Transport of malate and chloride into barley mesophyll vacuoles

Different carriers are involved

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Transport of malate and chloride across the tonoplast of barley mesophyll vacuoles has been compared. Whereas malate inhibited chloride uptake, 1,2,3-benzenetricarboxylic acid, a potent inhibitor of malate uptake which is assumed not to cross the tonoplast, was ineffective. In contrast to chloride uptake, malate uptake was inactivated by pyridoxal phosphate and phenylglyoxal. The malate transport system could be protected against pyridoxal phosphate when a substrate such as 1,2,3-benzenetricarboxylic acid was present in the preincubation medium. It is concluded that the transfer of malate and chloride across the tonoplast is mediated by different systems.

Vacuole; Transport; Malate; Chloride

1. INTRODUCTION

Fluxes of ions across the tonoplast (vacuolar membrane) of the plant cell are involved in the regulation of cell turgor and cytoplasmic homeostasis [1-3]. Malic acid accumulates in CAM plants during the night, and this process is reversed during the daytime [4]. In contrast, in leaves of C_3 plants, levels of malate are high at the end of the day and low in the morning [5]. Both examples reflect the dynamics of vacuolar compartmentation. Uptake and storage of inorganic ions enable the plant to maintain the required difference in water potential between soil and leaves without expending energy for the production of organic osmotica.

Both the vacuolar carriers for malic acid [6,7] and chloride [8-10] have recently been characterized to some extent. Malate uptake was inhibited by chloride [6,7], whereas data on chloride uptake in the presence of malate have not been published. Studies using the patch clamp technique [11] have demonstrated that at least two channels are present in the tonoplast [12]. Only the channel opening at high Ca^{2+} concentrations (SV-channel) has been characterized in detail [13-15] and shown to be permeable for potassium and sodium, but also for anions such as malate and chloride [14]. These observations raised the question whether or not the transfer of malic acid and chloride across the tonoplast is mediated by the same transport system.

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2. MATERIALS AND METHODS

Barley (*Hordeum vulgare* L.cv Gerbel) was grown in a growth cabinet with 12 h fluorescent light (45 μmol $\times$ $m^{-2}s^{-1}$) at 22°C and 12 h dark at 18°C; relative humidity was 75%.

Primary leaves of 8-day-old plants were harvested at the beginning of the light period. Mesophyll protoplasts were prepared as described [16]. Mesophyll vacuoles were isolated and purified by a slight modification of the method described by Martinoia et al. [17]. Protoplasts were suspended in a solution containing 500 mM sucrose, 3% (w/v) Ficoll, 1 mM $CaCl_2$ and 10 mM 4-morpholinoethanesulfonic acid (Mes) pH 6.0. This suspension was overlayered with medium A (400 mM sucrose, 3% (w/v) Ficoll, 2 mM EDTA, 1 mM Mg-gluconate and 30 mM hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes-KOH, pH 7.6)) and medium B (400 mM sorbitol, 30 mM K-gluconate, 2 mM EDTA, 1 mM Mg-gluconate and 20 mM Hepes-KOH, pH 7.6). After centrifugation for 3 min at 200 $\times$ *g* and 4 min at 1200 $\times$ *g* the protoplasts were recovered from the upper interphase, forced through a needle (100 mm $\times$ 0.8 mm) and the liberated vacuoles purified by flotation. For this the lysate was overlayered with medium B and medium C (as medium B but 400 mM glycinebetaine instead of 400 mM sorbitol). The vacuoles were collected from the upper interphase. The vacuoles recovered from several density gradients were mixed with Percoll (final concentration 10% w/v) containing 500 mM sorbitol and 15 mM Hepes-KOH, pH 7.6, and a gradient as described for the purification of the vacuoles was formed. The purified and concentrated vacuoles were again recovered from the upper interphase.

Uptake of ^{14}C-malate and $^{36}Cl^-$ was measured as described previously [6,8]. In experiments involving phenylglyoxal the vacuoles were preincubated with phenylglyoxal for 30 min at room temperature. Prior to the uptake experiment, intact vacuoles were separated from broken ones by the density gradient centrifugation as described above.

3. RESULTS AND DISCUSSION

Inorganic anions affect malate transport [6,7]. This inhibition may indicate that organic and inorganic

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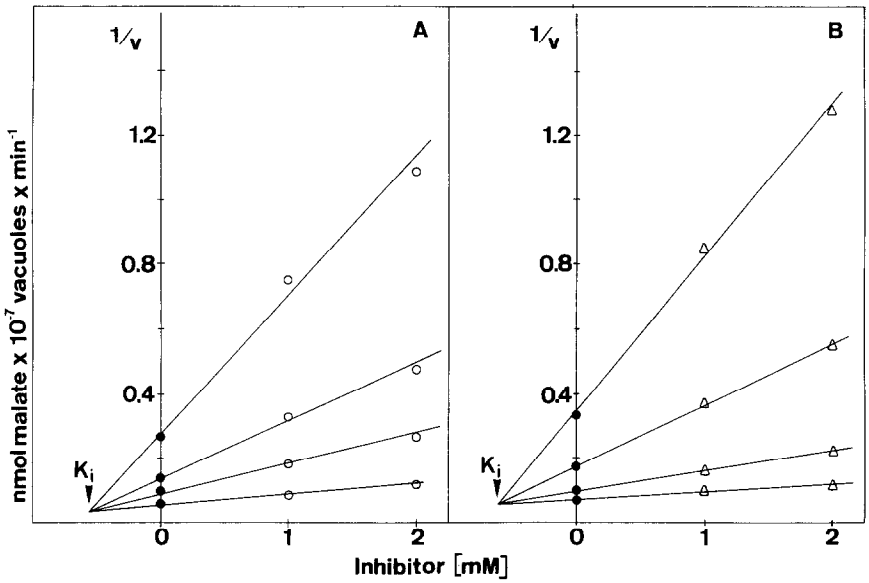

Fig.1. Dixon plots for the inhibition of malate uptake by benzenetricarboxylic acid (A) and by phenylsuccinic acid (B). K_m for malate was 1.1 mM.

anions compete for a common translocator. However, it may also be the result of a competition for the same energy source. The tonoplast contains two electrogenic pumps which generate a ΔpH (inside acidic) and a $\Delta\Psi$ (inside positive) [18,19]. Both of these gradients are still present in isolated vacuoles [20]. Therefore, we looked for a competitive inhibitor of the malate uptake system

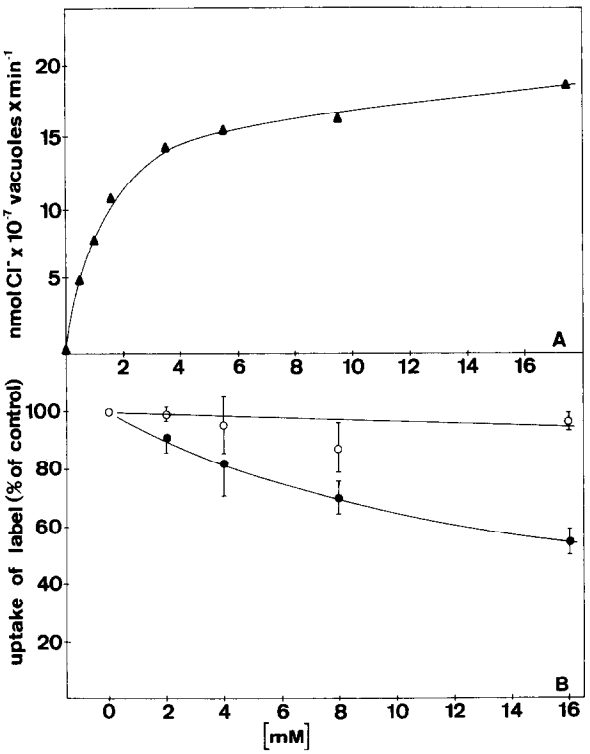

Fig.2. Concentration-dependent uptake of $^{36}Cl^-$ (A) and its inhibition by malate (●) and 1,2,3-benzenetricarboxylic acid (○) respectively (B). Chloride concentration in (B) was 1.5 mM. Control rates (100%) were 10.2 (●) and 9.8 (○) nmol × 10^{-7} vacuoles × min^{-1}, respectively. The values are expressed in means ± SD.

which would bind to the translocator but not be taken up by the vacuole in an energy requiring step. Mitochondria contain a dicarboxylate and a tricarboxylate carrier [21], for both of which competitive substrates have been described which are not transported [21]. Malate uptake into isolated vacuoles was also found to be affected by these compounds (fig.1). Interestingly, 1,2,3-benzenetricarboxylic acid, which is a strong inhibitor of the mitochondrial tricarboxylate carrier, is also a strong inhibitor of the malate carrier. The Dixon plot shown in fig.1A reveals a K_i of 0.6 mM, while the K_m for malate found in this experiment was 1.1 mM. Phenylsuccinate, which is a strong inhibitor of the mitochondrial dicarboxylate carrier and which has also a slight effect on the tricarboxylate carrier, also effectively inhibits malate uptake in barley vacuoles (K_i 0.7 mM, fig.1B). Phenylmalonate, however, which is a

Table 1

Uptake of [^{14}C]malate (1 mM) and $^{36}Cl^-$ (1.5 mM) into isolated vacuoles in the presence of protein modifying agents

Treatment	Uptake (% of control)	
	Malate	Chloride
Control	100 ± 4	100 ± 10
50 μM DIDS	11 ± 5	33 ± 4
200 μM DIDS	9 ± 5	2 ± 2
200 μM pyridoxal phosphate	28 ± 5	108 ± 12
500 μM pyridoxal phosphate	24 ± 2	106 ± 15
1 mM phenylglyoxal	88 ± 6	115 ± 15
7 mM phenylglyoxal	20 ± 4	97 ± 6

Preincubation at room temperature with 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) or pyridoxal phosphate was for 5 min, with phenylglyoxal 30 min (for details see section 2). The control rates were 7.1 ± 0.3 nmol × 10^{-7} vacuoles × min^{-1} for malate and 9.0 ± 0.9 nmol × 10^{-7} vacuoles × min^{-1} for chloride uptake; means of six replicates ± SD.

Table 2

Protection of the malate carrier against inhibition by pyridoxal phosphate

Pretreatment	nmol malate × 10^{-7} vacuoles × min^{-1}	% of control
None (control)	2.40 ± 0.21	100
0.2 mM pyridoxal phosphate	0.51 ± 0.15	21
0.2 mM pyridoxal phosphate + 40 mM benzenetricarboxylic acid	1.88 ± 0.11	78

Vacuoles were preincubated for 15 min and thereafter removed from the incubation medium by flotation. Uptake was measured in the presence of 0.5 mM [^{14}C]malate; means of six replicates ± SD; for experimental details see text.

good inhibitor of the mitochondrial dicarboxylate carrier, inhibits only slightly (data not shown). Uptake of $^{36}Cl^-$ into barley mesophyll vacuoles showed both a saturable and a linear component (fig.2A) as described before [8]. The magnitude of the linear component varies from one experiment to another. Malic acid inhibited the uptake of label from 1.5 mM $^{36}Cl^-$ into isolated vacuoles. In contrast, 1,2,3-benzenetricarboxylic acid inhibits $^{36}Cl^-$ uptake only negligibly (fig.2B). This result is a first indication that competition between malate and chloride is not for the binding site of a common carrier, but rather for the same energy source for uptake.

Using protein modifying reagents we have tried to further distinguish between the malate and the chloride carrier (table 1). Both carriers are inhibited by 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), a well known inhibitor of anion transport systems [22]. Inhibition by DIDS has also been observed for the SV-channel. Pyridoxal phosphate as well as phenylglyoxal inhibited malate transport strongly whereas chloride transport was not affected by this reagent. Pyridoxal phosphate forms a Schiff base with the ϵ-amino group of lysine residues. Phenylglyoxal preferentially binds to the guanidinium group of arginine. However, it can also bind to amino and thiol groups [23,24]. We can therefore presently not exclude the possibility that the inhibition of the malate carrier by phenylglyoxal occurs via modification of the same lysine residue(s) involved in the inhibition by pyridoxal phosphate. To investigate whether the lysine involved in the pyridoxal phosphate-dependent inactivation is possibly located at the binding site of the malate carrier, isolated vacuoles were preincubated with 0.2 mM pyridoxal phosphate in the presence or absence of 40 mM benzenetricarboxylic acid. After 5 min at room temperature sodium borohydride (5 mM) was added for 10 min to reduce the Schiff base and to stabilize the reversible bond. Subsequently the vacuoles were purified by a density gradient centrifugation. As can be seen in table 2, 1,2,3-benzenetricarboxylic acid protects the malate carrier against inactivation by pyridoxal phosphate, suggesting that a lysine residue may reside at the active site of the malate carrier.

4. CONCLUSIONS

Malic acid and chloride cross the tonoplast by different transport systems. The channel identified at the tonoplast and reported to be permeable for chloride and malate [13] may play a minor role in the uptake of anions or alternatively may be responsible for the release of anions from the vacuole into the cytosol.

REFERENCES

[1] Matile, P. (1978) Annu. Rev. Plant Physiol. 29, 193–213.
[2] Leigh, R.A. (1983) Physiol. Plant. 57, 390–396.
[3] Boller, T. and Wiemken, A. (1986) Annu. Rev. Plant Physiol. 37, 137–164.
[4] Osmond, C.B. and Holtum, J.A.M. (1981) in: The Biochemistry of Plants (Hatch, M.D. and Boardman, N.K., eds) vol. 8, pp. 283–328, Academic Press, New York.
[5] Gerhardt, R. and Heldt, H.W. (1984) Plant Physiol. 75, 542–547.
[6] Martinoia, E., Flügge, U.I., Kaiser, G., Heber, U. and Heldt, H.W. (1985) Biochim. Biophys. Acta 806, 311–319.
[7] Marigo, G., Bouyssou, H. and Laborie, D. (1988) Bontan. Acta 101, 187–191.
[8] Martinoia, E., Schramm, M.J., Kaiser, G., Kaiser, W.M. and Heber, U. (1986) Plant Physiol. 80, 895–901.
[9] Kaestner, K.H. and Sze, H. (1987) Plant Physiol. 83, 483–489.
[10] Pope, A.J. and Leigh, R.A. (1988) Planta 176, 451–460.
[11] Hamill, O.P., Marty, A., Neher, E., Sakman, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85–100.
[12] Hedrich, R. and Neher, E. (1987) Nature 329, 833–836.
[13] Coyaud, L., Kurkdijan, A., Kado, R. and Hedrich, R. (1987) Biochim. Biophys. Acta 902, 263–268.
[14] Hedrich, R. and Kurkdijan, A. (1988) EMBO J. 7, 3661–3666.
[15] Colombo, R., Cerana, R., Lado, P. and Peres, A. (1988) J. Membrane Biol. 103, 227–236.
[16] Kaiser, G., Martinoia, E. and Wiemken, A. (1982) Z. Pflanzenphysiol. 107, 103–113.
[17] Martinoia, E., Heck, U. and Wiemken, A. (1981) Nature 289, 292–294.
[18] Sze, H. (1985) Annu. Rev. Plant Physiol. 36, 175–208.
[19] Rea, P.A. and Sanders, D. (1987) Physiol. Plant. 71, 131–141.
[20] Barbier-Brygoo, H., Gibrat, R., Renaudin, J.P., Brown, S., Pradier, J.M., Grignon, C. and Guern, J. (1985) Biochim. Biophys. Acta 819, 215–224.
[21] LaNoue, K.F. and Schoolwerth, A.C. (1979) Annu. Rev. Biochem. 48, 871–922.
[22] Cabantchik, Z.I. and Rotstein, A. (1984) J. Membr. Biol. 15, 207–226.
[23] Phillips, M., Pho, D.B. and Pradel, L.A. (1978) Biochim. Biophys. Acta 566, 296–304.
[24] Kumar, S., Lennane, J. and Ratner, S. (1985) Proc. Natl. Acad. Sci. USA 82, 6745–6749.