A reversible carrier mediates the transport of malate at the tonoplast of *Catharanthus roseus* cells

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The conditions of malate transport were deamed in tonoplast vesicles purified from a microsomal homogenate of *Catharanthus roseus* cells by preparative free-flow electrophoresis. Isolated vesicles exhibited malate transport when the membranes were prepared by grinding the cells in a homogenisation medium only buffered in the acidic pH range. By using vesicles energized artificially by an imposed pH gradient (acid interior), it was shown that malate is actively accumulated in response to the generation of a proton-motive force. Several lines of evidence (saturation kinetics, action of malate analogs and protein modifiers) support the concept that malate transport is mediated by a protein carrier which could be implicated in the uptake process as its protonated form. The malate transported in the vesicles was released by lowering the external malate concentration. The release was prevented by the anion transport inhibitor DIDS indicating the reversibility of the carrier.

Tonoplast vesicle; *Catharanthus roseus*; Malate transport; Reversible carrier

1. INTRODUCTION

Malate is an abundant and a mobile storage form of carbon which is involved in a variety of physiological processes including carbohydrate breakdown, CAM and C_4 photosynthesis, maintenance of pH and electrical balance of the cytosol and stomatal movements [1]. To perform these different functions, malate is accumulated transiently in the vacuolar space of plant cells. The possible mechanisms implicated in malate transport have been considered in CAM plants by Lüttge and coworkers [2] who concluded that movement of malate is coupled to active H^+ transport. This concept was confirmed with vacuoles isolated from various species: (i) by the demonstration of an energy-dependent transport of malate, suggesting that tonoplast ATPase [3–5] and PPiase [4] are involved; (ii) by the characterization of a malate carrier with very similar properties in CAM [5] and C_3 plants [3,4].

Partial purification of the tonoplast-bound malate carrier was performed recently by Martinoia and Vogt [6], after its reconstitution in lipid vesicles, but this work was limited by the low yield of the tonoplast membrane available using isolated vacuoles as starting material. To overcome this problem, and because isolated vesicles have proven to be a useful tool for the uptake studies of a large variety of compounds [7], we used *Catharanthus roseus* cells to obtain tonoplast vesicles by preparative free-flow electrophoresis [8]. This technique allowed the isolation of highly sealed vesicles of known sidedness (cytoplasmic side out for a large part of the vesicle population) with a purity higher than 95%. The present study demonstrates that the isolated vesicles exhibited malate transport across the two faces of the tonoplast membrane (influx, effux) and that these transport processes are mediated by a protein carrier which could be activated by a protonation step.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; DIDS, 4,4-diisothiocyano-2,2-stilbene disulfonate; DCCD, *N,N'*-dicyclohexylcarbodiimide; pH_i, intravesicular pH; pH_e, external pH; Mes, 2[*N*-morpholino]ethane-sulfonic acid; Tris, Tris(hydroxymethyl)-aminomethane

2. MATERIAL AND METHODS

2.1. *Plant material*

Cell suspension cultures of *Catharanthus roseus* (L.) G. Don, cell line C_{20} were obtained as in [9], 4-day-old cells (exponential phase of growth) being used in all experiments.

2.2. *Preparation of tonoplast vesicles*

Tonoplast membrane vesicles were prepared by free-flow electrophoresis according to [8]. Briefly, cells (25 g) were homogenized in 25 ml of a medium containing 0.25 M sucrose, 25 mM Mes/KOH (pH 5.5, 6.5 or 8.3 according to the experiment), using a Moulinex blender (type 534). After discarding the cell walls and unbroken cells by differential centrifugation [8], the membranes were collected by centrifugation for 30 min at 45 000 × *g*. The final pellet, resuspensed in electrophoresis chamber buffer (0.25 M sucrose, 11 mM KCl, 1 mM $MgCl_2$, 10 mM Tris/boric acid, pH 7.5) was submitted to free-flow electrophoresis.

The distribution of membranes in each separation was monitored by the absorbance at 280 nm. The fractions collected nearest the anode were enriched in tonoplast; their purity determined using marker enzyme was shown to be greater than 95% [8]. Tonoplast vesicles were pelleted for 30 min at 45 000 × *g* and resuspended in a small volume (about 3 mg protein/0.5 ml) of 0.25 M sucrose containing 25 mM Mes/KOH adjusted to the pH of the grinding medium. They were incubated for at least 1 h in the resuspension medium to ensure pH equilibration inside and outside the vesicle.

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2.3. *Malate uptake assays*

Malate uptake and release were determined by measuring malate trapped inside vesicles. Transport experiments were conducted in a water bath at 23°C. An imposed pH gradient (acid interior) was used to energize the vesicles. Vesicles were isolated as previously described by grinding the cells in a solution buffered at pH 5.5 and then diluted in the transport solution at the desired pH value. The transport solution (300 μl) consisted of 50 mM Tris/Mes (adjusted to the desired pH) and 396 kBq [^{14}C]malate ml (5.5 GBq·mol^{-1}, CEA France) diluted to 200 μM with unlabelled malate. The reaction was started by the addition of 30 μl tonoplast vesicles (0.6 mg protein/ml). At the desired times, 100 μl aliquots (60 μg protein) were removed and collected on Millipore HAWP filters (0.45 μm) by vacuum filtration (Millipore 1225 sample collector). The filters were washed 10 times with 2 ml of the same buffer and counted for radioactivity in a liquid-scintillation counter (Packard Tri-Carb 460 c model).

2.4. *Vesicle volume determination*

Vesicle volumes were measured as described by Rottenberg [10]. After equilibration of the tonoplast membranes (0.6 mg/ml) for 10 min in the presence of 264 kBq ^{3}H$_2$O (CEA France) and 88 kBq [^{14}C]dextran-carboxyl (44 MBq·g^{-1}; NEN Research Products), the vesicles were separated from the incubation medium by pelleting (15 000 × *g*, 15 min) through a layer of silicon oil (silicon oil AP 100 Wacker Chemie, Munchen, FRG). The tips of the microfuge tube containing the vesicle pellet were cut off and the radioactivity of ^{3}H and ^{14}C counted by liquid scintillation.

2.5. *Protein determination*

Protein was determined by the method of Smith et al. [11] with bovine serum albumin as the standard.

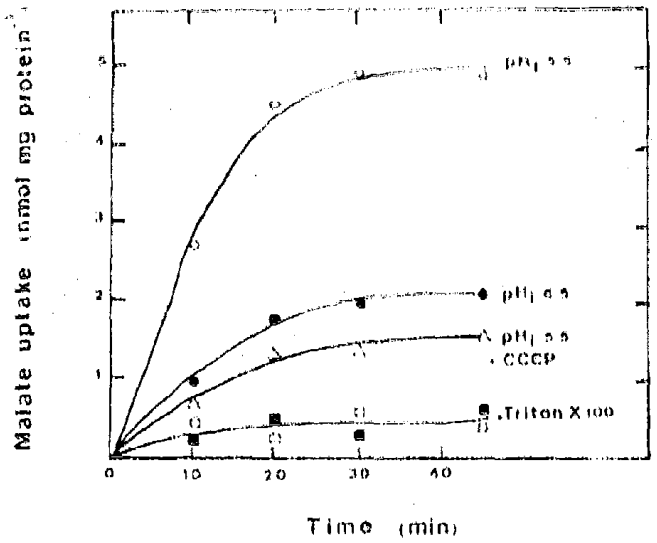

Fig. 1. Effects of the intravesicular pH (pH$_i$) on malate uptake into tonoplast vesicles of *C. roseus*. The vesicles were prepared by grinding the cells in a medium at pH 5.5 or 6.5. The transport experiments were made at an external pH of 6.5. An assay was performed with vesicles obtained at pH 5.5 including 10 μM CCCP (Δ—Δ). At the indicated times, aliquots were removed from the incubation medium and treated (pH$_i$ 5.5, □—□; pH$_i$ 6.5, ■—■) or not (pH$_i$ 5.5, ○—○; pH$_i$ 6.5 ●—●) by Triton X-100 (0.04%) they were collected on Millipore filters and washed 10 times with 2 ml of incubation mixture (with or without Triton X-100) prior to the determination of radioactivity.

3. RESULTS AND DISCUSSION

Initial experiments consisted of incubations of [^{14}C]malate with tonoplast vesicles obtained by grinding the cells in medium at different pH (5.5, 6.5, 8.3). As shown in Fig. 1, the radioactivity associated with the vesicles increased with time and reached a steady state after 20–30 min incubation according to the experiments. For vesicles obtained by grinding the cells at acidic pH (5.5 and 6.5), the radioactive tracer was easily released from the vesicles after a Triton treatment (0.04% final concentration), reflecting a real uptake of malate into the tonoplast vesicles rather than an adsorption onto the membranes. Surprisingly, an unexpected retention of [^{14}C]malate occurred when the vesicles were obtained at pH 8.3 (data not shown) due to covalent binding of the labelled compound to membrane polypeptides [8]. For the moment, the reason of this binding is unclear. However, this observation, already reported for L-lactate in vesicles obtained from hepatocytes [12], shows that artefactual covalent binding of solutes onto membranes has to be carefully checked in the course of the transport studies.

Malate was actively accumulated in the purified tonoplast vesicles when an imposed proton-electrochemical gradient (acid interior) was generated by isolating the vesicles in a grinding medium at pH 5.5 and assaying the transport in a medium at pH 6.5 (Fig. 1).

As illustrated by the low transport activity in the absence of gradient (pH 6.5 inside and outside the vesicles) or in the presence of the protonophore CCCP (10 μM), it appears that malate uptake was dependent on the generation of a H^+ gradient. Using the mean measured vesicle volume (8.0 ± 0.5 μl/mg protein, mean of 6 experiments ± SE), the intravesicular malate concentration was estimated leading to the determination of an accumulation ratio (C_i/C_e) between the intravesicular and the external malate concentrations. For vesicles used without an imposed pH gradient, the accumulation ratio is near 1 indicating a passive distribution of malate through the tonoplast to reach a concentration equilibrium. In contrast, in the presence of ΔpH, an accumulation ratio of about 3 was determined confirming that malate can be transported against a concentration gradient.

The pH dependence of the external medium was investigated using incubation media prepared in order to give final pH values of 5.5, 6.5, 7.5. The pH inside the vesicular space was 5.5 whatever the external pH. Malate uptake measured at pH 7.5 was very low (0.12 nmol/mg protein per min) whereas upon lowering the external pH a considerable enhancement was observed: double at pH 6.5, and 4 times at pH 5.5. An explanation for this pH dependence could be related either to the proportion of the different species of malate over the pH range (H_2·mal, H·mal$^-$, or mal^{2-}) assuming that malate is transported as its protonated form, or to

the protonation of a protein carrier as described for the pH dependence of sugar transport [13]. By examining the energetics of the transfer into isolated vacuoles [14] we concluded that, as in CAM plants [2], mal^{2-} was most likely the species being transported, excluding the first possibility. To test the second, we investigated in this work with tonoplast vesicles the kinetics of malate uptake at two external pH values (5.5 and 7.5). Lineweaver-Burk plots of the kinetic data showed (Fig. 2) that acidic pH of the medium increases the apparent K_m of uptake from about 20 mM (pH_e 7.5) to 2 mM (pH_e 5.5). The saturation kinetics were consistent with carrier-mediated transport with different affinities for the substrate depending on the external pH: a high affinity in the presence of H^+, a low affinity at neutral pH. To explain these findings we propose that protonation raises the affinity of the carrier for its substrate.

In further support of carrier-mediated transport, structural analogs of malate and several reagents that modify protein side chains were tested for their ability to inhibit the malate transport. The stereoisomer, D-malate, inhibited the initial rate of malate uptake by 85%, other analogs such as oxaloacetate, fumarate, succinate and malonate also being effective, but to a lesser extent (data not shown). Direct implication of a protein in the uptake process was demonstrated using Dansyl-chloride reagent which binds the terminal amino group of proteins; addition of 0.2 mM suppressed the initial malate uptake to 52% of the basal value. Among the usual anion transport inhibitors, DIDS, at a concentration of 0.2 mM, was the most effective causing a reduction of the initial malate uptake of 70%. Malate transport was also inhibited by DCCD (65% at 0.1 mM) a lipophilic reagent binding to carboxylic residues. It should be noted, moreover, that the treatments with the structural analogs (D-malate) or the chemical agents (DIDS) which, together, inhibit the initial rate of malate uptake, have no clear effect on the final steady state level.

In vivo, malate accumulation is known to be reversible. The most striking example is provided by the diurnal oscillations of malate in CAM plants [2], but we have also demonstrated in *Catharanthus roseus* that vacuolar malate remobilization occurs after transferring the cells into a fresh medium [15]. In order to examine if the malate remobilization also occurred via the same component, we designed experiments to test the reversibility of the carrier. Vesicles were first preloaded with [^{14}C]malate in conditions to ensure maximal malate accumulation (pH 5.5 inside and outside the vesicles); when the equilibrium distribution of [^{14}C]malate was reached after about 30 min, the vesicles were diluted with the same transport medium to lower the external malate concentration (Fig. 3). Following the dilution procedure, a sudden release of [^{14}C]malate occurred during the first 5 min. As demonstrated by the external malate concentration dependence, the [^{14}C]malate was released in the direction of its concentration gradient and this displacement was prevented by the action of DIDS (Fig. 3). These findings indicate that the malate carrier could also be directly involved in malate remobilization.

In conclusion, the data present the first demonstration of malate transport (influx and efflux) in tonoplast vesicles purified from plant material. The characteristics of the uptake which appear to be very similar to those described in isolated vacuoles [4,14] are

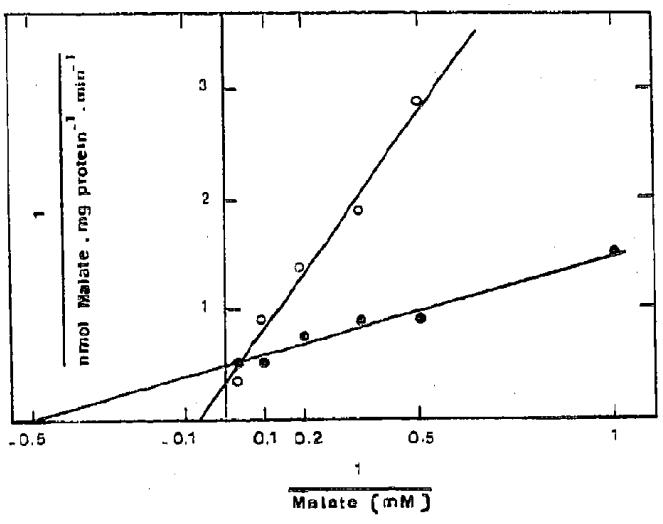

Fig. 2. Concentration dependence of malate uptake in *C. roseus* tonoplast vesicles. The Lineweaver-Burk plots were determined at two different external pH_e values: 5.5 (●—●) and 7.5 (○—○).

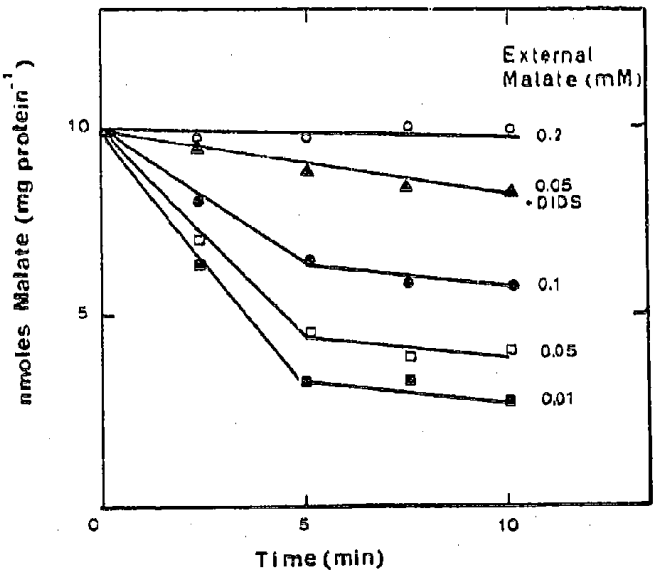

Fig. 3. Reversibility of the malate carrier. Vesicles were equilibrated for 30 min with 0.2 mM [^{14}C]malate. Subsequently (time 0 of the graph) the vesicles were diluted with the transport medium without malate to give a final malate concentration of 0.1 mM (●—●), 0.05 mM (□—□), 0.01 mM (■—■). An assay was made with an external concentration of 0.05 mM malate in the presence of 200 μM DIDS (▲—▲).

in favour of an energy-dependent transport of malate mediated by a protein carrier. Compared to intact vacuoles, tonoplast vesicles offer several advantages. Because of their high surface area/volume ratio, they showed a high rate of malate transport and they equilibrated rapidly with the [^{14}C]malate in the external medium. By lowering the external malate concentration a vesicular efflux of [^{14}C]malate can then be induced and it was demonstrated, with the anion transport inhibitor DIDS, that this release is also carrier-mediated. Another important observation was made in relation to the stability of the vesicles at low pH, suggesting that the carrier may respond to physiological stimuli such as H^+ availability. In this way, it should be stressed that the synthesized malic acid itself could regulate its vacuolar uptake by releasing $2H^+$ after dissociation in the cytoplasm.

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