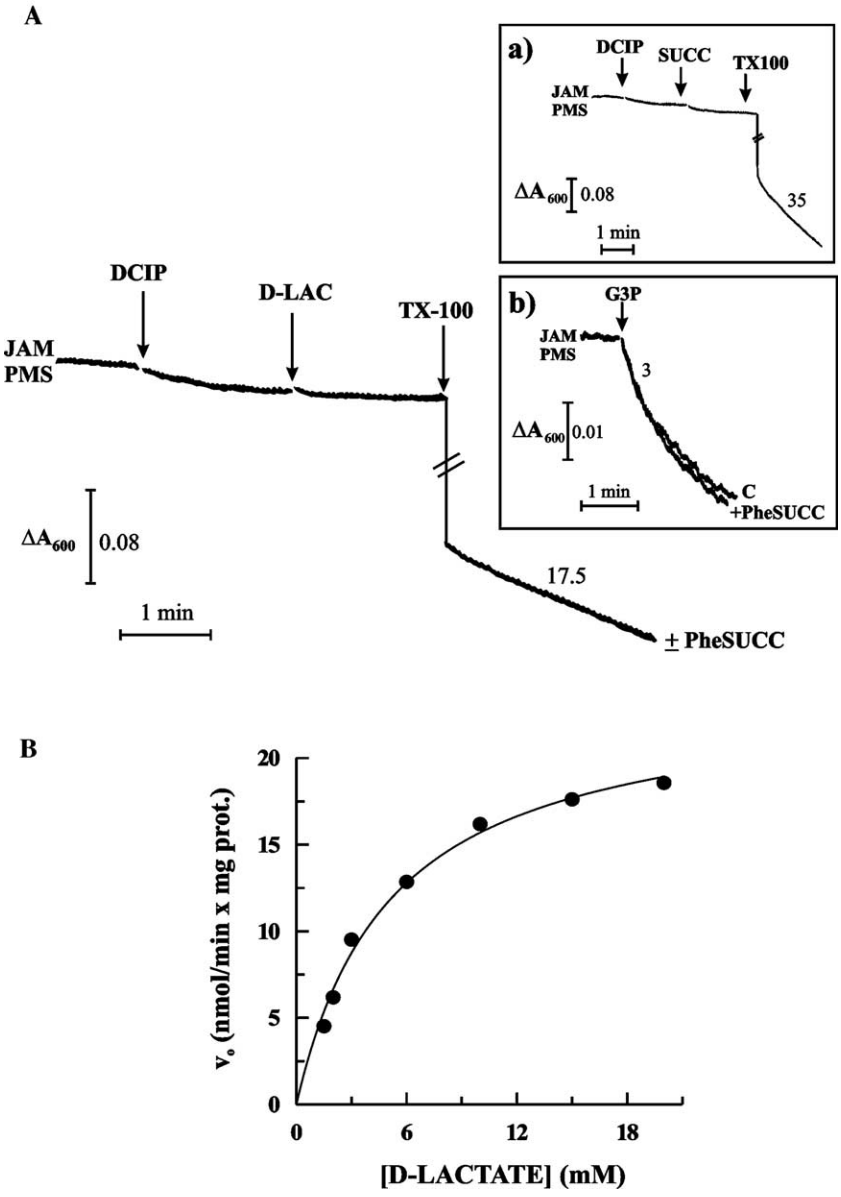


Fig. 3. D-LDH activity assay in JAM and the dependence of the rate of D-lactate oxidation on increasing D-lactate concentrations. (A) JAM (0.2 mg protein) were incubated in 2 ml of standard medium in the presence of PMS (30 μM) and either in the absence or presence of PheSUCC (10 mM). D-LDH activity was monitored at 600 nm as described in the Methods. At the arrows DCIP (50 μM), D-LAC (15 mM) and TX-100 (0.2%) were added. The insets report control experiments: in (a) at the arrows DCIP (50 μM), succinate (SUCC, 5 mM) and TX-100 (0.2%) were added to mitochondria added with PMS; in (b) at the arrows glycerol phosphate (G3P, 5 mM), in the presence or absence of PheSUCC (10 mM), added 2 min before, was added to mitochondria added with PMS and DCIP. Numbers along the curves are rates of D-lactate, succinate or glycerol-3-phosphate oxidation expressed as nmol DCIP reduced/min × mg protein. (B) D-lactate was added at the indicated concentrations to JAM treated with TX-100 (0.2%) with the rate (v_o) of DCIP reduction calculated as the tangent to the initial part of the progress curve and expressed as nmol DCIP reduced/min × mg protein.

carrier. It was confirmed that PheSUCC (10 mM) and MERS (0.1 mM) do not cause any non-specific effect on the mitochondrial membrane, by using as a substrate butyrate (BUT) (10 mM), which is known to enter mitochondria via diffusion (see inset to Fig. 4A). In this case the addition of BUT results in proton release in a manner insensitive to both inhibitors but sensitive to the uncoupler FCCP; however, when JAM were added with BUT in the presence of the inhibitors of the respiratory chain, *i.e.* rotenone, antimycin A and cyanide (RAC), proton uptake was found which proved to be insensitive to both PheSUCC (10 mM) and MERS (0.1 mM) (inset to Fig. 4A).

In the same experiment, uptake and metabolism of D-lactate was further investigated by determining the ability of externally added D-lactate to reduce intra-mitochondrial dehydrogenase cofactors. This was done by using fluorimetric techniques that have previously been used to monitor changes in redox state of pyridine nucleotides [18] and flavins [11]. No significant reduction of mitochondrial $NAD(P)^+$ was found when D-lactate was added to JAM

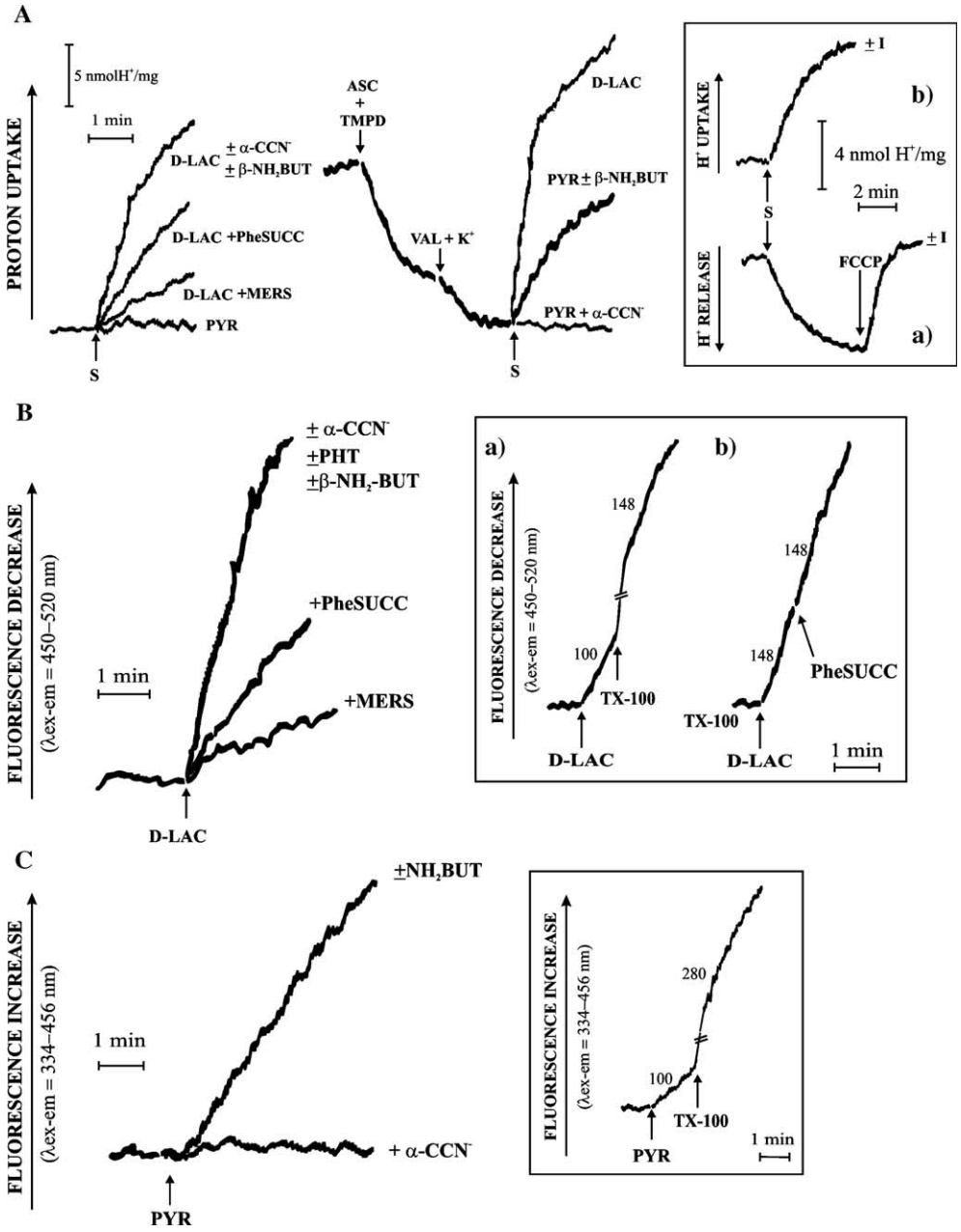


Fig. 4. Proton uptake and both mitochondrial flavin and pyridine nucleotides reduction. (A) Measurements of proton movement across the mitochondrial membrane. JAM (1 mg protein) were incubated for 3 min at 25 °C in 1.5 ml of proton medium in the presence of RAC cocktail. At the arrows ascorbate (ASC, 5 mM) plus *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD, 0.5 mM) followed by valinomycin (0.5 μg/mg) plus KCl (25 mM), D-LAC (5 mM) or pyruvate (PYR, 1 mM) indicated as S, i.e. substrate, were added. Where indicated PheSUCC (10 mM), β-amino-butyrate (β-NH_2-BUT, 10 mM), mersalyl (MERS, 0.1 mM) or α-cyano-4-hydroxycinnamate (α-CCN^-, 25 μM in the case of D-LAC and 5 μM in the case of PYR) were added to JAM 1 min before the addition of the substrate. In the inset butyrate (BUT) (10 mM), indicated as S, i.e. substrate, and FCCP (1.25 μM) were added to JAM (1 mg protein) in the presence or absence of the inhibitors (I) MERS (0.1 mM) or PheSUCC (10 mM) (a). The same additions were made with JAM previously added with RAC cocktail (b). Numbers along the curves are rates of proton efflux calculated as the tangent to the initial part of the progress curve and expressed as nmol H^+/min × mg protein. (B) Fluorimetric investigation of the change in redox state of mitochondrial flavin nucleotides caused by externally added D-lactate. JAM (1 mg protein) were incubated for 3 min at 25 °C in 2 ml of standard medium and added with RAC cocktail. At the arrow, D-LAC (5 mM) was added. Where indicated the following not penetrant inhibitors (added 1 min before D-LAC) were present: PheSUCC (10 mM), MERS (0.1 mM), α-CCN^- (25 μM), PHT (1 mM) or β-NH_2-BUT (10 mM). The inset reports TX-100 experiments: in a) TX-100 addition (0.2%) follows 5 mM D-LAC addition; in b) TX-100 (0.2%) addition precedes D-LAC. PheSUCC (10 mM) is added where indicated by the arrow. (C) Fluorimetric investigation of the change in redox state of mitochondrial pyridine nucleotides caused by externally added pyruvate. JAM (1 mg protein) were incubated for 3 min at 25 °C in 2 ml of standard medium in the presence of ascorbate (ASC) plus *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) followed by valinomycin (0.5 μg/mg) plus KCl (25 mM) and then ROT (2 μg) (for details see Method Section). Where indicated the following not penetrant inhibitors (added 1 min before PYR) were present: α-CCN^- (5 μM) or β-NH_2-BUT (10 mM). In the inset TX-100 (0.2%) experiment is reported. Reduction of either flavin (B) or pyridine nucleotides (C) was followed fluorimetrically (λ_{exc} 450 nm/λ_{em} 520 nm; λ_{exc} 334 nm/λ_{em} 456 nm, respectively) as a function of time. Numbers along the curves are rates of either flavin or pyridine nucleotide reduction expressed as a percentage of control (considered as the 100%).

previously incubated with ROT (not shown). On the other hand, addition of D-lactate (5 mM) to JAM pre-incubated with RAC cocktail (2 μg ROT plus 2 μg AA, plus 1 mM CN^-) resulted in a rapid decrease in fluorescence at wavelengths characteristic of oxidized flavins (450 nm/520 nm excitation/emission) (Fig. 4B). This reduction of intramitochondrial flavin was strongly inhibited when either PheSUCC (10 mM) or MERS (0.1 mM) were added before the addition of D-lactate. Contrarily, no inhibition was found in the presence of phthalonate (PHT, 1 mM), α-CCN^- (25 μM) or β-NH_2-BUT (10 mM). To determine whether the rate of oxidation of D-lactate via the putative D-LDH was limited by the rate of its uptake, TX-100 (0.2%) was added to the JAM. This increased the rate of flavin reduction showing that the rate of oxidation of D-lactate reflects the rate of D-lactate/H^+ symport across the mitochondrial inner membrane. In the same experiment, we found that PheSUCC (10 mM) was ineffective in inhibiting the D-lactate oxidation occurring in solubilized JAM (see inset to Fig. 4B).

When pyruvate was added to mitochondria energized with an increase of ΔpH, we found a significant reduction of mitochondrial $NAD(P)^+$; this was completely prevented by α-CCN^- (5 μM), but insensitive to β-NH_2-BUT (10 mM) (Fig. 4C). TX-100 (0.2%) addition proved to increase the rate of $NAD(P)^+$ reduction (inset to Fig. 4C).

PheSUCC proved to be competitive inhibitor of the rate of flavin reduction with K_i value equal to 9 mM (Fig. 5A). Interestingly, the y intercepts of the lines fitting the experimental points obtained in the presence of the inhibitor coincide with the experimental values obtained in the

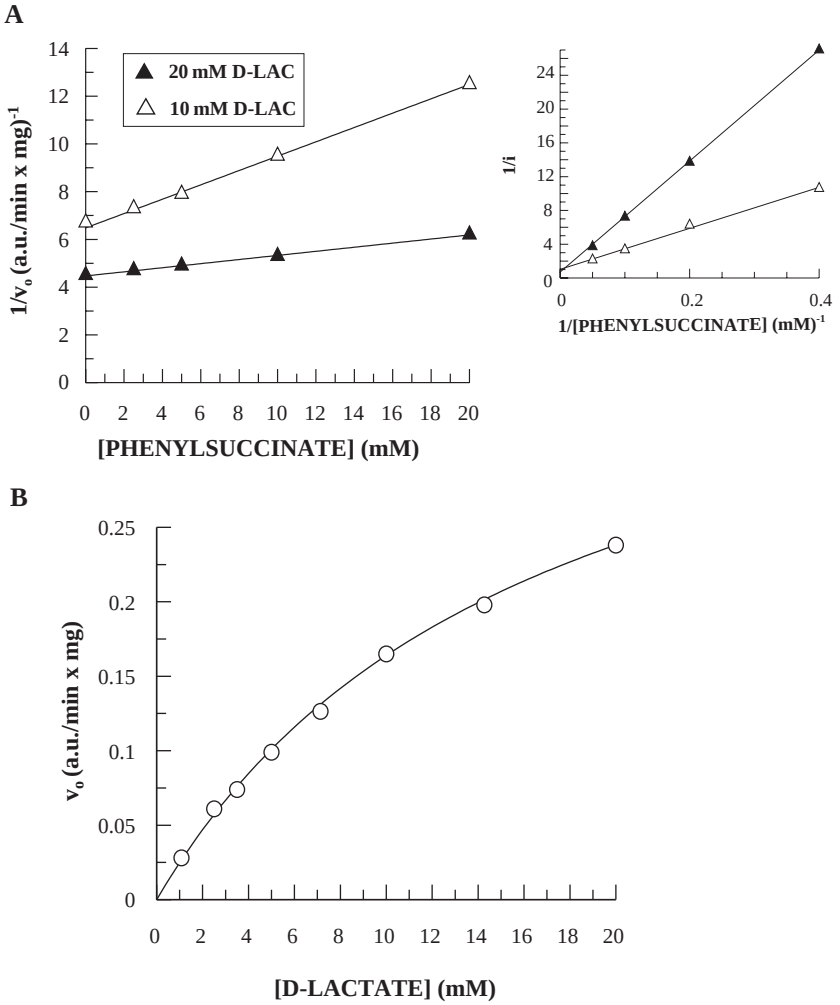


Fig. 5. Dixon plot of the inhibition by PheSUCC of the rate of intramitochondrial flavin nucleotides reduction due to externally added D-lactate and dependence of D-lactate/H^+ symport on increasing D-lactate concentrations. (A) JAM (1 mg protein) were incubated at 25 °C in 2 ml of standard medium. The reduction rate of intramitochondrial flavin nucleotide was measured as in Fig. 4B, by using 10 mM and 20 mM D-lactate either in the absence or presence of PheSUCC at the indicated concentrations. The rate (v_o), measured as the tangent to the initial part of the progress curve, is expressed in arbitrary units/min × mg mitochondrial protein. The inset reports the plot of $1/i$ against 1/[PheSUCC], where $i = 1 - v_i/v_o$; v_i and v_o being the rate of D-lactate uptake in the presence and in the absence of PheSUCC, respectively. (B) D-lactate was added to JAM (1 mg protein) at the indicated concentrations and the rate of flavin nucleotides reduction was measured as described in Fig. 4B. The rates (v_o), measured as the tangents to the initial part of the progress curves, are expressed as arbitrary units/min × mg mitochondrial protein.

absence of inhibitor. As expected in the light of the TX-100 experiments (see insets to Fig. 4B), this shows that PHESUCC-sensitive D-lactate transport controls the rate of the measured process, in accordance with the control strength criterion [11], *i.e.* the rate of the flavin reduction reflects the rate of the D-lactate uptake.

The data of Fig. 5A were also plotted as $1/i$ against 1/[Inhibitor], where the fractional inhibition i is $1 - v_i/v_o$ (see insets). The y intercept was unity, showing that PheSUCC can prevent D-lactate transport completely.

The dependence of the rate of D-lactate/H^+ symport on increasing D-lactate concentrations was measured. The results were analyzed by means of a Michaelis–Menten plot (Fig. 5B). Saturation kinetic was found with a K_m value equal to 16 mM.

4. Discussion

In this paper we show for the first time that D-lactate can be transported into plant mitochondria, in particular those from Jerusalem artichoke, and metabolized therein. The sequence of events involved in metabolism of D-lactate most likely includes D-lactate synthesis in cytosol due to glyoxalase II, uptake via a D-lactate/H^+ symporter, and oxidation of the D-lactate to pyruvate by a putative D-LDH located in the inner mitochondrial compartment.

The existence of glyoxalase II in Jerusalem artichoke cytosol confirms previous findings [7,8,10,20], thus suggesting that this enzyme occurs also in tubers.

In order to study the uptake of D-lactate by JAM, use was made of spectroscopic techniques under conditions in which mitochondrial metabolism is mostly active, thus allowing for monitoring of mitochondrial reactions and of the traffic of newly synthesized substrates across the mitochondrial membrane. Mitochondria can take up D-lactate with net carbon uptake in a proton-compensated manner. This was substantiated by the observed swelling in isotonic solutions of ammonium D-lactate which per se indicates that uptake of D-lactate is proton-compensated. Moreover, D-lactate/H^+ symport was investigated as in Ref. [11] by directly measuring proton movement across the mitochondrial membrane. D-lactate when added to JAM either in the absence or presence of ROT caused proton ejection in a manner inhibited by AA. In a set of parallel experiments, we have investigated pyruvate uptake by JAM, showing that D-lactate/H^+ and pyruvate/H^+ symports occur in a different manner as revealed by the effects of α-CCN^-. Interestingly, pyruvate uptake strictly requires energized JAM as obtained by an increase in ΔpH, whereas such a condition simply increases the rate of D-lactate uptake. This provides a further clear distinction between the two.

The results that we have reported are entirely consistent with the existence of a mitochondrial D-LDH (see Fig. 3). The enzyme is a flavoprotein capable of reducing complex III, as shown by the reduction of the intramitochondrial flavins in the presence of ROT. In this regard, the JAM D-LDH is similar to the enzymes found in mitochondria from *S. cerevisiae* [21] and rat liver [11] as well as that from *Escherichia coli* [22].

Moreover, we show that the putative D-LDH activity can be assayed after TX-100 addition to JAM thus suggesting the localization of the enzyme on the inner side of the mitochondrial inner membrane or in the matrix space. The possibility that D-lactate is oxidized on the external face of the inner membrane, with electrons transferred to the inner surface for reaction with DCIP (which requires Triton to gain access), can be ruled out on the basis of the lack of inhibition on D-LDH activity, as assayed, both fluorimetrically and using DCIP, in solubilized JAM, by PheSUCC which proved to inhibit completely D-lactate dependent flavin reduction. Thus we are forced to conclude that the inhibition is dependent on a carrier mediated transport, this strongly suggesting that the D-lactate must be taken up for its mitochondrial metabolism to occur.

The localization of the enzyme is consistent with intramitochondrial oxidation of D-lactate newly synthesized in the matrix from lactoyl-glutathione by the mitochondrial glyoxalase II [9,20,22–24], as well as for the cytosolic D-lactate taken up by mitochondria. In initial experiments we did not find any D-LDH activity in the cytosol fraction of Jerusalem artichoke tubers (data not shown), and so the possible occurrence of a cytosolic D-LDH remains for future investigation.

The picture emerging from the results described in this paper is as follow. The potentially toxic methylglyoxal is metabolized to D-lactate in cytosol by glyoxalase II (Fig. 1B). Uptake of D-lactate by JAM occurs in a proton-compensated manner via the D-lactate/H^+ symporter (Fig. 4A,B). In the matrix, the imported D-lactate, as well as D-lactate formed by the mitochondrial glyoxalase II (Fig 1A), is oxidized to pyruvate via the D-LDH (Fig. 3). Electrons flow to oxygen results in ATP synthesis as well as in generation of a membrane potential (Fig. 2A,B). Finally, the combined actions of cytosolic glyoxalase II, of the D-lactate transporter and of the putative D-LDH provide a route for removal of methylglyoxal which is toxic to plant cells [3].

Acknowledgements

The authors thank Prof Shawn Doonan for his critical reading. This work was partially financed by Fondi di Ricerca di Ateneo del Molise to S.P. and by PRIN 2004 “Cross talk between organelles in response to oxidative stress and programmed cell death in plants”.

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