A Malic Acid Permease in Isolated Vacuoles of a Crassulacean Acid Metabolism Plant[1]

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ABSTRACT

Vacuoles isolated from mesophyll protoplasts of the Crassulacean acid metabolism plant *Bryophyllum daigremontianum* take up [^{14}C]L-malic acid from the medium. The initial rates of uptake show saturation kinetics (apparent K_m about 1 millimolar). In competition experiments, the uptake rates of L-malic acid are greatly reduced in the presence of a surplus of D-malic and tartronic acid. A number of other acids are much less effective and aspartic and isocitric acid have virtually no effect. The uptake rate of malic acid is unchanged in the presence of ATP and the uncouplers dinitrophenol and carbonyl cyanide m-chlorophenyl hydrazone. When the vacuoles are isolated in the absence of bovine serum albumin or when 1 millimolar HgCl$_2$ is added, they are inactive in transport but retain their malic acid pool. The existence of a specific permease which catalyzes an exchange diffusion of malic acid across the vacuolar membrane is postulated.

CAM is a conspicuous feature of photosynthesis in various succulent plants. It is characterized by fixation of CO_2 and accumulation of malic acid at night, and the utilization of this acid as an endogenous source of CO_2 during the subsequent light period. Thus, CAM is associated with the rhythmic filling up and depletion of large storage pools of malic acid. For obvious reasons it has tacitly been assumed that during acidification at night, malic acid formed in the carboxylation reaction is continuously removed from the cytoplasm and accumulated in the central vacuole. Subcellular compartmentation of this kind appeared to be a prerequisite for homeostatic conditions at the cytoplasmic sites of metabolism. Indeed, vacuoles isolated from mesophyll protoplasts of *Bryophyllum daigremontianum* contained the bulk of malic acid (4). In *Sedum telephium*, a CAM plant which allows the preparation of protoplasts within only 2 h, it has been demonstrated that the diurnal fluctuation is in fact a fluctuation of the vacuolar malic acid. In contrast to malic acid, isocitric acid which is also abundant in CAM plants, was present in *Sedum* vacuoles at a constant level (6, 7).

Thus, in CAM, large quantities of malic acid presumably are transported in and out of vacuoles. Key problems concern on the one hand the mechanism of transport across the tonoplast and on the other, the mechanism generating the driving force for the daily reversible accumulation and depletion of large quantities of malic acid in vacuoles. It is an open question whether or not these two mechanisms are directly linked.

In his recent review, Osmond (9) questioned whether "isolated

vacuoles from leaves of CAM plants can be used to study the properties of malic acid influx at the tonoplast." The answer is yes: we established conditions that allow the assessment and characterization of malic acid transport in purified vacuoles of a CAM plant.

MATERIALS AND METHODS

The method for preparing protoplasts described earlier (4) was modified as follows. Leaves of *B. daigremontianum* (Solisb.) were harvested at 8 AM and kept at 4°C until noon. Peeled tissue slices were treated with 0.5% (w/v) Macerozyme (Onozuka, Welding, Hamburg, Germany) for 3 h and then with 0.5% (w/v) Cellulase SS (Onozuka) in the presence of 0.2 mM PEG 4000 overnight at room temperature. Protoplasts were then isolated as detailed in (4). This empirically developed procedure yielded protoplasts (Fig. 1a) suitable for the preparation of vacuoles of the purity and stability necessary for the transport experiments.

Lysis was induced by suspending the protoplasts in a medium containing 0.5 M mannitol, 25 mM Hepes-Tris buffer (pH 7.5), 5 mM EDTA, and 1 mg/ml BSA. Upon incubation at 37°C for 1 min and gentle agitation, practically all of the protoplasts were lysed and a high proportion of vacuoles (50–60% as determined by counting) remained intact. Inasmuch as no suitable media for separating protoplasts from vacuoles by density gradient centrifugation could be found, complete lysis was essential. To isolate the vacuoles, the product of protoplast lysis was layered onto a washing medium containing 0.7 M sucrose, 25 mM Hepes-Tris buffer (pH 7.5), 1 mg/ml BSA, 1.5 mM MgSO$_4$, 5 mM KOH (neutralized with Hepes). The vacuoles were allowed to settle, and the supernatant was sucked off after 2 min before the chloroplasts had moved into the washing layer. On the average, 40% of the malate present in the protoplasts, corresponding to an equal proportion of vacuoles (4), was recovered at the bottom of the washing layer after 5 min. A representative sample of isolated vacuoles is shown in Figure 1b.

Uptake of [^{14}C]L-malic acid into vacuoles was measured using the sucrose medium described above. The standard incubation mixture contained 0.67 μCi of [^{14}C]malate (uniformly labeled, Amersham, 50 mCi mmol^{-1}) and 5 × 10^4 vacuoles in a total volume of 1 ml. The actual specific radioactivity in the incubation media was lower because of bursting of a few vacuoles. It was determined each time as indicated below and used for the calculation of uptake rates. After incubation at 20°C under gentle agitation, the vacuoles were separated from the incubation media by allowing them to settle across a discontinuous Ficoll gradient consisting of 1 ml 5% (w/v) Ficoll (Pharmacia) at the bottom and 1 ml 2% (w/v) Ficoll at the top and prepared in 55 × 10 mm plastic tubes. The Ficoll was dissolved in the aforementioned buffered sucrose medium. This slow separation at 1 g was necessary as *Bryophyllum* vacuoles are easily destroyed upon centrifugation. On the average, 50% of the vacuoles had moved into the 2% Ficoll layer after 1.5 min. Hence, the gradients were loaded at
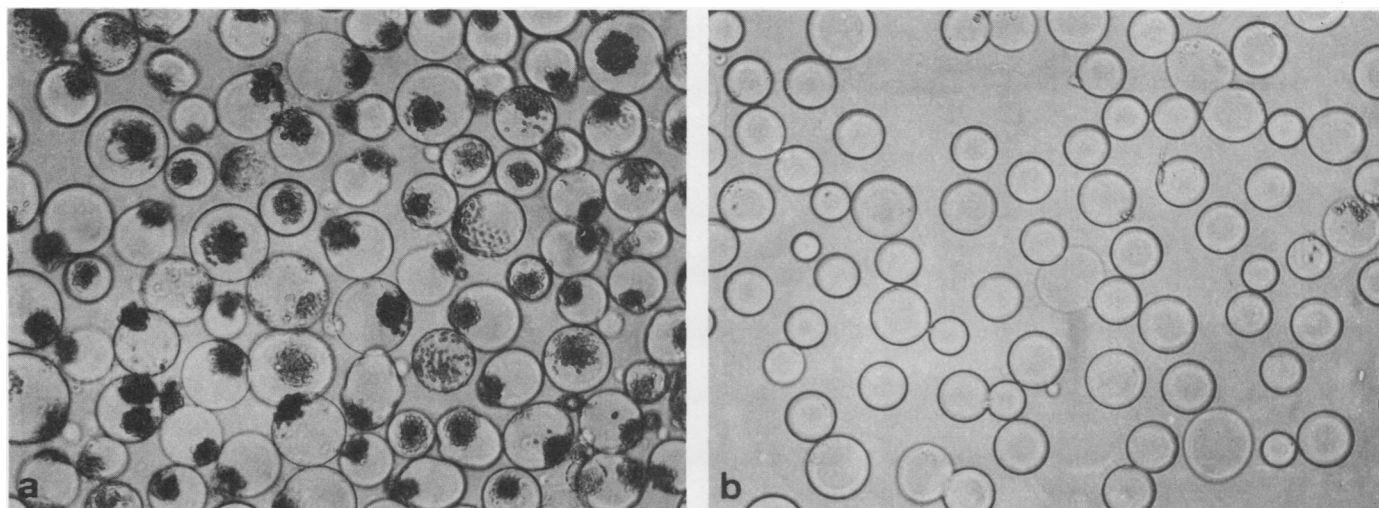
FIG. 1. (a), Protoplasts prepared from mesophyll tissue. Only from these polarized protoplasts vacuoles could be obtained which were pure and stable enough for transport studies. Phase contrast ×120. (b), Vacuoles isolated from mesophyll protoplasts. Phase contrast ×120.

X minus 1.5 min after the addition of labeled malate (X = desired incubation period). For the assessment of the initial rates of uptake the incubation periods were 2, 4, and 6 min. Thereafter, the vacuoles were allowed to settle for 15 min. After rapid freezing of the gradients in liquid N_2, the bottom of the tubes containing 0.5 ml of the 5% (w/v) Ficoll layer was cut off and used for liquid scintillation counting (Scintillator: Rialuma, Lumac AG, Basel, Switzerland; counting efficiency: 85%) as well as for the determination of malate as a measure of vacuoles recovered. Irrespective of incubation periods up to 6 min, about 95% of the vacuolar malate present in the incubation mixtures settled to the bottom of the gradients. When the incubation time was extended, the recovery of malate in the sedimented vacuoles slowly decreased to a value of 84% after 35 min. Uptake of radioactivity was expressed per unit of malate present in the preparation of settled vacuoles. Aliquots of the supernatant were also withdrawn after the separation of vacuoles, in order to determine the concentration in the incubation mixture of free malate stemming from burst vacuoles. With this value, the specific radioactivity present in the uptake assay was calculated.

Chloroplasts were collected by centrifugation (5 min, 1,000g) from the supernatant after the settling of the vacuoles. They were washed three times in a medium containing 0.5 M mannitol, 25 mM Hepes-Tris buffer (pH 7.5), and 1 mg/ml BSA. For the assessment of the rates of uptake of malic acid, chloroplasts were suspended in the standard incubation mixture. After incubation, they were layered onto the Ficoll gradients and sedimentated (5 min, 3,000g).

Activities of some marker enzymes and substances were determined as indicated in the legend of Table I.

RESULTS AND DISCUSSION

Transport activity of isolated vacuoles could never be observed unless BSA was added to the medium in which protoplasts were lysed and to all media used subsequently. Although in the absence of BSA about 75% of the vacuoles, exposed to radioactive malate for 6 min, settled to the bottom of the separation gradients, the radioactivity in this bottom fraction was always practically background. When BSA is present in the media, uptake of labeled malic acid (50 μM at the beginning) into vacuoles occurs at a linear rate over a period of 5 to 6 min; it then slows down and levels off after about 35 min of incubation (Fig. 2). Therefore, initial uptake rates had to be measured within the first 6 min. Because in the presence of BSA about 95% of the vacuolar malic acid settles to

Table I. Vacuolar and Extravacuolar Markers in Isolated Vacuoles

	mU/10⁴ Proto-plasts[a]	mU/10⁴ Vacu-oles[a]	% in Vacu-oles
Vacuolar markers			
Acid phosphatase[b]	41 – 100%	35.0	85
α-Mannosidase[b]	2	1.4	70
α-Galactosidase[b]	2	1.4	70
Isocitrate (nmol)[b]	390	341	87
Extravacuolar markers			
PEP carboxylase[c]	35	<1	<3
Aldolase[b]	69	<1	<1
Cyt c reductase (NADH)[d]	21	<1	<5
Cyt c oxidase[d]	14	<1	<7
Glucose-6-P dehydrogenase[b]	25	<1	<4
Hexose-6-P isomerase[b]	103	1.9	2
Malate dehydrogenase[e]	1237	51.6	4
Chl (μg)[e]	0.875	0.03	<3

[a] The content of malate[b]/10⁴ protoplasts and 10⁴ isolated vacuoles, respectively, was found repeatedly to be about equal (510 nmol × 10⁻⁴ protoplasts, 500 nmol × 10⁻⁴ vacuoles) showing that at least 95% of the malate is sequestered in the vacuoles. Having established this, the number of vacuoles could be determined on a basis of malate contents in protoplasts. For calculation, it was assumed that 100% of the malate is located in the vacuoles. 1 mU = 1 nmol substrate used or product formed per min. [b,c,d,e]determined according to ref. 2, ref. 10, ref. 5, and ref. 1, respectively.

the bottom of the Ficoll gradients after an incubation period of 6 min (compared with 75% in its absence), it is suggested that BSA has also a beneficial effect on the stability of the isolated vacuoles.

Protoplasts and chloroplasts are possible contaminants of vacuolar preparations which may simulate a transport activity of vacuoles. Both are capable of malate transport. Although the analysis of isolated vacuoles for marker enzymes of cytosol and chloroplasts suggested a satisfactory purity of the preparations (Table I), it was important to exclude the possibility of the falsification of the results by contaminating protoplasts and chloroplasts. The initial rate of malic acid transport per protoplast is about 5 times lower than the rate per vacuole (1 pmol × 10⁻⁴ vacuoles·min⁻¹) under the same assay conditions (50 μM malic acid). Determinations of a cytosolic marker, hexose-P-isomerase, and counting in the microscope showed that the proportion of protoplasts in suspensions of isolated vacuoles never exceeded 5%. Hence, protoplasts were not responsible for the malic acid uptake.
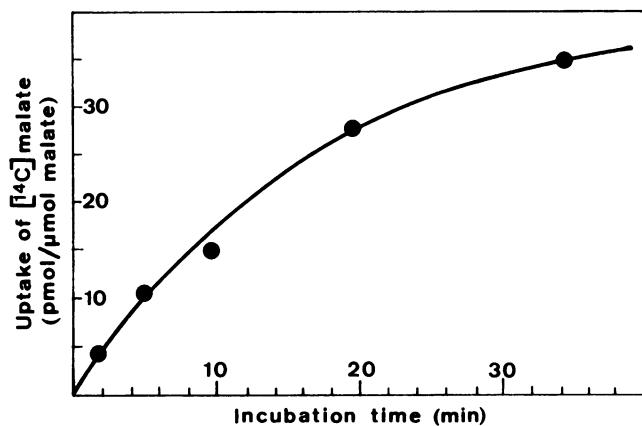
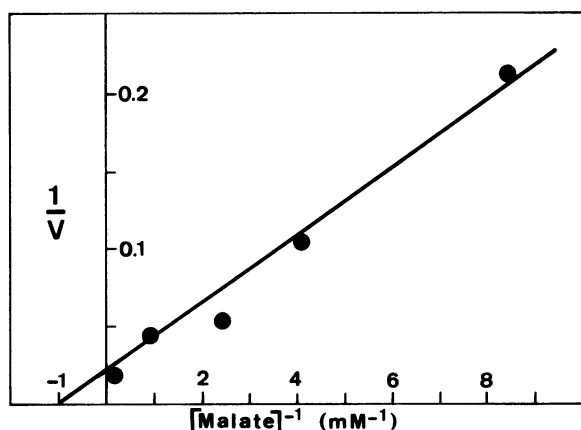FIG. 2. Dependence on time of the uptake of [^{14}C]L-malic acid (50 μM) by isolated vacuoles at 20°C (pH 7.5).

FIG. 3. Double reciprocal plot of the initial rates of malic acid uptake (pmol·μmol malate^{-1}·min^{-1}) as a function of malic acid concentration in the incubation medium by isolated vacuoles (20° C [pH 7.5]). The malic acid content of 10^4 vacuoles is 500 nmol.

The initial rate of malic acid uptake per number of chloroplasts is 70 times lower than per number of vacuoles, measured under the same conditions (50 μM malic acid). As in the purified preparations of vacuoles in the worst case where three chloroplasts were counted per vacuole, the contribution of chloroplasts to the measured rates of uptake is less than 5%.

A V_{max} of about 25 pmol malic acid × 10^{-4} vacuoles·min^{-1} was calculated (Fig. 3). Under the assumption that all the malic acid present in the vacuoles (500 nmol × 10^{-4} vacuoles) is transported during one night period (12 h), the necessary rate of transport is approximately 700 pmol malic acid × 10^{-4} vacuoles·min^{-1}. This comparison may lead to the conclusion that the malic acid transport rates observed *in vitro* are insignificant with regard to CAM. However, the comparison is not very meaningful as the conditions of transport *in vitro* are certainly widely different from the conditions within the living cell. Moreover, the preparation of vacuoles according to our technique requires 24 h and may be associated with a loss of transporting activity. The use of rapidly isolated vacuoles such as those prepared from *Sedum telephium* (7) would perhaps provide further insight.

The uptake measured with the isolated vacuoles is not dependent on ATP (added as the 5 mM Tris-salt). It is also practically unaffected by 2,4-dinitrophenol (0.1 mM) and carbonylcyanide *m*-chlorophenylhydrazone (5 μM). These uncouplers did not affect the stability of the vacuoles as determined by counting or by assessing the sedimentability of malic acid. The involvement of a carrier protein is suggested by the complete inhibition of transport

Table II. *Effectors on the Initial Rates of* L-*Malic Acid Transport in Isolated Vacuoles*

Effectors[a]	Rate of Transport[a] (Relative Values)
Control[b]	100
D-Malic acid	10
Tartronic acid	9
Citric, oxaloacetic, L-lactic acid	25–30
Succinic, fumaric, malonic acid	50–70
L-Aspartic acid	98
DL-Isocitrate-(Na$_3$$^+$)	63
Na$^+$(Hepes), 30 mM	71
ATP (5 mM Tris-salt, 1.5 mM Mg^{2+}, 5 mM K$^+$)	104
2,4-Dinitrophenol, 0.1 mM	93
Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (5 μM)	81
HgCl$_2$ (1 mM)	0

[a] Initial rates of uptake of [^{14}C]L-malic acid (20–50 μM; 6–12 μCi/umol) were measured in the presence of effectors (10 mM).

[b] The control rate (100%) represents the difference between the rate of uptake of [^{14}C]L-malic acid (50 μM) measured in the presence and absence of additional 10 mM unlabeled L-malic acid (pH adjusted with Tris). It was 1.6 pmol·μmol malate^{-1}·min^{-1}. The uptake of the labeled L-malic acid in the presence of 10 mM unlabeled L-malic acid and in the presence of HgCl$_2$ was identical (0.4 pmol·μmol malate^{-1}·min^{-1}). It was attributed to unspecific uptake of radioactivity.

in the presence of 1 mM HgCl$_2$, whereas malate still sedimented with vacuoles to 70% of the control. Saturation kinetics of malic acid transport (Fig. 3) provide a further indication of the involvement of a permease. The apparent K_m value is about 1 mM. Moreover, the malic acid transport system appears to be specific, as shown by competition experiments. When the incubation mixtures were supplemented with an excess (10 mM) of nonradioactive analogs of L-malic acid, the uptake rate of [^{14}C]L-malic acid (20–50 μM) was reduced to various degrees (Table II). The stereoisomer D-malic acid and the C$_3$-dicarboxylic acid analog of malic acid, tartronic acid, reduced the rate of uptake by 90%. Reduction of the rate was much less in the case of a number of other acids, and L-aspartic and isocitric acid had practically no effect.

Concerning the pH dependency of transport, the range of conditions was limited by the stability of vacuoles between pH 6 and 8.5 (Mes-Tris buffer [pH 6–6.5], Hepes-Tris buffer [pH 6.5–8.5]). Optimal stability is at pH 7.5. From pH 6 to 7.5, the transport activity decreases by about 25% and at pH 8.4 it is reduced to about 30% of the activity measured at pH 6.

The transport of malic acid in *Bryophyllum* vacuoles has features in common with the transport of arginine in yeast vacuoles by the action of a specific permease (3). Although the concentration of arginine in the yeast vacuoles is up to 1,000 times higher than the concentration in the suspending medium, labeled arginine is transported readily into the isolated vacuoles. This transport is due to the exchange of external arginine with vacuolar arginine as demonstrated by double labeling experiments (3). In a similar fashion, the uptake of labeled malic acid by isolated vacuoles of *Bryophyllum* appears to reflect a catalyzed exchange diffusion across the tonoplast. The independence of malic acid transport on ATP and uncouplers support this hypothesis as an exchange reaction does not depend on an energy source. It is interesting that electrophysiological considerations have led to the suggestion that malic^{2-} anions may diffuse passively across the tonoplast (8). For reasons of experimental difficulties, it was impossible to demonstrate the

efflux of [^{14}C]malate from preloaded vacuoles. Nevertheless, we propose that the permease measured catalyzes a facilitated exchange diffusion across the tonoplast which by itself does not result in a net uptake of malic acid into the vacuoles. Therefore, the mechanism generating the driving force for the reversible accumulation and depletion of large quantities of malic acid in vacuoles of CAM plants remains to be elucidated.

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