L-[U-¹⁴C]Lactate binding to a 43 kDa protein in plasma membranes of *Candida utilis*

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To identify the putative lactate transporter protein of *Candida utilis*, plasma membranes from cells grown either on lactic acid (presence of lactate proton symport) or glucose (absence of lactate proton symport) were incubated with L-[U-¹⁴C]lactic acid and the membrane proteins were then separated by SDS-PAGE. A well-defined peak of radioactivity occurred in the lane of the gel containing plasma membrane proteins from lactic-acid-grown cells but not from glucose-grown cells. Binding was inhibited by unlabelled pyruvate and lactate, whereas succinate and citrate were not inhibitory. The monocarboxylate transporter inhibitor of animal cells, 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate, competitively inhibited the lactate proton symport in the whole yeast and also inhibited lactate binding to proteins of isolated plasma membranes. The polypeptide pattern of plasma membranes from lactic-acid-grown cells revealed a 43 kDa polypeptide associated with the peak of labelled lactate. Altogether the results suggest that this polypeptide is either the lactate transporter or a component of it.

Keywords: affinity labelling, lactate transporter, *Candida utilis*

INTRODUCTION

Candida utilis is one of the many yeast species able to use short-chain carboxylic acids as the sole carbon and energy source. As a consequence and from a practical point of view, *Candida* species are often found as food-contaminating flora (Fleet, 1990) and have been proposed for single-cell protein production from waste organic acids (Sinskey & Batt, 1987). Previous studies have demonstrated that in *C. utilis* the uptake of short-chain carboxylic acids is mediated by carrier proteins (Leão & Van Uden, 1986; Cássio & Leão, 1991, 1993; Cássio *et al.*, 1993). In particular, lactate is transported by a monocarboxylate proton symport which is inducible and subject to glucose repression (Leão & Van Uden, 1986). This system, beyond D- and L-lactate, is able to accept pyruvate, acetate and propionate. The activity of this carrier was reconstituted in hybrid vesicles prepared by fusion of plasma membranes of the yeast with *Escherichia coli* liposomes (Gerós *et al.*, 1996). However, despite all the work which has been carried out on the transport of carboxylic acids across the plasma membrane of *C. utilis* and other yeasts, the

identification of the protein(s) involved has not been achieved yet.

The transport of monocarboxylates into animal cells has been better characterized than transport into other systems and several approaches have been used to identify the carrier proteins. In this respect, labelling experiments using a variety of compounds, such as the stilbene disulfonate DIDS and L-[U-¹⁴C]lactic acid, proved to be useful (reviewed by Poole & Halestrap, 1993; Juel, 1997). Additionally, studies involving molecular biological methods have led to the proposal of a family of monocarboxylate transporters (MCTs) in membranes from animal cells which comprises about 13 proteins (see Price *et al.*, 1998; Pao *et al.*, 1998; Paulsen *et al.*, 1998). In *Saccharomyces cerevisiae*, four potential MCT homologues have been identified. Furthermore, in this yeast species, it was found that the gene *JEN1*, included by Paulsen *et al.* (1998) in the sialate proton symporter family, encodes a lactate permease (Casal *et al.*, 1999).

To our knowledge, the present work is the first report of an attempt to identify a plasma membrane protein responsible for lactate transport in yeast cells. We have selected the yeast *C. utilis* IGC 3092 since the lactate proton symport of this yeast displays a high capacity to transport lactate, suggesting high expression of the

Abbreviations: DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonate; MCT, monocarboxylate transporter.

protein. Labelling experiments performed with L-[U-¹⁴C]lactic acid led to the identification in SDS-PAGE of a 43 kDa polypeptide, which is likely to be the lactate transporter protein.

METHODS

Strain and growth conditions. *Candida utilis* IGC 3092 (CBS 890) was grown in a mineral medium containing vitamins (Leão & Van Uden, 1986), supplemented with either 0.5% (v/v) DL-lactic acid (pH 4.8) or 2% (w/v) glucose (pH 4.0), at 26 °C with shaking (120 r.p.m.).

Preparation of plasma membranes from *C. utilis*. *C. utilis* cells grown in either lactic acid or glucose medium were harvested in the mid-exponential growth phase (OD₆₄₀ 0.5–0.6), and washed twice with ice-cold distilled water and once with buffer A (0.3 mM KCl, 0.1 M glycine, pH 7.0). Cells (15 g) were suspended in buffer A (15 ml) containing 0.1 mM PMSF and plasma membranes were prepared as described by Van Leeuwen *et al.* (1991). Briefly, cells were homogenized (Braun Cell Homogenizer) with 35 g glass beads (0.25–0.32 mm) for 2 min, and the suspension was separated from the glass beads by filtration (glass filter) and centrifuged at 2100 g for 10 min. The supernatant was filtered through a glass-fibre filter (Sartorius; 13400-47-S) and centrifuged at 6200 g for 20 min. The supernatant was re-centrifuged (6200 g, 20 min) and the two pellets were combined and washed once with buffer A. For the aggregation of mitochondrial membranes the pellet was resuspended in 15 ml buffer A with 0.1 mM PMSF and titrated to pH 4.9 with 40 mM HCl in buffer A. Aggregated mitochondrial membranes were removed by centrifugation at 2100 g for 10 min and the supernatant was brought to pH 7.0; the pellet was resuspended in buffer A, acidified to pH 4.9 and re-centrifuged (2100 g for 10 min). The supernatants were combined, acidified to pH 5.0 and centrifuged at 7700 g for 3 min. The resulting supernatant was adjusted once more to pH 7.0, frozen in liquid N₂ and thawed at room temperature. This suspension was then centrifuged for 25 min at 100 000 g, and the pellet was washed with 50 mM potassium phosphate, 1 mM MgCl₂, pH 6.2, and finally resuspended in this buffer to a final protein concentration of about 5 mg ml⁻¹. Aliquots were stored in liquid N₂.

Protein was assayed by the Lowry method, using BSA as a standard.

L-[U-¹⁴C]Lactate binding to plasma membranes, and SDS-PAGE analysis. Binding assays were performed according to the method of Welch *et al.* (1984). Portions of plasma membrane suspensions (10–20 µl), containing approximately 100 µg protein, were incubated with 0.01 mM L-[U-¹⁴C]lactic acid (2220 Bq), pH 7.4, for 2 h at 30 °C, in a final volume of 40 µl. Prior to loading onto a polyacrylamide gel, samples were mixed with the same volume of 125 mM Tris/HCl buffer, 20% (v/v) glycerol, 4% (w/v) SDS, 0.01% (w/v) bromophenol blue and 1% (v/v) 2-mercaptoethanol, pH 6.8, and then heated to 100 °C for 5 min.

Slab polyacrylamide gels (10%, 1 mm thickness) with a 2.5% stacking gel were prepared as described by Laemmli (1970) in a Sturdi SE 400 (Hoefer) system. The run was performed at 100 V for 1 h and 250 V thereafter. Calibration markers of known molecular mass (14.4–94 kDa) were included in each gel. Electrophoresis was carried out until the dye was about 0.5 cm from the bottom of the gel. At the end of the run, the lanes containing the radioactive samples were sliced at 2.5 mm intervals, unless otherwise stated, and the slices were placed in vials containing 5 ml scintillation fluid. The vials were left for

24 h prior to counting (Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer). In addition, non-radioactive membrane protein samples were also separated by SDS-PAGE and gels were stained for protein with either silver nitrate (Merril *et al.*, 1994) or Coomassie brilliant blue R.

Inhibition assays of lactate transport in intact cells by DIDS.

Cells of the yeast grown on lactic acid were harvested in the mid-exponential growth phase, washed twice with cold distilled water, and suspended in distilled water at a final concentration of about 40 mg (dry weight) ml⁻¹. The initial uptake rates of labelled lactic acid were estimated as described previously (Leão & Van Uden, 1986). Briefly, 10 µl yeast suspension was mixed with 30 µl 0.1 M potassium phosphate buffer, pH 5.0, and, after 2 min incubation at 26 °C, the reaction was started by addition of 10 µl 0.025–0.4 mM labelled lactic acid (67 Bq nmol⁻¹). Samples were taken after 0, 5 and 10 s, time periods over which the uptake of labelled lactic acid was linear. The reaction was stopped by dilution with 4 ml ice-cold water and the mixtures were immediately filtered through GF/C filters (Whatman). The filters were washed with 8 ml ice-cold water, introduced into vials containing scintillation fluid and radioactivity was measured. For the non-specific ¹⁴C adsorption, at zero time, labelled lactic acid was added after cold water. To evaluate the inhibitory effect of DIDS on lactic acid uptake, the compound was included in the reaction mixture 30 s before adding labelled lactic acid, as indicated in Results.

Chemicals. L-[U-¹⁴C]Lactic acid, sodium salt (5.62 GBq mmol⁻¹), was purchased from the Radiochemical Centre (Amersham). Scintillation fluid OptiPhase HiSafe II and DIDS were from LKB FSA Laboratory Supplies and Sigma, respectively.

RESULTS

Characteristics of yeast plasma membranes

Cells of the yeast *C. utilis* IGC 3092 grown with either 0.5% (v/v) lactic acid or 2% (w/v) glucose as the sole carbon and energy sources were used to prepare plasma membranes. Following the work previously published on this yeast strain (Gerós *et al.*, 1996), the purity of the plasma membrane preparations was assessed by the measurement of the activities of the marker enzymes NaN₃-sensitive mitochondrial ATPase (optimum pH 8.5) and vanadate-sensitive plasma-membrane ATPase (optimum pH 6.0). The results showed that the ATPase activity at pH 6.0 was inhibited by more than 90% by 0.1 mM vanadate and by less than 5% by 10 mM NaN₃. At pH 8.5, there was also no significant inhibition by NaN₃, which suggested that the plasma membrane preparations were essentially free of mitochondrial contamination.

L-[U-¹⁴C]Lactate binding to yeast plasma membranes

Labelling experiments were performed with L-[U-¹⁴C]lactic acid since this substrate, after prolonged incubation (above 30 min), binds firmly to the MCT of animal cells (Welch *et al.*, 1984; McCullagh & Bonen, 1995). Plasma membranes isolated from *C. utilis* grown with either lactic acid (presence of lactate proton symport) or glucose (absence of lactate proton symport) were then incubated with L-[U-¹⁴C]lactic acid and

Fig. 1. Binding of L-[U- 14 C]lactic acid to plasma membranes of *C. utilis* IGC 3092 and corresponding protein electrophoretic pattern. Plasma membranes (100 μ g protein) were incubated with 2220 Bq L-lactic acid and proteins were separated by SDS-PAGE. Gels were sectioned into 2.5 mm slices. (a) Plasma membranes from cells grown on 0.5% (v/v) lactic acid ($\bullet$) or 2% (w/v) glucose ($\circ$); $\blacktriangle$, absence of membrane protein. (b) Molecular mass markers. Also shown is the protein electrophoretic pattern of plasma membranes from cells grown on 0.5% (v/v) lactic acid (c) and 2% (w/v) glucose (d). The gel was stained with silver nitrate.

separated by SDS-PAGE, as indicated in Methods. A sample without membrane protein was included as a control. A typical pattern of labelled lactate binding to plasma membrane proteins is shown in Fig. 1. The results showed a well-defined peak of radioactivity corresponding to the sample containing membrane proteins from lactic-acid-grown cells. Maximum binding was associated with a protein(s) with a molecular mass of approximately 43 kDa. In contrast, no defined peak of radioactivity was obtained with the sample of plasma membranes from glucose-grown cells and no radioactivity above background was detected when L-[U- 14 C]lactic acid was run on the gels in the absence of protein. Furthermore, when plasma membrane proteins were subjected to the SDS-PAGE denaturing treatment (see Methods), before incubation with labelled lactic acid, no peak of radioactivity was detected (not shown). To rule out the possibility that the radioactive peak was an artefact due to a contaminant bacterial protein, L-[U- 14 C]lactate-binding assays were performed in the presence of streptomycin (100 μ g ml $^{-1}$) and penicillin (100 U ml $^{-1}$). Under these conditions, a radioactive peak similar to that presented in Fig. 1 was obtained (data not shown). Overall, the results suggested that the peak of radioactivity observed corresponded to a binding of labelled lactate to a plasma membrane protein of *C. utilis*, possibly the lactate transporter.

Inhibition of L-[U- 14 C]lactate binding by inhibitors of lactate transport

To evaluate the specificity of binding of labelled lactate to plasma membrane proteins from lactic-acid-grown cells of *C. utilis*, labelling experiments using 0.01 mM L-[U- 14 C]lactic acid were performed in the absence or presence of 10 mM unlabelled lactic, pyruvic, succinic or citric acids. For each substrate, the ability to block lactate binding was assessed by the magnitude of the radioactive peak observed after the proteins had been

separated by SDS-PAGE. The results showed that pyruvate and lactate inhibited binding of labelled lactate completely, whereas succinate and citrate inhibited labelled lactate binding by only 5 and 3%, respectively (data not shown). This was in agreement with the inhibition pattern by carboxylates of the lactate proton symport of *C. utilis*, found in transport studies performed on either intact cells (Leão & Van Uden, 1986) or membrane vesicles (Gerós *et al.*, 1996).

The stilbene disulfonate DIDS is one of the most effective inhibitors of lactate transport in animal cells (Poole & Halestrap, 1993) and its inhibitory action has been used as an index for the presence of a specific monocarboxylate carrier. To substantiate the presence of a transporter protein of the MCT type, the effect of this inhibitor on the transport of lactic acid in whole cells of *C. utilis* was investigated. Estimates of the initial uptake rates using 0.025–0.5 mM labelled lactic acid, in the presence and absence of 0.1–10 mM DIDS, showed that this compound behaved as a competitive inhibitor of lactate transport (not shown) and an inhibition constant (K_i) of 0.26 mM was obtained. Based on these results, and to confirm that the radioactive peak was associated with the protein of the lactate proton symport of *C. utilis*, lactate binding to plasma membranes was studied in the presence of 0.02–10 mM DIDS. As shown in Fig. 2, lactic acid binding was inhibited by DIDS and this effect was dependent on its concentration.

The radioactive peak is associated with a 43 kDa polypeptide

Non-radioactive samples of plasma membranes from cells of *C. utilis* grown with either 0.5% (v/v) lactic acid or 2% (w/v) glucose were separated by SDS-PAGE and stained for protein (Fig. 1c, d). Analysis of the polypeptide pattern of plasma membranes from lactic-acid-

Fig. 2. Inhibition by DIDS of L-[U- 14 C]lactic acid binding to plasma membrane proteins of *C. utilis* IGC 3092 grown with 0.5% (v/v) lactic acid, determined by SDS-PAGE. Incubations of plasma membranes with labelled lactic acid were performed in the presence of 0.02–10 mM DIDS, as indicated in Methods. Gels were sectioned into 10 mm slices. Insert shows the dependence on DIDS concentration of labelled lactate binding to plasma membrane proteins; data were calculated from the peak areas and fitted by a non-linear regression analysis (GraphPad software; San Diego, CA, USA).

grown cells showed that associated with the peak of labelled lactic acid was a 43 kDa polypeptide, which was absent in plasma membranes isolated from glucose-grown cells. On the whole, the results suggested that the peak of radioactivity corresponded to a specific binding of labelled lactate to the protein, or at least part of it, involved in the lactate proton symport of *C. utilis*.

DISCUSSION

In the present work, the lactate proton symport of *C. utilis* IGC 3092 was revealed to be a reliable working model for biochemical studies aiming at the identification of the lactate carrier. Firstly, as this transport system is inducible and subject to glucose repression (Leão & Van Uden, 1986), it was possible to compare the L-[U- 14 C]lactic acid binding pattern and the protein distribution on SDS-PAGE of plasma membranes isolated from cells grown with either glucose (absence of lactate proton symport) or lactic acid (presence of lactate proton symport). Secondly, the high affinity of this carrier for lactate, as well as its high capacity to transport this substrate, were probably important factors for the effectiveness of the L-[U- 14 C]lactic acid binding experiments. In fact, when plasma membranes from derepressed cells were used, a well-defined peak of radioactivity was obtained which corresponded to a polypeptide in SDS-PAGE that stains readily with either silver nitrate or Coomassie blue. We have also performed similar labelling experiments with *S. cerevisiae*, which also exhibits activity for an inducible lactate proton symport (Cássio *et al.*, 1987). Nevertheless, binding of lactate to plasma membranes isolated from lactic-acid-grown cells was not observed. A possible explanation for this result could be related to the $V_{\max}$ value reported for the lactate proton symport in *S. cerevisiae*, which is about 10-fold lower than that of *C. utilis* (see Leão & Van Uden, 1986; Cássio *et al.*, 1987).

In *C. utilis*, it appears that L-[U- 14 C]lactate displays the capacity to covalently bind its own carrier, since this binding was stable to the procedures involved in SDS-PAGE. It has also been reported that labelled lactate binds to plasma membranes from rat hepatocytes (Welch *et al.*, 1984) and rat skeletal muscle (McCullagh & Bonen, 1995) in a similar way. As discussed by these authors, this type of binding is unlikely to be physiological since it would make for an energetically unfavourable transport mechanism. Similar behaviour was described for the MCT inhibitor DIDS, which initially binds reversibly to the lactate carrier in mammalian erythrocytes, but upon prolonged incubation becomes irreversibly bound (Poole & Halestrap, 1991). Indeed, our results indicated that DIDS binds reversibly to the lactate carrier during short incubations since it behaved as a competitive inhibitor of lactate transport in whole cells of *C. utilis*. However, the chemical nature of the binding of these compounds to plasma membranes upon prolonged incubation is not completely understood.

A possibility that has arisen from the present work is that the metabolism of L-[U- 14 C]lactate by contaminating bacteria during prolonged incubation could cause labelling of a bacterial protein, which could be responsible for the peak of radioactivity observed. However, this does not appear to be the case since no significant L-[U- 14 C]lactate binding to plasma membranes from glucose-grown cells (absence of lactate proton symport) was detected, and the inclusion of

antibiotics in the labelling assays did not prevent the appearance of the peak of radioactivity.

The data presented here showed that the characteristics of L-[U-¹⁴C]lactate binding to plasma membrane proteins of *C. utilis* were similar to those of the lactate proton symport exhibited by the yeast. First, the presence of the radioactive peak in labelling experiments performed with plasma membranes from lactic-acid-grown cells and absence of the peak from glucose-grown cells was in accordance with the inducibility of this carrier. Second, both lactate and pyruvate were able to inhibit binding of labelled lactate while succinate and citrate did not show significant inhibitory effect. This inhibition pattern of lactate binding by carboxylates was identical to that of the lactate proton symport system of *C. utilis*, which is able to accept monocarboxylates but not di- or tricarboxylates (Leão & Van Uden, 1986; Gerós *et al.*, 1996). In addition, the MCT inhibitor DIDS inhibited, in a dose-dependent manner, lactate binding to plasma membranes and lactate uptake in intact cells.

The polypeptide, identified in SDS-PAGE, associated with the peak of radioactivity had an apparent molecular mass of 43 kDa, which was similar to the values reported for the MCTs of animal cells (for a review see Poole & Halestrap, 1993; Juel, 1997). Altogether, the present study strongly supports the idea that we have identified a protein involved in the symport of lactate with protons in the plasma membrane of *C. utilis*, which can probably be included in the MCT family. Nevertheless, conclusive proof that this protein is the lactate proton symporter will require its purification and functional reconstitution in membrane vesicles. These approaches are in progress in our laboratory. Amino acid sequence analysis of the protein would also be important, enabling its comparison with sequences of the MCT family.

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