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Identification of an Essential Histidine Residue at the Active Site of the Tonoplast Malate Carrier in *Catharanthus roseus* Cells

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Summary. The involvement of a histidyl residue in the binding or translocation step was investigated in the malate carrier at the tonoplast of *Catharanthus roseus* cells. The transport rate was strongly stimulated when the pH of the incubation medium was decreased from pH 7.0 to 5.0. The histidine-specific reagent diethylpyrocarbonate (DEPC) efficiently inhibited the activity of the malate carrier. Inhibition developed rapidly and was completed after 5 min at a concentration of 2 mM DEPC. The original substrate, malate, partially protected the carrier from inactivation by DEPC. Other organic acids (citrate, quinate) which are known to affect the malate transport of isolated vacuoles or tonoplast vesicles also showed protective properties. Inhibition of malate transport on tonoplast vesicles can also be achieved by photooxidation in the presence of the dye Rose Bengal. Malate also proved to protect against inactivation.

The results strongly support the notion that a histidyl residue(s) is involved either in the binding or translocation of malate and that the protonation of the histidyl residue is essential to provide a high rate of malate transport.

Key Words *Catharanthus roseus* · tonoplast · malate transport · histidine · diethylpyrocarbonate (protein modification) · Rose Bengal (photooxidation)

Introduction

The translocation of organic acids across the tonoplast, the vacuolar membrane of plant cells, is a crucial step in controlling metabolic processes. The influx and efflux of organic acids across the tonoplast allow the plant to maintain a constant cytoplasmic pH (Raven, 1985) and to play a role in the ionic and osmotic homeostasis of the cells (Marigo, Bouyssou & Boudet, 1986). The most striking example is provided by crassulacean acid metabolism plants (Winter, 1985). In this typical pattern of carbon assimilation, malate is stored in the vacuole during the night and subsequently mobilized the next day to supply CO_2 for photosynthesis.

During recent years, different transport systems have been shown to conduct malate across the tonoplast using isolated vacuoles or tonoplast vesicles from various plants (Buser-Suter, Wiemken & Matile, 1982; Martinoia et al., 1985; Nishida & Tominaga, 1987; White & Smith, 1989; Martinoia, Vogt & Amrhein, 1990). In particular, we have shown that malate uptake results from electrophoretic movement in response to a positive potential difference (Marigo & Bouyssou, 1990) and that the malate carrier is reversible, being involved in both influx and efflux of malate (Bouyssou, Canut & Marigo, 1990).

The tonoplast-bound malate carrier was recently partially purified after its reconstitution in lipid vesicles (Martinoia et al., 1991), but no identification or structural characterization of the carrier protein was made.

In *Catharanthus roseus* tonoplast vesicles, we have shown that the affinity of the carrier towards its substrate (malate) changed according to the pH of the incubation medium. The K_m decreased from 20 mM at pH 7.0 to 2 mM at pH 5.5 (Bouyssou et al., 1990). These results allowed us to suggest that protonation of the carrier is involved in increasing its affinity towards its substrate. The only amino acid with a side chain pK value in the range of 5.5 to 7.0 is histidine with a value of 6.0.

Diethylpyrocarbonate (DEPC) is a histidine-specific reagent that modifies the imidazole ring of the histidyl residue to yield N-carbethoxyhistidyl derivatives (Miles, 1977). This compound was successfully used to demonstrate the involvement of histidyl residues at the active site of proteins (Padan, Patel & Kaback, 1979; Bauer, Buki & Kun, 1990; Bertran et al., 1991).

Therefore, we investigated the sensitivity of the malate transport system to modification by DEPC

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together with Rose Bengal, another histidine-specific reagent (Fahnestock, 1975; Bauer et al., 1990), in order to gain information on the structural basis of the pH dependence of the malate transport system.

Abbreviations

DEPC: Diethylpyrocarbonate
DTT: Dithiothreitol
MES: Morpholino ethane sulfonic acid
TRIS: Tris [hydroxymethyl] aminomethane

Materials and Methods

Cell Culture and Preparation of Tonoplast Membranes

Cell suspension cultures of *Catharanthus roseus* (L.) G. Don., cell line C 20 were performed as previously described (Marigo et al., 1983). Three- to seven-day-old cells (exponential phase of growth) were used in all experiments. Tonoplast vesicles were prepared from microsomal membranes following the procedure of Canut et al. (1991) by preparative free-flow electrophoresis.

The protein content of the membrane preparation was determined as reported by Smith et al. (1985) with bovine serum albumin for standardization.

Malate Uptake by Tonoplast Vesicles

Uptake of ^{14}C-malate was determined by measuring the malate trapped inside the tonoplast vesicles (Bouyssou et al., 1990). Membranes corresponding to 50 μg of protein were added to a final volume of 55 μl. The reaction mixture contained 100 mM MES-KOH (pH 5.5), 250 mM sucrose and other compounds as indicated in the legend of the figures and tables. The malate concentration was adjusted as indicated. ^{14}C-malate was present at a radioactivity of about 30×10^3 Bq ml^{-1}. Uptake was terminated after 10 min of incubation by pipetting 50 μl of the reaction mix on Millipore HAWP filters (0.45 μm). The filters were washed six times with 2 ml of rinsing buffer (10 mM MES-KOH, pH 5.5, 250 mM sucrose) and counted for radioactivity.

Treatment of Tonoplast Vesicles with DEPC

Both time and concentration dependence of inactivation of malate transport by DEPC were determined under the conditions described for the uptake experiments. For the experiments where the carrier-active site was protected, the organic acids were added 5 min prior to the addition of DEPC. In all samples, the incubation of the membranes with DEPC was terminated after 5 min by addition of imidazole (20 mM) to bind the free DEPC; for the nonprotected samples, 5 mM malate was added together with imidazole. After 10 min of incubation, the vesicles were sedimented at 10,000 $\times$ g for 15 min at 4°C. Thus all samples were exposed to an identical malate concentration for 25 min to reach equilibrium of the malate concentration inside and outside the vesicles. After two washes of the membrane pellet with 200 μl of uptake buffer, malate uptake was determined as before.

Treatment of Tonoplast Vesicles with Rose Bengal

Photooxidation in the presence of the dye Rose Bengal was initiated by placing open vials, containing the tonoplast vesicles in 100 mM MES-KOH (pH 5.5), 250 mM sucrose, in an ice bath 20 cm underneath a 150 W, white bulb lamp. After 10 min of illumination, the samples were transferred to darkness. Controls with the same dye concentration were kept in darkness throughout. Then, malate uptake was determined as before.

To test the protective effect of malate, and different organic acids, on carrier inactivation by Rose Bengal, a preincubation system similar to that described for DEPC was employed.

Reaction of DEPC with Imidazole

To test the effect of the added organic acids on the reaction rate of DEPC with histidyl residues, a spectrophotometric model assay with imidazole as substrate was used as described by Miles (1977). The formation of N-carbethoxyhistidine derivative is followed by an increase in absorbance between 230 and 250 nm ($\varepsilon_{240} = 3200$ M^{-1} cm^{-1}). To mimic our reaction conditions with the tonoplast malate carrier, the assay contained 100 mM MES-KOH (pH 5.5) and 250 mM sucrose. Imidazole was added to a final concentration of 0.2 mM. The reaction was started by addition of 2 mM DEPC and recorded at 240 nm.

Materials

Diethylpyrocarbonate and Rose Bengal were obtained from Sigma (St Louis, MO). Stock solutions of DEPC were prepared fresh every day in absolute ethanol. The final ethanol concentration in the uptake assay was below 2%. Rose Bengal was dissolved in water. The solution was stored in darkness.

Results

Inhibition of Malate Uptake by DEPC

Figure 1 shows the time-dependent uptake of malate by tonoplast vesicles in the presence or absence of 2 mM DEPC in the incubation medium. A strong inhibition of malate uptake by DEPC occurred. After 15 min of incubation, the DEPC-treated vesicles revealed only 38% of the radioactivity of the control. It is noteworthy that the inhibition by DEPC develops to its maximum within the first 5 min of incubation. For this reason, we routinely used preincubation times of 5 min.

Malate Protection of DEPC Inhibition

As a next step towards the characterization of the DEPC inhibition, we investigated the effect of the substrate, malate, on carrier inactivation by DEPC

Table 1. Protection of the tonoplast malate carrier by the presence of substrate malate against DEPC-mediated inactivation

Experiment No.	Control	DEPC	DEPC + malate	Protection (%)
	(uptake rate in pmol mg^{-1} protein min^{-1})			
1	34.8	8.8	20.0	43
2	21.8	7.3	12.6	37
3	155.2	47.3	70.6	22

Tonoplast vesicles were pretreated with 2 mM DEPC for 5 min in the presence or absence of 5 mM malate. The reaction was stopped by the addition of imidazole, and the vesicles were used for the uptake experiments. The malate concentration in the uptake assays was 10 μM (experiments 1 and 2) and 50 μM (experiment 3).

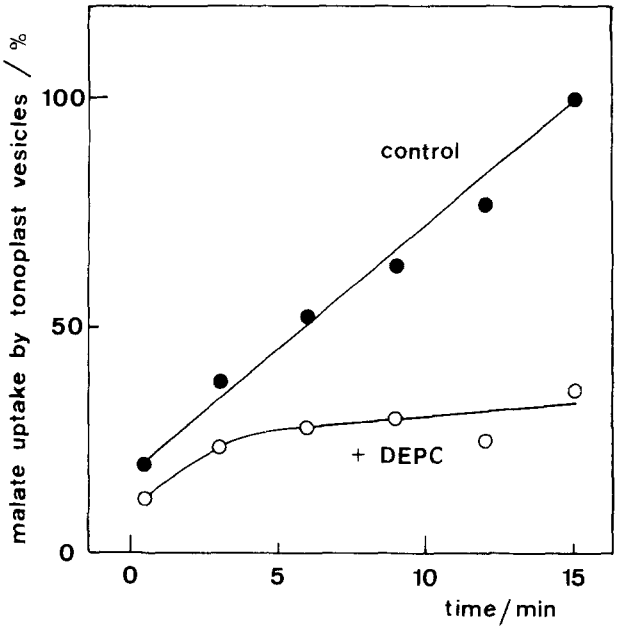

Fig. 1. Effect of DEPC on time-dependent uptake of ^{14}C-malate by tonoplast vesicles. DEPC was present at a final concentration of 2 mM during the actual uptake experiments. The malate concentration was 10 μM. The figure shows the mean of two experiments with identical inhibition characteristics but with different absolute rates; 100% corresponded to an uptake rate of either 20 or 8 pmol mg^{-1} protein min^{-1}.

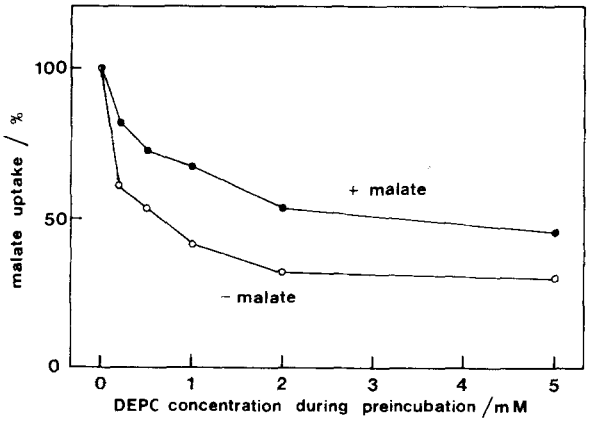

Fig. 2. Concentration dependence of inhibition of the tonoplast malate uptake by DEPC. The tonoplast vesicles were preincubated with (5 mM) or without malate in the incubation mixture for 5 min. DEPC was then added for another 5 min (at concentrations between 0 and 5 mM). Following this treatment, the vesicles were freed from the surrounding medium by sedimentation, washed twice and suspended in uptake medium containing 50 μM malate. Uptake of ^{14}C-malate was terminated after 6 min of incubation.

(Table 1). Malate presented protective properties; in this set of experiments, protection was in the range of 22–43%.

These observations led us to investigate the inhibitory effect of DEPC during preincubation for different DEPC concentrations (Fig. 2). The assays were performed in the absence or presence of 5 mM malate. Without malate in the preincubation mix, maximum inhibition was reached by 2 mM DEPC. Malate showed the already described effect of protection. Protection of the malate carrier by its substrate was greater at low DEPC concentrations. Protection efficiencies up to 50% and higher were observed at DEPC concentrations below 1 mM. Protection decreased to 23% with 5 mM DEPC in the assay.

In other sets of experiments, protection of the malate carrier by its substrate was investigated for different malate concentrations with constant 2 mM DEPC (Fig. 3). Malate concentrations below 0.1 mM showed no significant protection against the inhibitory effect of DEPC. The degree of protection observed in the uptake experiment increased with the malate concentration (from 0.2 to 5 mM) present during the preincubation. At malate concentrations higher than 5 mM, less protection was observed. The subsequent uptake assays were performed at three different malate concentrations, i.e., 10, 100 and 1000 μM, to test the effect of possible malate gradients across the vesicle membrane as a consequence of the preincubation with malate. For all the concen-

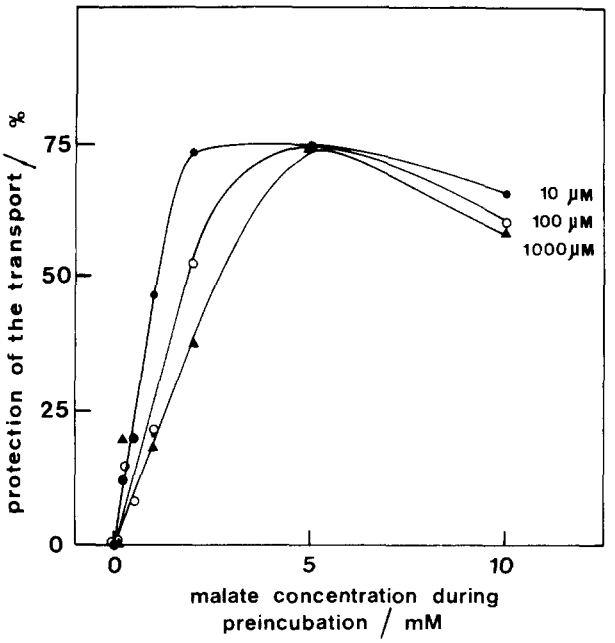

Fig. 3. Protection of the tonoplast malate carrier against DEPC-inactivation *versus* malate concentration. The tonoplast vesicles were preincubated with malate at concentrations as indicated on the abscissa. DEPC was added at 2 mM concentration for a further 5 min. The reaction was terminated by addition of imidazole (20 mM) and malate (5 mM). The malate concentration in the uptake assay was varied between 10, 100 and 1000 μM to ensure that no major effects of the malate concentration inside the vesicles influenced the uptake. 100% corresponds to 11.8 (10 μM), 58 (100 μM) or 990 pmol malate mg^{-1} protein min^{-1} (1 mM). The results are the mean of two experiments.

trations, malate protected the carrier from inactivation. The slope of the malate concentration against protection plot was less steep when the uptake experiment was performed at higher malate concentrations. This may reflect the influence of preloading of the vesicles with malate. Otherwise, it could be indicative of a change in substrate affinity upon DEPC treatment. A comparison of the K_m of the carrier for malate with the dissociation constant of malate in its protective role would allow further characterization of the site of inhibition of DEPC. As a first approximation of the dissociation constant, we used the data of Fig. 3 and calculated, as mean from all three curves, the malate concentration needed for 50% protection. The value of 1.3 mM is quite close to the reported K_m for transport of 2 mM, suggesting identity of the histidine residue modified by DEPC and the histidine residue involved in binding of the transported substrate.

Protection of the Malate Carrier by other Organic Acids

Different organic acids were tested as protective compounds of the malate carrier during incubation with 2 mM DEPC (Table 2). Succinate, fumarate and maleate showed little or no effect on DEPC inhibition. On the contrary, two other organic acids (citrate and quinate) which are known to accumulate together with malate in the vacuole (Alibert, Carrasco & Boudet, 1982) protected, like malate, the carrier against inactivation by DEPC.

To demonstrate that the protection of the carrier by organic acids was not due to a direct effect on the reaction of DEPC with histidyl residues, the formation of N-carbethoxyhistidine derivatives was checked in the presence of the organic acids (Table 2, column 2). In these experiments, fumarate and maleate could not be tested because of their high absorption at 240 nm. However, it was shown that the other organic acids did not affect the rate of N-carbethoxyhistidine-derivative formation. Thus, the decreasing inhibition of DEPC in the presence of malate, citrate and quinate is a real protective effect at the active site of the carrier.

Inhibition of Malate Uptake by Rose Bengal and Protective Effect of Malate

To confirm the involvement of a histidine residue for the activity of the malate carrier, we performed experiments to photooxidize the carrier in the presence of the dye Rose Bengal. Figure 4 plots the activity of the carrier against the concentration of Rose Bengal. As a control, tonoplast vesicles were treated with identical dye concentrations in the dark. It can be seen that even the dark-treated vesicles lost activity at high dye concentrations. At 80 $\mu g \cdot ml^{-1}$, both light and dark treatment led to complete inhibition of malate transport. Under these conditions, we assume destruction of the vesicles by some detergent-like properties of the dye. However, at low concentrations of Rose Bengal (1 $\mu g \cdot ml^{-1}$), a clear and pronounced inhibition can be seen in the light (~80%; *see* Fig. 4). Like for the DEPC treatment, a protective effect of malate against inactivation by Rose Bengal was evidenced; protection by 5 mM malate was 50% (*data not shown*).

pH Dependence of Catalyzed Malate Uptake

Figure 5 shows the effect of the external pH on the rate of malate upake in the presence or absence of 2 mM DEPC. Malate uptake was inhibited whatever the pH of the incubation medium: 70% inhibition at pH 6.0.

At acidic pH values, malate is also transported in its protonated form by passive diffusion (Lüttge

Table 2. Protection of the tonoplast malate carrier by different organic acids against the DEPC-mediated inactivation

	Protection of the carrier (%)	Reaction rate with imidazole (nmol min^{-1})
Control	100	—
DEPC	0	31 ± 3
Succinate + DEPC	0 ± 12	29 ± 1
Fumarate + DEPC	7 ± 7	ND[a]
Maleate + DEPC	11 ± 5	ND[a]
Quinate + DEPC	28 ± 9	29 ± 2
Citrate + DEPC	29 ± 4	31 ± 3
Malate + DEPC	55 ± 17	31 ± 4

Control uptake activity was 14 pmol mg^{-1} protein min^{-1} (= 100%).
The concentration of the organic acids was 5 mM. The results are means of four determinations ± SD.
Malate was present during the uptake assays at a concentration of 10 μM.
ND: not determined.
[a] Fumarate and maleate could not be tested for their effect on the reaction rate of DEPC with imidazole due to their high absorption at 240 nm.

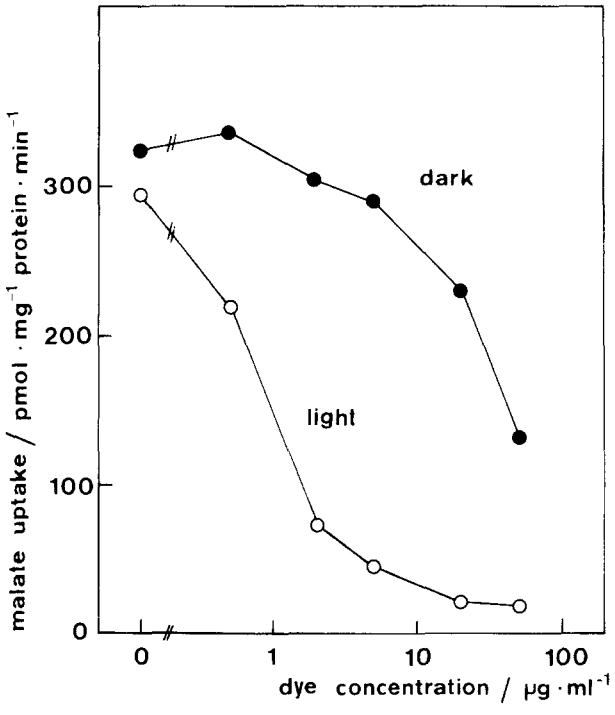

Fig. 4. Inactivation of the malate carrier by Rose Bengal. Vesicles were treated with Rose Bengal at the indicated concentrations either in the light for the photooxidative modification of the histidine residues or—as controls—in the dark. After 10 min of pretreatment, the malate uptake was initiated by addition of labeled malate (final concentration 100 μM) and terminated after 9 min by filtration and subsequent washing of the filters.

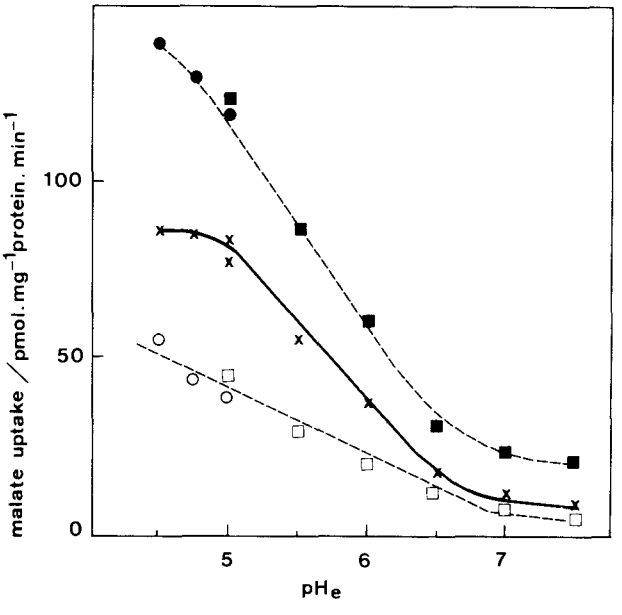

Fig. 5. Effect of external pH (pH*e*) on malate uptake. Tonoplast vesicles were incubated in the presence of 100 μM [^{14}C]-malate in a medium buffered with a mixture of 25 mM acetic acid, 50 mM Tris/MES (●,○) or 50 mM Tris/MES (■,□) and adjusted to the indicated pH values. Uptake was in the presence (open symbols) or in the absence (filled symbols) of 2 mM DEPC. Catalyzed uptake by the carrier was calculated by subtracting the values obtained in the presence of 2 mM DEPC from those obtained in its absence (×——×). The data are the mean of two independent experiments.

& Smith, 1984), explaining the lack of total inhibition by DEPC. So, the real catalyzed uptake was calculated from the difference between label taken up in the presence and that taken up in the absence of the histidine-reactive compound DEPC. The resulting calculated curve (Fig. 5, continuous line) is similar in appearance to a titration curve with a midpoint at pH 5.8, a value close to the pK of histidine (pK = 6.0).

Discussion

In the present study we used DEPC and the dye Rose Bengal, two histidine-specific reagents, to modify the malate carrier at the tonoplast, the vacuolar membrane of plant cells. Both compounds inhibited malate transport. The inhibition was partially prevented by addition of the substrate (malate). Moreover, the midpoint of the pH dependence curve of catalyzed malate uptake is close to the pK of histidine. These results, together with the large increase in substrate affinity at acidic pH (Bouyssou et al., 1990) suggest the involvement of a histidine residue in the binding or the translocation step of malate transport.

The results obtained working with DEPC have to be carefully evaluated due to the large number of reactions which can take place in the presence of excess DEPC. This reagent not only modifies histidine but also lysine, tryptophan and cysteine residues, although histidine is considered to be the most susceptible (Miles, 1977). However, a reaction of DEPC with cysteine residues is not likely to be the cause for inactivation of the carrier. Indeed, *p*-chloromercuriphenyl-sulfonic acid or N-ethylmaleimide, two efficient SH-group modifiers, were without effect on malate uptake by isolated vacuoles from *Catharanthus* (Marigo & Bouyssou, 1989). In addition, DEPC has been shown to be more specific for histidine residues when used at pH values below 6.0 (Miles, 1977; Lundblad & Noyes, 1984). In our experiments, greater inhibition of malate uptake was shown at low pH, suggesting that histidine, rather than another amino acid, is the target of DEPC in the carrier at the tonoplast.

In previous reports, the specificity of the DEPC effect on histidine residues was tested by its reversibility in the presence of hydroxylamine (Padan et al., 1979; Bauer et al., 1990), since the carbethoxy moiety is displaced from the imidazole nitrogen of histidine regenerating the initial native structure and function of the proteins (Miles, 1977). However, this approach requires high hydroxylamine concentrations and extended incubation periods (20 hr). Under these conditions, the tonoplast vesicles were no longer suitable for malate uptake assays due to a large increase of basal permeability towards malate.

The dye Rose Bengal, another histidine-modifying reagent, has been reported to be more specific because of its total lack of reactivity towards other amino acids (Fahnestock, 1975). Photooxidation of the tonoplast vesicles in the presence of Rose Bengal also led to a huge decrease in malate uptake. Maximum inhibition was achieved at very low concentrations of the dye as compared to previous reports (Fahnestock, 1975), confirming the presence of an essential histidine residue in the malate carrier.

An additional set of key experiments involved the use of substrate during studies of reactions with DEPC. The finding that inactivation of the malate carrier by DEPC was prevented by the presence of the substrate (malate) proved that a histidine residue is located at the active site of the carrier. The results did not exclude the possibility of other reactive histidine residues outside the active center. In fact, malate protection of the carrier against inactivation by DEPC varied from 25% to values as high as 75%, but the protection was never complete. This may be taken as evidence that other histidine residues susceptible to DEPC modification are present in the carrier protein. Their derivation also leads to conformational changes of the carrier and decreases of the uptake rate, thus explaining the lack of complete protection.

Moreover, protection of malate transport against inhibition by DEPC was also given by different organic acids. First of all, we showed that the tested organic acids did not prevent protein modification by inactivating the protein reagent itself, since no effect of the various organic acids was detected on the reaction of DEPC with the imidazole ring of the histidine residue. Furthermore, malate, citrate and quinate which were reported to accumulate in the vacuole (Alibert et al., 1982) presented a strong protective effect against DEPC inactivation. This coincides with a high efficiency of these compounds to inhibit malate uptake by isolated vacuoles (Marigo, Bouyssou & Laborie, 1988). Maleate, which did not affect malate uptake, had only little protective properties against DEPC inactivation. Thus, a positive correlation appears between the protection of the carrier and the inhibition of the uptake by organic acids. However, succinate and fumarate provided no or less protection than expected on the basis of their inhibitory behavior (Marigo et al., 1988). These organic acids are not accumulated in the vacuole, and their poor protection might be related to the lower capacity of the carrier to transport them.

As argued before, protonation of the transporter protein seems to be important for high activity (Marigo & Bouyssou, 1990). Protonation of histidine resi-

dues leads to a positive charge which may be required for binding the anionic substrates. After binding of malate (or other organic acids) the site is no longer accessible for the inhibitors. Therefore, competition between either malate or other organic acids and DEPC or Rose Bengal in binding to the histidyl residue causes a decrease of inhibition.

In conclusion, the present data indicate that a histidine residue(s) performs an essential function in the malate carrier at the active site of the protein and consequently provides a structural basis for the pH dependence of the malate transport system.

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