Biochimica et Biophysica Acta 806 (1985) 311–319
Elsevier

BBA 41706

Energy-dependent uptake of malate into vacuoles isolated from barley mesophyll protoplasts

Enrico Martinoia [a], Ulf Ingo Flügge [b], Georg Kaiser [a], Ulrich Heber [a] and Hans Walter Heldt [b]

[a] *Lehrstuhl Botanik I der Universität Würzburg, Mittlerer Dallenbergweg 64, 8700 Würzburg and* [b] *Institut für Biochemie der Pflanze der Universität Göttingen, Untere Karspüle 2, 3400 Göttingen (F.R.G.)*

(Received August 14th, 1984)

Key words: Malate uptake; Energy dependence; Protoplast vacuole; (Barley)

The uptake of malate into vacuoles isolated from barley mesophyll protoplasts has been studied by silicone layer flotation filtering. The transport shows saturation characteristics allowing the determination of K_m (2.5 mM) and V_{max} (2.6 μmol/mg Chl per h). In the presence of MgATP the uptake is markedly enhanced (V_{max}, 8 μmol/mg Chl per h), while the K_m value remains constant. The MgATP-dependent malate transport has been characterized also with respect to anion and cation dependence, nucleotide specificity, pH dependence and its sensitivity to ATPase inhibitors and ionophores. It is concluded that this energy-dependent transport can occur against a transmembrane concentration gradient and that the transport is coupled to the action of a largely specific H^+-translocating ATPase which can be clearly distinguished from mitochondrial and plasma membrane ATPases.

Introduction

In a leaf performing photosynthesis, fixed carbon is exported as triose phosphate and 3-phosphoglyceric acid from the chloroplast stroma to the cytosol, where it is converted to secondary products of photosynthesis, which, in the case of assimilating protoplasts from mesophyll cells of wheat, spinach and barley, are mainly sucrose and, apart from this, also organic acids [1–3]. Although in a leaf usually most of the products of photosynthesis will be translocated through the phloem system to other parts of the plant, where they are utilized (e.g., for growth or for the supply of respiring tissues), a certain portion of the fixed carbon is stored in the mesophyll cells in order to be consumed in the subsequent dark period. In the mesophyll cell there exist at least three different ways in which the fixed carbon can be accumulated: the accumulation of (i) starch in the chloroplast stroma and of (ii) sucrose and (iii) malate in the extrachloroplast compartments. The relative sizes of these stores vary in different plants. In leaves of spinach 280, 100 and 25 μatom C/mg Chl of starch, sucrose and malate, respectively, have been found after the end of a 9 h illumination period, and all these stores are almost depleted at the end of the subsequent night [4]. Most of the sucrose and malate is stored in the vacuole. Studies of ^{14}C incorporation with *Vicia faba* leaflets showed that newly formed sucrose had been transferred into the vacuole [5]. In barley mesophyll protoplasts a large portion of the fixed carbon was found in the vacuole as sucrose, and some part also as malate [3]. Subcellular fractionation of

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DES, diethyl stilbestrol; Mes, 4-morpholineethanesulphonic acid; CAM, crassula acid metabolism.

spinach leaves by a nonaqueous procedure not only demonstrated that both sucrose and malate are mainly located in the vacuole, but also indicated that the two substances are transported into the vacuole in a different mode [4]. In the case of sucrose the concentrations in the cytosol and in the vacuole were found to be similar, indicating that loading of the vacuole may proceed by passive transport. This concurs with direct measurements of sucrose transport into vacuoles from barley showing that this transport was not stimulated by ATP [6]. In the case of malate, however, the vacuolar concentration was found to be at least one order of magnitude higher than in the cytosol [4] strongly indicating that the uptake of malate into the vacuole proceeds by active transport.

In CAM plants, where malate plays a central role as an assimilatory intermediate [7], an active transport of malate into the vacuole has been postulated on the ground that with the very high amounts of malic acid accumulated in these plants during the night, the metabolism in the cytosol would be strongly inhibited unless this malic acid was sequestered in the vacuole [8]. Although it is an obvious possibility that an active transport of malate into the vacuole may be driven by an ATP-dependent proton transport, experimental evidence for this is still lacking. Investigations of malate transport with isolated vacuoles of a CAM plant yielded evidence for a passive transport of malate only [9]. A vacuolar ATPase has been identified in a CAM plant, with properties similar to proton-translocating ATPases [10], but its participation in malate transport so far is speculative only. In vacuole-like lutoids from the latex of the rubber tree *Hevea brasiliensis*, an MgATP-dependent transport of citrate could be correlated with an MgATP-driven proton transport into the vacuoles, suggesting that the protonmotive force created by an ATPase is the driving force of citrate uptake [11].

In the present article the transport of malate into vacuoles from barley mesophyll protoplasts has been studied. Evidence is presented for an MgATP-dependent active malate transport, being driven by a proton-translocating ATPase.

Materials and Methods

Preparation of vacuoles

Protoplasts from young barley leaves were prepared as described previously [3]. Vacuoles were isolated and purified from the protoplasts by a modification of other methods of Martinoia et al. [12] and Kaiser and Heber [6]. All steps were carried out at 4°C. Protoplasts (3–4 mg chlorophyll) in 6 ml medium (0.45 M sorbitol/30 mM Hepes-imidazol (pH 7.4)/0.1% bovine serum albumin (w/v)/0.1% polyvinylpyrrolidone (w/v, 10 kDa) containing 18% (v/v) Percoll (Pharmacia) were lysed by forcing the suspension through a syringe (0.7×100 mm). In order to purify the released vacuoles by flotation, the lysate (phase 1) was overlayered with 8 ml of the above-mentioned medium containing 2% (v/v) Percoll only (phase 2) and 2 ml medium containing no Percoll and 0.45 M glycine betaine instead of sorbitol (phase 3). After centrifugation (2 min at $100 \times g$ followed by 3 min at $1100 \times g$) the vacuoles accumulated at the upper interphase and were collected from there. Afterwards, the remainder of phase 3 and also phase 2 were removed, and those protoplasts which had remained intact in phase 1 were again subjected to the lysis treatment and the subsequent purification procedure as described above. For further purification, the collected vacuoles, suspended in 3 ml medium containing 8% (v/v) Percoll, were overlayered with 2 ml of phase 2 and 2 ml of phase 3 (see above) and centrifuged for 4 min at $650 \times g$. The purified vacuoles were removed from the upper interphase and stored on ice.

Measurement of malate transport into the vacuoles

The uptake of [^{14}C]malate into vacuoles was measured at 20°C by silicon layer filtering flotation as previously described [6] or by the following slight modification. To a suspension of vacuoles ($5 \cdot 10^5$ vacuoles/ml) in a medium containing 0.45 M sorbitol, 25 mM Hepes-imidazole (pH 7.4) and other solutes as indicated in tables and legends, [^{14}C]malate (specific activity, 0.9 Ci/mol (Amersham-Buchler, Braunschweig, F.R.G., concentration as indicated) was added. For measuring the kinetics of the uptake, at various times 100-μl aliquots were pipetted into a 400-μl polypropylene

microcentrifugation tube. In order to avoid that some of the pipetted volume was adhering to the wall of the tube, it was centrifuged very shortly (about 1 s) at 10000 × *g* in a microfuge (Beckman Instruments) and the sample was then overlayered with 200 µl phenylmethyl silicone oil (AR 200, Wacker Chemie, München, F.R.G.) and 20 µl of 2% Triton X100 were added on top of this. The uptake of [^{14}C]malate was terminated by centrifugation (10000 × *g*, 30 s), by which the vacuoles immediately flotated through the silicone layer above into the aqueous phase containing Triton X100. To this, 50 µl of water were added and the total aqueous phase above the oil was collected for measurement of radioactivity by scintillation counting. In parallel experiments [^{14}C]ferricyanide, a substance known not to permeate membranes and also ^3H_2O were added instead of malate to the vacuolar suspension. From measuring the resultant radioactivity in the supernatant fraction after centrifugation, the small amount of medium adhering to the surface of the vacuoles, as they migrate through the silicone layer, can be corrected for. By evaluation of the ferricyanide impermeable ^3H_2O space, the internal volume of the filtered vacuoles is defined. The rates of malate uptake into the vacuoles are related to the number of vacuoles, as evaluated from the ferricyanide impermeable ^3H_2O space and the assumption that $1 \cdot 10^7$ vacuoles have a volume of 130 µl.

For a comparison with the rates of photosynthesis, the rates of vacuolar transport have been related also to the chlorophyll contained in the protoplasts. The amount of chlorophyll corresponding to a vacuolar suspension was determined from assay of the activity of the vacuolar marker enzyme α-mannosidase [12] in the vacuolar preparation and in the protoplasts according to the method of Boller and Kende [13]. For the assay of chlorophyll in the protoplasts, see Ref. 14.

Since relatively large samples are required for the assay of α-mannosidase, in some experiments acid phosphatase was used as a marker instead, taking into account that on average 75% of this activity is of vacuolar origin. This figure had been earlier determined from a comparison of the subcellular distribution of α-mannosidase and acid phosphatase. Acid phosphatase was assayed according to Leigh and Walker [15].

Results and Discussion

Time-course and kinetic parameters of the MgATP dependent malate transport into the vacuoles

Malate transport into vacuoles shows a requirement for MgATP. As shown in Fig. 1, the rate of transport in the absence of MgATP not only is much lower than in its presence, but it also further decreases after a period of about 5 min. In contrast to this, malate transport in the presence of MgATP is shown to be linear for at least 20 min. In the experiment of Fig. 1, after 20 min the internal concentration of [^{14}C]malate (12 mM) is more than 10-times higher than the external concentration (1.1 mM) initially added, whereas in the absence of MgATP, the internal malate concentration exceeds the external one only by a factor of 2–3. This slight accumulation observed in the absence of externally added ATP could be further depressed by the addition of nigericin or CCCP/valinomycin (not shown). This may indicate that there was a residual ion gradient driving malate transport into the vacuoles even in the absence of a metabolic energy supply. ATP in the presence of EDTA did not stimulate malate trans-

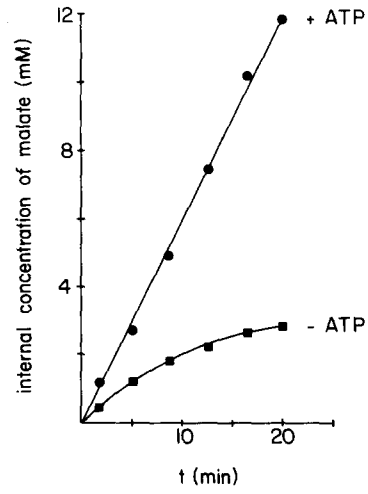

Fig. 1. Time course of [^{14}C]malate uptake to vacuoles isolated from barley mesophyll protoplasts. Isolated vacuoles (equivalent to 35 µg Chl/ml) were incubated at 20°C with 1.1 mM [^{14}C]malate (specific activity, 0.9 Ci/mol). At the time indicated, the reaction was terminated by centrifugation of 100 µl aliquots as described in Material and Methods. ■———■, without MgATP; ●———●, in the presence of 10 mM MgATP.

port to any considerably extent indicating that MgATP and not the free ATP is the true substrate for the stimulated malate uptake. In order to ensure that MgATP did cause a net accumulation of malate, rather than an exchange with unlabelled malate, we measured the uptake of malate into the vacuoles by enzymatic assay employing malic dehydrogenase/citrate synthase. With a malate concentration of 9.2 mM in the vacuolar space before the addition of malate (1.5 mM for 20 min), the internal concentration increases to 12.6 mM in the absence of MgATP and to 17.5 mM in the presence of 10 mM MgATP. These results indicate that there is indeed net uptake of malate against a concentration gradient in the presence of MgATP. Also in the absence of MgATP some accumulation is observed, which could be due to an exchange with an existing gradient of other anions, e.g., chloride. These results clearly demonstrate that vacuoles of the C3 plant barley have a system for an active transport of malate, which is driven by ATP. In a very similar mode citrate is taken up by barley vacuoles (Fig. 2). This uptake is also stimulated by MgATP, whereas ATP alone had no effect (data not shown).

For studying the specificity of vacuolar malate transport, the competition of other carboxylates on the transport was investigated. As shown in Fig. 3, the transport of L-malate is competitively inhibited by D-malate and citrate. In the inset of Fig. 3, a Dixon plot reveals a K_i of 2.7 mM for citrate. Oxaloacetate is also a competitive inhibitor. Likewise, tartronate, malonate and succinate are inhibitory, although the competition is less pronounced (not shown in the figure). The K_i values for the various dicarboxylates, as obtained in a number of similar experiments are shown in Table I. It appears from these results that the observed transport of malate into the vacuoles may be rather unspecific. Probably the malate transporter also mediates the observed uptake of citrate.

In order to define the effect of MgATP on the malate transport, we measured the concentration dependence of this transport in the presence and absence of MgATP (Fig. 4). A double reciprocal plot of the data reveals that MgATP does not affect the apparent affinity of the malate transporter toward its substrate. With MgATP being present or absent, the K_m for malate is found to be about 2.5 mM, which is in the range of the cytosolic malate concentrations in spinach leaves [4]. The V_{max} for malate transport in the presence of MgATP (8.0 μmol per mg Chl/h) is about 3-times

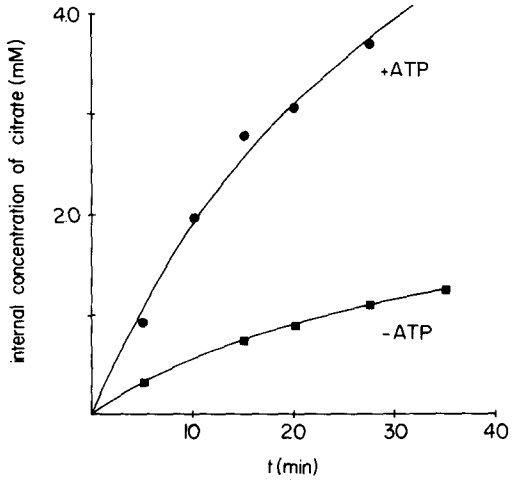

Fig. 2. Time-course of [^{14}C]citrate uptake into vacuoles from barley mesophyll protoplasts. See legend Fig. 1. 1.4 mM [^{14}C]citrate (specific activity, 1.2 Ci/mol) were added.

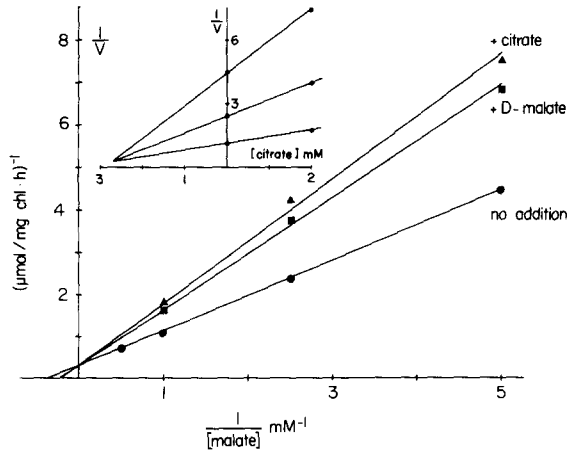

Fig. 3. Concentration dependence of the uptake of [^{14}C]malate into isolated vacuoles in the presence of D-malate or citrate (2 mM external concentration). The evaluation of the curve yields for L-malate, K_M = 2.6 mM; for D-malate, K_i = 3.2 mM and for citrate, K_i = 2.7 mM.

TABLE I

COMPETITIVE INHIBITION OF [^{14}C]MALATE TRANSPORT INTO ISOLATED VACUOLES BY CARBOXYLATES

The results have been obtained from the experiment of Fig. 3 and similar experiments. n, the number of experiments. The average value for the K_m of L-malate was 2.5 mM.

Treatment	K_i (mM)	n
D-malate	2.0–3.2	3
Citrate	1.7–2.7	3
Oxaloacetate	3.6–4.8	2
Tartronate	4.0–7.0	3
Malonate	9.0–12.0	3
Succinate	9.0–12.0	2

higher than that in the absence of MgATP (2.6 μmoles per mg Chl/h), which demonstrates that the stimulation of malate transport by MgATP is due to an increase of V_{max}. It may be noted that maximum rates of transport obtained with the isolated vacuoles are more than sufficient to sustain the import of malate into the vacuoles of mesophyll cells which was calculated to occur at an apparent rate of 0.6 μmol per mg Chl/h [3,4].

The data shown so far suggest that the stimulation of malate transport by MgATP is due to an ATPase located in the vacuolar membrane. In order to characterize this process, we studied the dependence of the MgATP-stimulated malate transport, defined as the difference between the rates of malate transport observed with and without MgATP, on the concentration of MgATP. A double reciprocal plot of such data, as shown in Fig. 5, yields a V_{max} of 4.5 μmol per mg Chl/h, which is comparable to the V_{max} observed in the experiment of Fig. 4, taking into account that in the experiment of Fig. 5 a half-saturating concentration of malate was employed. The apparent K_m for MgATP is evaluated as 2.5 mM. It is considerably higher than the published values of other tonoplast ATPases [11,16,17].

pH dependence of malate transport

In the experiment of Fig. 6, the pH dependence of the malate transport in the presence and absence of MgATP was investigated. The transport measurements carried out in the presence of MgATP are complicated by the fact that there are

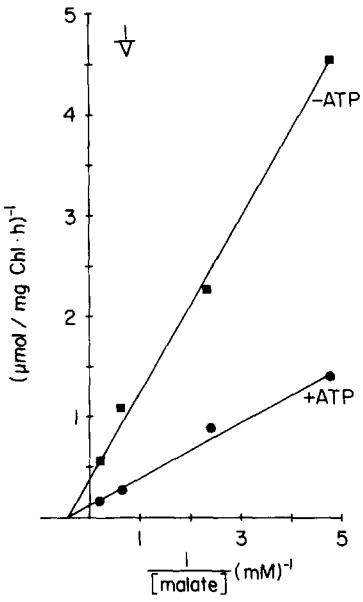

Fig. 4. Concentration dependence of the uptake of [^{14}C]malate into isolated vacuoles. Temperature, 20°C. In order to confirm that initial rates were measured the linearity with time was checked for the malate concentration used. ■———■, without MgATP; ●———●, in the presence of 10 mM MgATP.

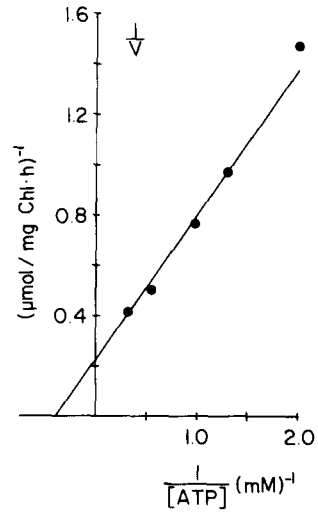

Fig. 5. Concentration dependence of the uptake of [^{14}C]malate into isolated vacuoles by varying the external MgATP concentration. The malate concentration was kept constant at 2.5 mM. From the initial rates measured, the initial transport rates measured in the absence of MgATP were subtracted. For details, see Materials and Methods and other sections as well.

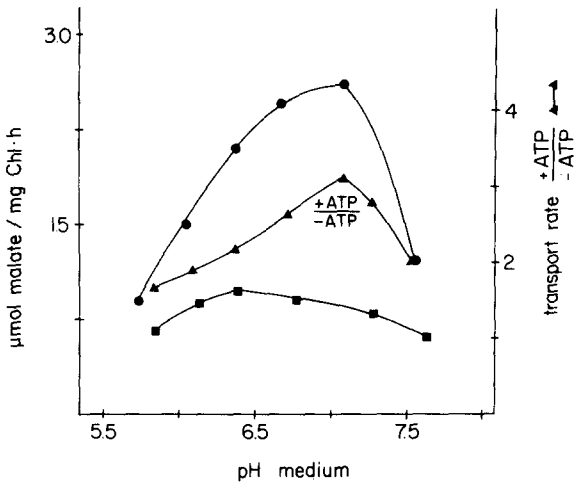

Fig. 6. pH dependence of the [^{14}C]malate uptake into isolated vacuoles. The incubation mixture contained 80 mM Hepes/Mes buffer adjusted to the indicated pH values and vacuoles equivalent for 40 µg Chl/ml. For measuring the MgATP-dependent malate transport, the incubation mixture contained 10 mM MgATP/3 mM creatine phosphate/25 µg/ml creatine kinase. Malate uptake is expressed in terms of µmol per mg Chl/h (calculated with the relation that $1 \cdot 10^7$ vacuoles are equivalent to 1 mg Chl, see Ref. 12). ■———■, without MgATP; ●———●, in the presence of MgATP and an ATP-regenerating system.

always some ruptured vacuoles in the suspension, releasing acid phosphatase to the medium, which, at lower pH, will cause the hydrolysis of ATP. To compensate for ATP hydrolysis, we used in these experiments an ATP regenerating system, which even in the acidic pH range maintained an ATP concentration of at least 5 mM.

In the absence of MgATP, the transport of malate is only slightly affected by the pH in the medium. Malate transport in the presence of MgATP, however, has a relatively broad pH optimum around pH 7. A plot of the ratio between the transport rates with and without MgATP yields a relatively sharp optimum at pH 7.0, a value which is thought to reflect the pH in the cytosol of a plant cell [18]. It is feasible that this pH optimum reflects the optimum of the ATPase driving the malate transport. pH optima in the neutral region have also been found with other vacuolar ATPases, e.g., the H^+-translocating ATPase of Hevea lutoids [11] or of red beet vacuoles [16].

Influence of ions on the MgATP stimulated transport of L-malate

Malate transport in the absence of MgATP is little affected by either cations or anions (Table II). Only SCN^- caused 20–30% inhibition of transport. The MgATP-dependent transport, however, was slightly stimulated by sodium and potassium ions, and severely inhibited by anions. The inhibition was most pronounced with SCN^-, followed by NO_3^-, Cl^- and SO_4^{2-}. An inhibition by NO_3^- has been earlier observed with tonoplast ATPase from storage roots of red beet [16,19,20] and from CAM plants [10], but in these cases Cl^- and other anions had a stimulatory effect. Direct measurements of ATPase activity in barley mesophyll vacuoles, carried out under the same conditions as outlined in the legend of Table II confirmed that this ATPase was stimulated by Cl^- and SO_4^{2-} and inhibited by SCN^- and NO_3^- (Flügge, U.I. and Martinoia, E., unpublished data). It is obvious from these findings that the above-mentioned inhibition of MgATP-dependent malate transport by Cl^- and SO_4^{2-} cannot be due to an inhibition of the ATPase. It is therefore feasible that not only the uptake of malate but also the

TABLE II

INFLUENCE OF DIFFERENT IONS ON THE INITIAL RATES OF [^{14}C]MALATE UPTAKE

Anions (40 mM) were applied as salts of choline base, except for the SCN^- ion which was the potassium salt. In the control, choline base was neutralized with Mes-buffer. The malate and the MgATP concentrations were 1.1 and 8.0 mM, respectively. Cations (40 mM) were applied as gluconate salts. the control rates, obtained without K^+ and Na^+ ions being present in the medium were 0.75 µmol per 10^7 vacuoles/h (about equivalent to µmol per mg Chl/h) in the absence of MgATP and 2.50 in the presence of MgATP. Measuring time, 3 min. The values are averages of results obtained in four different experiments.

Treatment	Malate transport (% of control)	
	−MgATP	+MgATP
K^+	102	124
Na^+	101	110
SO_4^{2-}	108	51
Cl^-	94	50
NO_3^-	94	20
SCN^-	77	15

transport of Cl^- and SO_4^{2-} can be driven by an electrochemical force created by the action of the vacuolar ATPase. Preliminary results indeed show that the uptake of Cl^- into the vacuoles is stimulated by MgATP. It is tempting to speculate that by competition for the driving force, the presence of these anions would lower the uptake of malate.

Nucleotide specificity of the energy-dependent malate uptake

The nucleotide specificity was tested by measuring the stimulated malate transport (defined as in the experiment of Fig. 4) in the presence of 10 mM Mg^{2+} as the gluconate salt plus 10 mM of one of the following: ATP, ADP, GTP, CTP, UTP and pyrophosphate. The relative rates of the stimulated transport were 1.0, 0.12, 0.31, 0.56, 0.32 and 0.07, respectively. Thus, for driving the active transport of malate, MgATP is the best substrate, although the other nucleoside triphosphates are also reacting. This shows that the specificity of the vacuolar ATPase involved in malate transport is not very high. It may be noted that also for some other vacuolar ATPases a relatively broad nucleotide specificity has been found [10,15].

Effect of inhibitors on the MgATP-stimulated malate transport

N,N'-dicyclohexylcarbodiimide (DCCD) and diethyl stilbestrol (DES) are effective inhibitors of MgATP-dependent malate transport (Table III). DCCD is known as an inhibitor of proton-translocating ATPases, whereas DES has been considered in the past to be a typical inhibitor of plasmalemma ATPase [21,22]. It was shown recently, however, that DES also inhibited the vacuolar ATPases from storage roots of red beet [16] and from CAM plant [10]. Sodium vanadate, which inhibits plasma membrane ATPases by preventing the formation of a phosphorylated enzyme intermediate, had no effect on the MgATP-dependent malate uptake. Oligomycin and azide, both powerful inhibitors of mitochondrial ATPase, had little or no effect on the MgATP-dependent malate transport. In summary, the results of Table III indicate that the MgATP-dependent malate transport into barley vacuoles involves a proton-translocating ATPase which is similar to other known vacuolar ATPases and which can be clearly distinguished from mitochondrial and plasmalemma ATPases.

TABLE III

EFFECT OF INHIBITORS ON THE INITIAL RATES OF MgATP DEPENDENT [^{14}C]MALATE TRANSPORT INTO ISOLATED VACUOLES

The measurements were done in the presence of 1.1 mM malate and, if not stated otherwise, 8 mM MgATP. Measuring time, 4 min. Mean values of four experiments. 1 μmol per 10^7 vacuoles/h is equivalent to 1 μmol per mg Chl/h.

Treatment	Transport rates (μmol per 10^7 vacuoles/h)	Percentage
Control	1.96	100
+ DCCD (100 μM)	0.73	37
+ DES (250 μM)	0.75	38
+ Vanadate (200 μM)	1.92	98
+ Azide (1 mM)	1.92	98
+ Oligomycin (40 μg/ml)	1.82	93
+ pHMB (50 μM)	0.25	13
Without MgATP	0.66	34
Without MgATP, with pHMB (50 μM)	0.22	11

Relatively low concentrations of the SH-reagent *p*-hydroxymercuribenzene sulfonate inhibit the transport of malate in the absence as well as in the presence of MgATP. Apparently, by modification of an SH-group, the inhibitor acts on the malate transporter, although an additional effect of this relatively unspecific inhibitor on the ATPase cannot be excluded.

Effect of ionophores on the MgATP stimulated malate transport

The results shown so far suggest that a proton-translocating ATPase is involved in the active transport of malate. If this is correct, and if uphill transport is driven by an electrochemical gradient, malate uptake should be influenced by ionophores which degrade a membrane potential and/or ion gradients. In order to check this, we studied the effects of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), of the potassium ionophore valinomycin and of nigericin, catalyzing a H^+/K^+ exchange, on the MgATP-dependent malate transport. The experiments shown in Table IV have been carried out in the presence and in the absence of externally added K^+ ions.

TABLE IV

EFFECT OF IONOPHORES ON THE MgATP DEPENDENT [^{14}C]MALATE TRANSPORT INTO ISOLATED VACUOLES

The measurements were done in the presence of 1.1 mM malate and, if not stated otherwise, 8 mM MgATP. If indicated, K^+ was present as the gluconate salt (50 mM). The control rates were 2.1 μmol per 10^7 vacuoles/h (equivalent to μmol per mg Chl/h) without external K^+ and 2.5 in the presence of K^+. The effective concentrations of the ionophores employed are presumably much lower than indicated due to the presence of bovine serum albumin in the assay which is known to bind lipophilic solutes. The incubation was terminated after 5 min by centrifugation. Mean values of four different experiments.

Treatment	Transport activity	
	K^+ present	Without K^+
Control	100	100
Nigericin (15 μM)	40	125
Valinomycin (15 μM) + CCCP (15 μM)	44	61
Valinomycin (15 μM)	69	97
CCCP (15 μM)	85	96
Control without MgATP	30	36

The MgATP-stimulated malate transport was largely abolished if in the presence of external K^+ either nigericin alone or a combination of CCCP and valinomycin were added, the remaining rate of malate transport being only little above the rate observed in the absence of MgATP. It is a general experience that in presence of K^+ a combination of CCCP plus valinomycin or nigericin alone, both enabling a H^+/K^+ exchange, are very effective in decreasing the electrochemical potential of a proton gradient. The complete abolishment of the MgATP-stimulated malate transport by these agents strongly indicates that the vacuolar malate transport is driven by a protonmotive force.

In the absence of external K^+, nigericin has the opposite effect in even stimulating malate uptake. Vacuoles have been found to contain high concentrations of K^+ ions. Thus in vacuoles isolated from petals of *Hippeastrum* and *Tulipa* a K^+ concentration in the order of 150 mM has been measured [23]. It is feasible that, by electroneutral K^+/H^+ exchange, nigericin enables the uptake of protons into the vacuolar space at the expense of the existing K^+ gradient across the vacuolar membrane. In this way, an increase of the proton gradient would also increase the rate of malate uptake. This explanation is supported by the recent observation of Weigel and Weis [24] that the vacuolar pH measured by fluorescence in *Valerianella locusta* is increased by nigericin in the absence of K^+ and is dissipated in its presence.

The inhibitory effects of either CCCP or valinomycin alone, especially in the absence of K^+ were very small. These results emphasize the relative impermeability of the tonoplast membrane for ions. Even in the presence of valinomycin or the protonophore CCCP the flux of charge-compensating ions seems to be too low to account for a significant efflux of potassium and protons. An efflux of these ions would lead to a diffusion potential preventing its further efflux. Only in the presence of both ionophores can potassium act as the charge compensating ion facilitating the efflux of protons and causing inhibition of energy-dependent malate uptake.

Conclusion

We have shown that the malate transport into vacuoles isolated from barley mesophyll protoplasts can occur against a transmembrane concentration gradient. This transport is energized by coupling to the electrogenic transfer of a proton, catalyzed by the action of a largely ATP-specific ATPase. It remains to be elucidated, whether a proton gradient or a membrane potential is the driving force. The velocity of the energized transport is high enough to account for the influx of malate into the vacuoles as observed during the light period, when malate is accumulated inside the vacuolar space [4].

Addendum

After completion of this manuscript, an abstract was published by Kenyon and Black [25], showing an ATP-stimulated malate uptake into vacuoles isolated from the CAM plant *Sedum telephium.*

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft and the Alexander von

Humboldt-Stiftung. We are grateful for the excellent technical assistance of Mrs. Claudia Zink and Mrs. Jutta Gerber-Nolte.

References

1 Giersch, C., Heber, U., Kaiser, G., Walker, D.A. and Robinson, S.P. (1980) Arch. Biochem. Biophys. 205, 246–259
2 Stitt, M., Wirtz, W. and Heldt, H.W. (1980) Biochim. Biophys. Acta 593, 85–102
3 Kaiser, G., Martinoia, E. and Wiemken, A. (1982) Z. Pflanzenphysiol. 107, 103–113
4 Gerhardt, R. and Heldt, H.W. (1984) Plant Physiol. 75, 542–547
5 Fisher, D.B. and Outlaw, W.H. (1979) Plant Physiol. 64, 481–483
6 Kaiser, G. and Heber, U. (1984) Planta 161, 562–568
7 Osmond, C.B. and Holtum, J.A.M. (1981) in The Biochemistry if Plants (Hatch, M.D. and Boardman, N.K., eds.), Vol. 8, pp. 283–328, Academic Press, New York
8 Lüttge, U. and Ball, E. (1979) J. Membrane Biol. 47, 401–422
9 Buser-Suter, C., Wiemken, A. and Matile, Ph. (1982) Plant Physiol. 69, 456–459
10 Smith, J.A.C., Uribe, E.G., Ball, E., Heuer, S. and Lüttge, U. (1984) Eur. J. Biochem. 141, 415–420
11 Marin, B., Smith, J.A.C. and Lüttge, U. (1981) Planta 153, 486–493
12 Martinoia, E., Heck, U. and Wiemken, A. (1981) Nature 289, 292–294
13 Boller, T. and Kende, H. (1979) Plant Physiol. 63, 1123–1132
14 Arnon, D.J. (1949) Plant Physiol. 24, 1–15
15 Leigh, R.A. and Walker, R.R. (1980) Planta 150, 222–229
16 Walker, R.R. and Leigh, R.A. (1981) Planta 153, 140–149
17 Aoki, K. and Nishida, K. (1984) Physiol. Plant. 60, 21–25
18 Smith, F.A. and Raven, J.A. (1979) Annu. Rev. Plant Physiol. 30, 289–311
19 Bennet, A.B., O'Neill, S.D. and Spanswick, R.M. (1984) Plant Physiol. 74, 538–544
20 Poole, R.J., Briskin, D.P., Kratky, Z. and Johnstone, R.M. (1984) Plant Physiol. 74, 549–556
21 Dufour, J.P. and Goffeau, A. (1980) Eur. J. Biochem. 105, 145–154
22 Scarborough, G.A. (1976) Proc. Natl. Acad. Sci. USA 73, 1485–1488
23 Lin, W., Wagner, G.J., Siegelman, H.W. and Hind, G. (1977) Biochim. Biophys. Acta 465, 110–117
24 Weigel, H.J. and Weis, E. (1984) Plant Sci. Lett. 33, 163–175
25 Kenyon, W.H. and Black, C.C. (1984) Plant Physiol. 75, suppl. p. 47