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Characteristics of $MgATP^{2-}$-dependent electrogenic proton transport in tonoplast vesicles of the facultative crassulacean-acid-metabolism plant *Mesembryanthemum crystallinum* L.

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Abstract. Membrane vesicles were isolated from mesophyll cells of *Mesembryanthemum crystallinum* in the C_3 state and in the crassulacean acid metabolism (CAM) state. The distribution of ATP-hydrolysis and H^+-transport activities, and the activities of hydroxypyruvate reductase and Antimycin-insensitive cytochrome-*c*-reductase on continuous sucrose gradients was studied. For isolations carried out routinely a discontinuous sucrose gradient (24%/37%/50%) was used. Nitrate-sensitive ATP-hydrolysis and H^+-transport activities increased several-fold during the transition from C_3 photosynthesis to CAM. Nitrate-sensitive ATPase showed a substrate preference for ATP with an apparent K_m ($MgATP^{2-}$) of 0.19–0.37 mM. In both C_3 and CAM states the ATPase showed a concentration-dependent stimulation by the anions chloride and malate. However, the pH optima of the two states were different: the ATPase of C_3-*M. crystallinum* had an optimum of pH 7.4 and that of CAM- *M. crystallinum* an optimum of pH 8.4. The optical probe oxonol-VI was used to demonstrate the formation of $MgATP^{2-}$-dependent electric-potential gradients in tonoplast vesicles.

Key words: ATP hydrolysis – Crassulacean acid metabolism (induction) – Membrane potential (tonoplast) – *Mesembryanthemum* (H^+ transport) – Mg^{2+}-ATPase – Proton transport.

Abbreviations: Bistris-Propane = 1,3-bis [tris(hydroxymethyl)-methylamino] propane; CAM = Crassulacean acid metabolism; DIDS = 4,4-diisothiocyano-2,2-stilbene disulfonic acid; DTT = dithiothreitol; ER = endoplasmic reticulum; Hepes = 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; HPR = hydroxypyruvate reductase; IDPase = inosine 5′-diphosphatase; OX-VI = oxonol VI; Tris = 2-amino-2-(hydroxymethyl)-1,3-propanediol

Introduction

Plants performing Crassulacean acid metabolism (CAM) show a large nocturnal accumulation of malic acid in the vacuoles of the photosynthetic cells. Transport of malic acid across the tonoplast into the vacuole is assumed to be driven by a proton-pumping ATPase (Lüttge and Ball 1979; Lüttge et al. 1982). The uptake of the $malate^{2-}$ anion is secondarily coupled to primary active H^+ transport via the proton-electrochemical gradient established by the ATPase with a stoichiometry of 2 H^+ pumped and 1 $malate^{2-}$ anion transported per 1 ATP hydrolyzed (Lüttge et al. 1981; Smith et al. 1982).

Recently, an ATPase associated with vacuoles from *Kalanchoe daigremontiana* was characterized (Smith et al. 1983, 1984a, b; Aoki and Nishida 1984; Lüttge et al. 1984) and Mg-ATP-dependent H^+ transport into vacuoles was demonstrated (Jochem et al. 1984; Jochem 1986).

In *Mesembryanthemum crystallinum* (Mesembryanthemaceae), a species where CAM is induced by drought stress and salinity (Winter and von Willert 1972; Winter 1973, 1979; Winter and Lüttge 1979) an increase of vacuolar ATPase activity during the transition from C_3 photosynthesis to CAM was shown (Struve et al. 1985). Vacuolar ATPase activity in the C_3 state is not sufficient to drive malic-acid accumulation in the CAM state. This clearly demonstrates the physiological relevance of the vacuolar ATPase for the process of CAM in vivo.

In the present study, tonoplast vesicles were isolated from *M. crystallinum* in the C_3 and CAM states with the aim of comparing the characteristic properties of tonoplast ATPase activity and H^+

transport and the formation of an electric potential gradient at the tonoplast in the two states.

Material and methods

Plants. Plants of *Mesembryanthemum crystallinum* L. were grown from seeds in glasshouses in soil culture (Einheitserde type ED-73 DIN 11540-80 T, see Struve et al. 1985). Crassulacean acid metabolism was induced by NaCl stress (see Winter and Lüttge 1979; Heun et al. 1981).

Protoplast isolation. Protoplasts were isolated by enzymatic digestion as described by Struve et al. (1985) according to the method of Winter et al. (1982) with the following modifications: Only the upper epidermis was removed; 15 g of tissue were transferred to 40 ml of enzyme medium with Cellulase "Onozuka" RS, 0.5% (w/v), from Yakult Honsha Co., Tokyo, Japan, and Pectolyase Y-23, 0.02% (w/v), from Seishin Pharmaceutical Co, Tokyo, Japan. The suspension of protoplasts and cell fragments was diluted with 500 mM sucrose solution (1000 mM for salt-treated plants) at a ratio of 3:5 (suspension: sucrose solution). About 16 ml of the diluted suspension were placed in centrifuge vials and covered with a layer of 0.5 ml 400 mM mannitol (800 mM for salt-treated plants), 100 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) (pH 7.8, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)) and 1 mM dithiothreitol (DTT) (=buffer P).

Membrane preparation. Protoplasts of C_3-*M. crystallinum* were diluted with buffer P, those of salt-treated CAM-*M. crystallinum* with buffer P without mannitol, both at a ratio 1:3 to get the same final mannitol concentration. Protoplasts were homogenized by two passages through the steel needle of a syringe (internal diameter 370 µm). Chloroplasts were removed by centrifugation at $1000 \cdot g$ for 5 min at 4° C. An aliquot of 18 ml of the resulting supernatant was layered on a continuous sucrose gradient (15–50% sucrose, w/w, in medium G: 5 mM Hepes/Tris pH 7.6, 2 mM DTT, volume 20 ml) in cellulose-nitrate tubes. The gradient was centrifuged in a Beckman SW 27 rotor (Beckman, München, FRG) at $100000 \cdot g$ for 2 h at 4° C. Fractions of 1.5 ml were taken from the bottom, frozen in liquid N_2 and stored at −70° C.

For isolations carried out routinely a discontinuous gradient (24%, 37%, 50% sucrose w/w, 5 ml each, in medium G) was used. An aliquot of 23 ml of the $1000 \cdot g$ supernatant was layered on this gradient, centrifugation was the same as with the continuous gradient. Membranes at the interfaces of the discontinuous gradients were collected with a Pasteur Pipette (fraction A=interface supernatant: 24% sucrose; fraction B=24%:37% sucrose; fraction C=37%:50% sucrose), diluted fourfold (medium G_m: 300 mM mannitol in medium G) and centrifuged in a Beckman 50.2 Ti rotor at $100000 \cdot g$ for 90 min at 4° C. The supernatant was discarded and the pellet was suspended in 300 mM sucrose in medium G to a final protein concentration of 70–100 $\mu g \cdot ml^{-1}$ (P_A=fraction A, pelleted). All membrane fractions were frozen in liquid N_2 and stored at −70° C.

Assay of ATPase. The ATPase assays were performed essentially as described previously (Smith et al. 1984a; Struve et al. 1985) with the following modifications: the final assay volume was 225 µl including 50 µl of membrane suspension (diluted 1:10 for C_3-*M. crystallinum*, 1:20 for CAM-*M. crystallinum*). The reaction mixture contained 0.7 mM sodium molybdate ($Na_2MoO_4 \cdot 2\ H_2O$) instead of 0.1 mM ammoniummolybdate ($(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$) because ammonium acts as a permeant weak base, relieving pH gradients across the membranes (Kleiner 1981; DuPont et al. 1982). The standard mixture was incubated at 37° C for 45 min. In studies of the kinetic parameters of ATP hydrolysis the reaction medium was incubated at 25° C for 30 min to prevent a substrate deficiency.

For determinations of the pH-dependence of ATPase activity the N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine (Tricine)/Tris buffer in the standard test medium was replaced by a mixture of 2-(N-morpholino)ethane sulfonic acid (Mes) and 1,3-bis[tris(hydroxymethyl)-methylamino]propane (Bistris-Propane) buffer with pH values between pH 5.5 and pH 9.0. Temperature effects were avoided by determining the pH values at 25° C and incubating the reaction mixture at 25° C. The reaction was stopped by the addition of 500 µl 10% (w/v) sodium dodecylsulfate and P_i determined as in Smith et al. (1984a).

Fluorescence assays. Acid interior pH gradients were measured as the degree of fluorescence quenching of the permeant amine dyes, quinacrine and 9-aminoacridine (Deamer et al. 1972). Vesicles were added to an assay buffer of 220 mM sucrose, 1.5 mM DTT, 37 mM Tricine/Tris pH 8.0, 3 µM quinacrine or 30 µM 9-aminoacridine and various salt concentrations to give a final volume of 1.5 ml. Quinacrine was stored as a 5 mM stock solution (H_2O) at −20° C. 9-Aminoacridine was stored as a 3 mM stock solution (10% ethanol) in a refrigerator. Typically, 30–100 µl vesicles (3–10 µg protein) or 100–200 µl vesicles were added for assays with quinacrine and 9-aminoacridine, respectively. Fluorescence was measured at 25° C with a Sigma ZWS-II photometer (Biochem, München, FRG) equipped with a fluorescence attachment. Excitation was at 427 nm with quinacrine and at 422 nm with 9-aminoacridine and emission was measured through an interference filter (quinacrine: maximal transmission 61% at 530 nm, 9-aminoacridine: 55% at 470 nm). Measurements of ATP-dependent H^+ influx were initiated by the addition of 30 µl of 150 mM $MgSO_4$/ATP, adjusted to pH 7.0 with Bistris-Propane.

*Absorption spectroscopy of oxonol VI.*The formation of transmembrane electric potential gradients was measured with the optical probe bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol (OX-VI). Absorption changes of OX-VI were measured with a Sigma ZWS II dual-wavelength photometer. As shown by Bashford et al. (1979), Bennett and Spanswick (1983) and Scherman and Henry (1980) a decrease in the absorption difference $A_{590nm-610nm}$ indicates an increase in membrane potential (interior positive). Assays were performed in a final volume of 1.2 ml inclusive MgATP, which contains: 220 mM sucrose, 1.5 mM DTT, 2.5 µM OX-VI, 33 mM Tricine/Tris pH 8.0, 20–30 µg membrane protein. The reaction was started by addition of 3 mM MgATP. The optical probe OX-VI was kept as a 0.25 mM stock solution in the refrigerator.

Proteins. Proteins were measured by the method of Sedmark and Grossberg (1977) using bovine serum albumin as standard.

Assay of hydroxypyruvate reductase (HPR). The activity of HPR (marker enzyme for peroxisomes) was determined according to Zelitch (1955) using the following reaction mixture: 0.083 mM NADH, 0.1% (w/v) Triton X-100, 50 mM Hepes, pH 7.5 (KOH). The final test volume of 1.2 ml contained 50–150 µl vesicles. Reaction was started by adding 1 mM hydroxypyruvate and absorption was measured at 340 nm.

Assay of cytochrome-c-reductase (CCR). The activity of CCR (marker enzyme for endoplasmic reticulum (ER)) was measured following Lord et al. (1973). The reaction mixture (final volume 1.2 ml, including 50–150 µl vesicles) was composed of 0.02%

(w/v) Triton X-100, 1.6 mM KCN, 0.04 mM cytochrome *c*, 0.8 µg·ml^{-1} Antimycin-A, 80 mM Hepes, pH 7.5 (KOH). The reaction was started by adding 0.08 mM NADH.

Before addition of NADH it was necessary to oxidize contaminating DTT (from isolation solutions) with ferricyanide until the absorption was constant. We measured the decrease in the absorption difference $A_{550nm-541nm}$ with a Sigma ZWS-II dual-wavelength spectrophotometer.

Assay of inosine 5'-diphosphatase (IDPase). The activity of latent IDPase (marker enzyme for Golgi vesicles) was measured as the difference of activity in the presence and absence of 0.01% Triton X-100 (Chanson et al. 1984). The reaction mixture (50 mM Mes/Tris pH 6.5, 50 mM KCl, 3 mM $MgSO_4$, 3 mM IDP, 50 µl vesicles) was incubated for 45 min at 37° C. The reaction was stopped by the addition of 500 µl 10% (w/v) sodium dodecylsulfate and P_i determined as in Smith et al. (1984a).

Results and discussion

Preparation and purification of tonoplast vesicles

Isolation of membranes. It was chosen to prepare isolated tonoplast vesicles from *M. crystallinum* in the C_3- and the CAM-states because this made it possible to obtain greater amounts of membrane protein with greater purity compared with the isolation of vacuoles (see Struve et al. 1985). It is important to note that during the preparation procedure we did not use the centrifugation step (6000·*g*, 20 min) according to Nagahashi and Hiraike (1982) to separate mitochondria because this resulted in the loss of nitrate-sensitive ATP-hydrolysis activity and H^+transport. The supernatant of the 1000·*g* centrifugation step was layered directly on the sucrose gradient. This had the advantage that membranes did not have to be pelleted and thus avoided the danger of membrane sticking (Table 1).

Isopycnic distribution of ATPase activity. Figure 1A shows the distribution of ATP-hydrolysis activity on a continuous sucrose gradient. The ATP-hydrolysis activities of different membrane systems were calculated on the basis of analyses of differential inhibition. It is known that vanadate and azide rather specifically inhibit the ATPases of plasmalemma and mitochondria, respectively (Bowman et al. 1978; Cocucci et al. 1979; Perlin and Spanswick 1981; Walker and Leigh 1981; Gallagher and Leonard 1982; Stout and Cleland 1982; O'Neill et al. 1983; O'Neill and Spanswick 1984). Nitrate inhibits tonoplast ATPase and mitochondrial ATPase (d'Auzac 1977; Grubmeyer and Spencer 1979; Walker and Leigh 1981; Bowman and Bowman 1982; O'Neill et al. 1983). Hence, the sum of the nitrate-inhibited and the azide-in-

Table 1. Distribution of ATP-hydrolysis and H^+transport activities after differential centrifugation (5 min, 1000·*g*; 20 min, 6000·*g*)

Fraction	ATP hydrolysis			H^+transport (total)
	Total	Azide sensitive	Nitrate sensitive	
Activity in the homogenate = 100%. Distribution (%) after 5 min at 1000·*g*				
Supernatant	69	35	64	–
Pellet	35	15	25	–
Activity in the 1000·*g* supernatant (above) = 100%. Distribution (%) after 20 min at 6000·*g*:				
Supernatant	25	28	36	59
Pellet	65	54	68	32

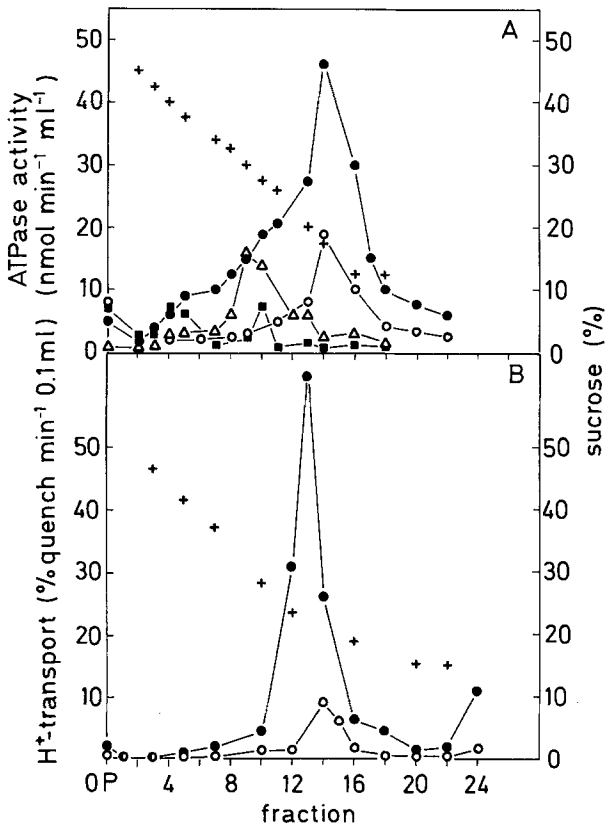

Fig. 1. **A** Distribution of ATP-hydrolysis activity of tonoplast (●–●), mitochondria (■–■) and plasmalemma (△–△) of membrane vesicles from CAM-*M. crystallinum* and of tonoplast from C_3-*M. crystallinum* (○–○) on a continuous sucrose gradient (+–+). The ATP-hydrolysis activities were estimated from differential inhibition of membrane vesicles at 37° C and at pH 8.0 for tonoplast and mitochondria and at pH 6.5 for plasmalemma in the presence of 50 mM KCl. **B** Distribution of H^+transport activity of membrane vesicles from CAM-*M. crystallinum* (●–●) and C_3-*M. crystallinum* (○–○) on a continuous sucrose gradient (+–+) at 25° C and pH 8.0 in the presence of 50 mM KCl

hibited ATPase minus the azide-inhibited ATPase corresponds to the tonoplast ATPase (determined at pH 8.0). The plasmalemma ATPase activity was estimated from vanadate-inhibition of the membrane fractions (determined at pH 6.5).

On the gradient, nitrate-sensitive, azide-insensitive ATPase (tonoplast ATPase) was localized at 15–20% sucrose which corresponded to a density of 1.06–1.08 $g \cdot cm^{-3}$. Only small amounts were found in the pellet and in the rest of the gradient.

The greatest amount of azide-sensitive ATPase (mitochondrial ATPase) was found at about 40% sucrose and in the pellet. Vanadate-sensitive ATPase (plasmalemma ATPase) was localized at about 30% sucrose. The distribution of ATPase activities in the continuous sucrose gradient of vesicles isolated from *M. crystallinum* in the C_3 and CAM states was comparable. However, the activity of the nitrate-sensitive, azide-insensitive ATPase of *M. crystallinum* (tonoplast ATPase) in the CAM state was clearly higher than the ATPase activity in the C_3 state. With a comparable number of protoplasts taken as a basis, tonoplast ATPase activity was two to three times higher in the CAM state. It was not possible to determine the specific activity because the vesicle preparations were contaminated with bovine serum albumin from the isolation medium.

Fluorescence quenching of the amine-dyes quinacrine and 9-aminoacridine was used to measure ATP-dependent H^+ transport into membrane vesicles of *M. crystallinum*. For routinely carried-out measurements the use of quinacrine instead of 9-aminoacridine was preferred, because of the greater sensitivity (Bennett and Spanswick 1983; own results). These fluorescent amines are weak bases distributing across a membrane according to the pH-gradient and accumulating inside the vesicles if the intravesicular compartment is relatively acid. After accumulation, the fluorescence intensity becomes quenched. This requires tightly sealed vesicles capable of maintaining ion gradients.

Figure 1B shows the distribution of H^+transport activity of vesicles from C_3- and CAM-*M. crystallinum* in similar gradients. The H^+transport activity of vesicles derived from CAM-*M. crystallinum* was sevenfold higher than that of vesicles from C_3-*M. crystallinum*. This also corresponds to the higher amount of ATP hydrolysis.

Vanadate-sensitive (plasmalemma) H^+ transport was not detectable. This may have been due to the orientation of the plasmalemma vesicles which are originally formed inside-in; the binding site for ATP is therefore not accessible (Hager and Biber 1984). Thus, under the present conditions, H^+ transport was mainly the result of tonoplast transport activity.

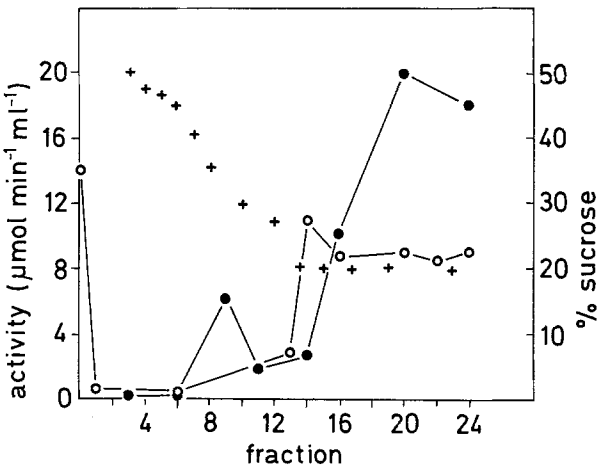

Fig. 2. Activity of hydroxypyruvate reductase (o–o) and of Antimycin-A-insensitive cytochrome-*c*-reductase (•–•) of membrane vesicles from C_3-*M. crystallinum* on a continuous sucrose gradient at 25° C. Marker-enzyme activity of membrane vesicles from CAM-*M. crystallinum* was identical (not shown)

Marker enzymes. Figure 2 depicts the isopycnic distribution of hydroxypyruvate reductase (HPR), a marker enzyme for peroxisomes and of the Antimycin-A-insensitive cytochrome-*c*-reductase, a marker enzyme of ER.

About 50% of the peroxisomal activity was sedimented. The soluble activity in the supernatant resulted from destroyed peroxisomes because HPR is a soluble enzyme of the peroxisomal lumen and is not membrane-bound (Quail 1979).

According to Quail (1979) the ER should be localized at a density of 25–35% sucrose. In Fig. 2 there is only a small accumulation of ER in this region, most of the activity is detectable in the supernatant. This distribution could not be affected by addition of Mg^{2+} which should shift the ER to regions of higher density because of ribosome binding (Lord et al. 1973; Ray 1977; see references in Quail 1979). There are two possible explanations: first, this phenomenon is typical for *M. crystallinum;* second, the specific isolation condition for tonoplast vesicles does not permit a clear separation of ER. An appreciable accumulation of IDPase (marker for Golgi-vesicles) was also not detected, possibly because of its inactivation under the given isolation conditions.

Discontinuous sucrose gradients. Based on the distribution of ATPase activities on continuous sucrose gradients, a discontinuous sucrose gradient

Table 2. Substrate specificity of ATP-hydrolysis and H^+transport activities associated with the P_A membrane fraction (see *Material and methods)* from *M. crystallinum* in the C_3 and CAM states. Data expressed as % of controls with ATP (means ±SD (n))

	H^+ transport		ATP hydrolysis	
	C_3	CAM	C_3	CAM
ATP	100	100	100	100
ADP	0 (1)	0 (1)	18 (2)	12 (2)
GTP	9 (2)	14±30 (3)	32±7 (3)	33±6 (3)
CTP	0 (3)	0 (3)	16 (2)	12 (2)
UTP	0 (1)	2 (2)	12 (2)	26 (2)

was designed to separate membranes enriched in the following ATPase activities: i) nitrate-sensitive, azide-insensitive activity, ii) vanadate-sensitive activity, and iii) azide-sensitive ATPase activity. In addition, soluble proteins were eliminated from the membranes by pelleting the membranes in a further centrifugation step.

Fraction A (see *Material and methods*) was found to be enriched in tonoplast ATPase, fraction B was enriched in plasmalemma ATPase, fraction C and the pellet were enriched in mitochondrial ATPase.

Properties of ATP hydrolysis and H^+ transport

Substrate specificity. The substrate specificity of the ATPase associated with the isolated vesicles was investigated by comparing the rates of hydrolysis of ATP and ATP-driven H^+ transport with those observed in the presence of other nucleotides (Table 2).

The results presented in Table 2 show that ATP was the most effective substrate, both for the release of inorganic phosphate and for H^+ transport. Guanosine 5′-triphosphate (GTP) gave substantial phosphohydrolase activity and could also energize H^+ transport, though to a much lesser extent than ATP. With ADP, cytidine 5′-triphosphate (CTP) and uridine 5′-triphosphate (UTP), relatively little hydrolysis and no H^+ transport was obtained. The greater specificity of transport as compared with hydrolysis could be explained by the presence of unspecific phosphatases in the assays. There was no difference in substrate specificity between C_3- and CAM-*M. crystallinum.*

Dependence on Mg-ATP^{2-}. Figure 3 shows the dependence of the tonoplast ATPase from *M. crystallinum* in the C_3 and CAM states on the concentration of its substrate, $MgATP^{2-}$. The curves show Michaelis-Menten-type saturation kinetics. Specific activities were calculated for these data. It can be concluded that the specific activity of the tonoplast ATPase increased about 1.5-fold during the transition from the C_3 to the CAM state. But this increase in specific activity was smaller than the increase of ATP-hydrolysis and H^+transport activities obtained when results were expressed on the basis of numbers of protoplasts, i.e. 3–7-fold (see above). At present it is not possible to decide whether this effect is the result of an increased amount of ATPase after CAM induction or of contamination by other membrane proteins which obscure the results.

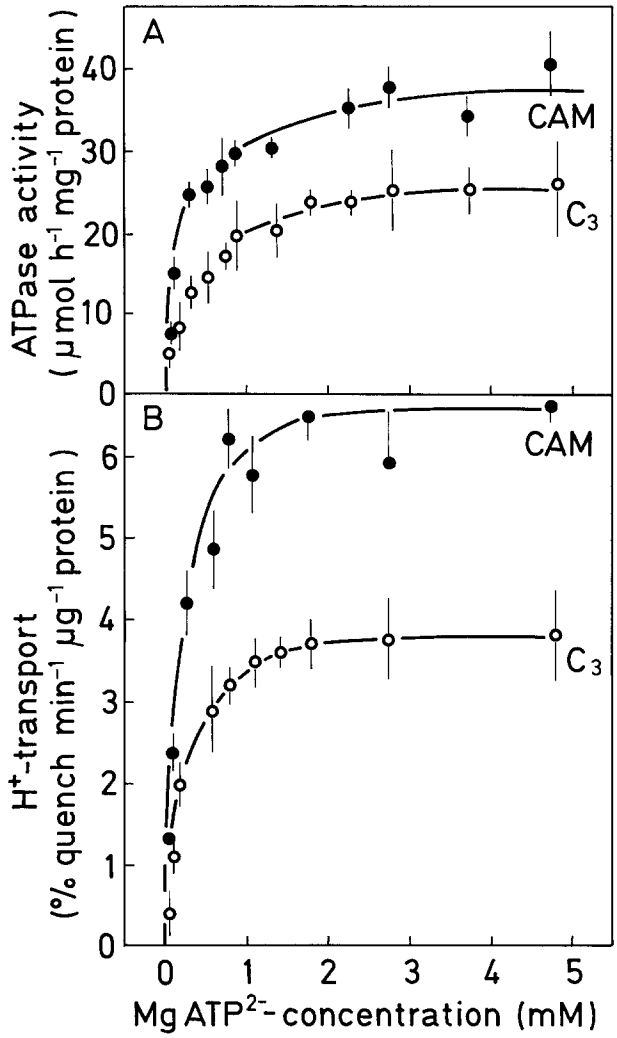

Fig. 3A, B. The ATP-hydrolysis activities (**A**) and H^+transport activities (**B**) of tonoplast preparations (fraction P_A) of *M. crystallinum* in the CAM and C_3 states at various concentration of the substrate $MgATP^{2-}$, a test temperature of 25° C and at pH 8.0 in the presence of 50 mM KCl. Vertical bars = 2 × SD

The substrate affinity of the tonoplast ATP-hydrolysis and H^+transport activities was determined under the conditions recommended by Cor-

Table 3. Apparent K_m of the tonoplast (fraction P_A) ATP-hydrolysis and H^+transport activities of *M. crystallinum* in the CAM and C_3 states. Data obtained from tests at various $MgATP^{2-}$ concentrations and 25° C (means ±SD (n))

	K_m ($MgATP^{2-}$) (mM)	
	CAM	C_3
ATP hydrolysis	0.30 (1)	0.37±0.14 (3)
H^+ transport	0.19±0.05 (7)	0.32 (1)

nish-Bowden (1979), in which the total $MgSO_4$ concentration is kept at a constant excess over the total ATP concentration. The equations of Wolf and Adolph (1969) were used to calculate the actual concentrations of $MgATP^{2-}$ in the reaction media.

Table 3 summarizes the results of some experiments with varied substrate concentrations (e.g. Fig. 3) giving the values for the apparent K_m ($MgATP^{2-}$) estimated according to the method of Eisenthal and Cornish-Bowden (1974). The K_m values appear to be comparable in the C_3 and the CAM state. It is noteworthy that these K_m values measured in vesicle preparations are comparable to those obtained with isolated vacuoles (Struve et al. 1985).

Nitrate inhibition. Figure 4 shows the concentration-dependence of nitrate inhibition. A marked inhibition became discernible above 5 mM NO_3^-. There was no difference between the inhibition of the tonoplast ATPase in the C_3 and the CAM states. Proton-transport activity (Fig. 4B) seemed to be somewhat more sensitive to nitrate than ATP hydrolysis (Fig. 4A). In the case of H^+ transport at 100 mM NO_3^-, no activity was detectable. Nitrate inhibition was nearly similar in the absence and presence of chloride (not shown). This means that Cl^- has no effect in protecting the ATPase against NO_3^-. Since 5 mM NO_3^- was effective in decreasing H^+ transport (see also Churchill and Sze 1984) and nitrate inhibition remained after solubilisation (Mandala and Taiz 1985; own results, data not shown), NO_3^- probably acts by directly altering enzyme activity rather than by a chaotropic effect.

Stimulation by anions. In contrast to plasmalemma ATPases (Leonard and Hodges 1973; Leonard and Hotchkiss 1976; Perlin and Spanswick 1981) tonoplast ATPases (d'Auzac 1977; Walker and Leigh 1981) seem to be stimulated by anions. The effects of KCl, NaCl, D-(−)-malate and L-(−)-malate on

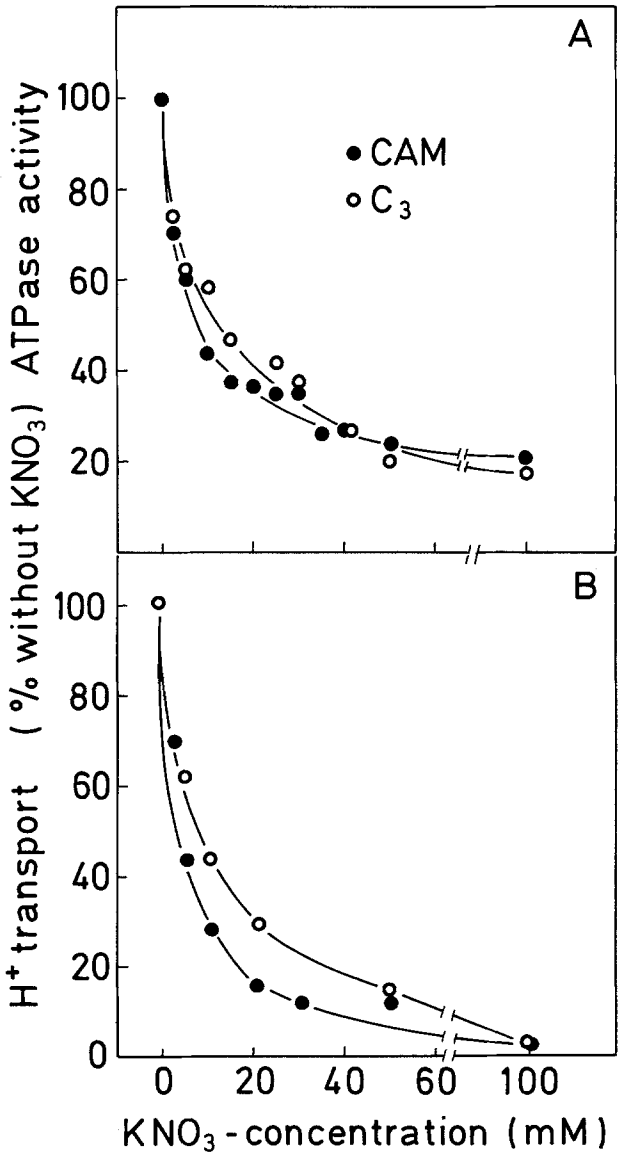

Fig. 4A, B. Effect of increasing KNO_3 concentrations on ATP-hydrolysis activity (**A**) and H^+-transport activity (**B**) of tonoplast vesicles (fraction P_A) from *M. crystallinum* in the CAM and C_3 states at pH 8.0 in the presence of KCl and at a test temperature of 37° C for ATP hydrolysis and 25° C for H^+ transport

C_3 and CAM ATPase activities and H^+ transport were examined.

The effects of salt on ATP-hydrolysis activity are shown in Fig. 5A, B. Chloride stimulated ATP hydrolysis in the CAM and the C_3 states in the presence and absence of Triton X-100. This indicates that Cl^- not only acts as a permeant anion by relieving the electrical gradient but also has a direct role in activating the ATPase. The use of detergents and ionophores is a common practice for differentiating between such direct and indirect

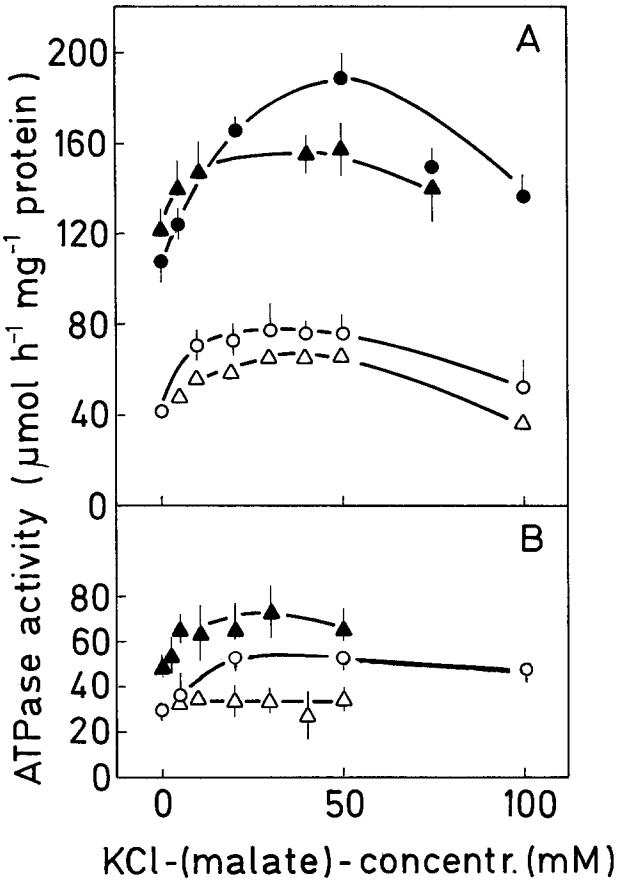

Fig. 5A, B. Effect of increasing KCl and L-(−)-malate concentrations on ATP-hydrolysis activity in the absence and presence of Triton X-100 (0.01%), respectively, of tonoplast vesicles (fraction P_A) from *M. crystallinum* in the CAM state (**A**) and the C_3 state (**B**) at pH 8.0 and a test temperature of 37° C. o–o, KCl; ●–● KCl + Triton; △–△ malate; ▲–▲, malate + Triton. Vertical bars = 2 × SD

effects. The detergents abolish the electrochemical gradient across the membrane and eliminate the effects of permeant anions (DuPont et al. 1982; Bennett and Spanswick 1983; Churchill and Sze 1984; Jochem et al. 1984). A different situation was obtained with malate. The hydrolysis of ATP by tonoplast-derived vesicles from *M. crystallinum* in the C_3 state was only stimulated by malate in the presence of Triton X-100. Conversely, in the CAM state, stimulation was obtained in the absence and the presence of Triton X-100. At the moment it is not possible to explain the absence of a stimulation of the C_3-ATPase when no Triton X-100 was added. In the case of tonoplast ATPase in the CAM state the malate effect was comparable to the Cl^- effect, with the exception that stimulation by malate was smaller.

The kinetics of chloride and malate stimulation of tonoplast H^+ transport in the C_3 and CAM

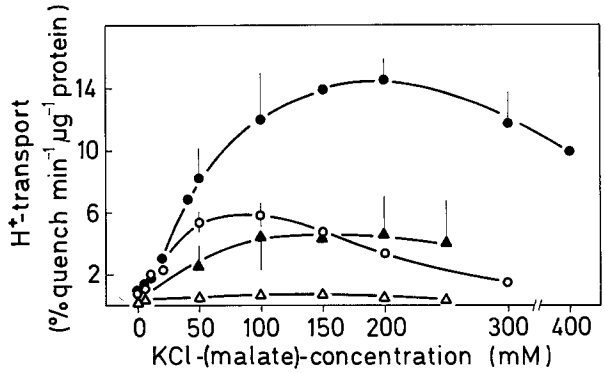

Fig. 6. Effect of increasing KCl and malate concentrations on H^+transport activity of tonoplast vesicles (fraction P_A) from *M. crystallinum* in the CAM and C_3 states at pH 8.0 and 25° C. ●–●, KCl-CAM; o–o, Mal-CAM; ▲–▲, KCl-C_3; △–△, Mal-C_3. Vertical bars = 2 × SD

states are shown in Fig. 6. Independently of the salt used, H^+transport activity in the CAM state was always greater than in the C_3 state. Maximal activity was obtained with 200 mM Cl^- and 100 mM malate. No differential stimulation was found using NaCl instead of KCl (not shown). D-(−)-malate had the same effect as L-(−)-malate (not shown). Altogether malate stimulated H^+ transport to a lesser extent than chloride.

Effect of 4,4-diisothiocyano-2,2-stilbene disulfonic acid (DIDS) on H^+ transport. Like other vacuolar ATPases (Bennett and Spanswick 1983; Churchill and Sze 1984) nitrate-sensitive H^+ transport was inhibited by the anion-channel blocker DIDS in the absence and the presence of chloride. The inhibition was 62% in vesicles from CAM-*M. crystallinum* and 45% in vesicles from C_3-*M. crystallinum*, but this difference in inhibition was not statistically significant. The fact that DIDS inhibition was independent of Cl^- concentration provides further evidence for a direct effect of Cl^- on the ATPase.

Dependence on pH. The pH-dependence of the ATPase is shown in Fig. 7. Most interestingly, the pH optimum of tonoplast vesicles in the C_3 state was close to pH 7.4, whereas in the CAM state it was about pH 8.4, both in the ATPase assay (Fig. 7A) and in H^+ transport (Fig. 7B). Hydrolysis of ATP exhibited a broader pH optimum than H^+ transport. It is possible that this was due to the activity of unspecific phosphatases (see also substrate specificities, Table 2).

The pH-dependence of H^+ transport was determined with both quinacrine and 9-aminoacridine because quinacrine changes its fluorescence depen-

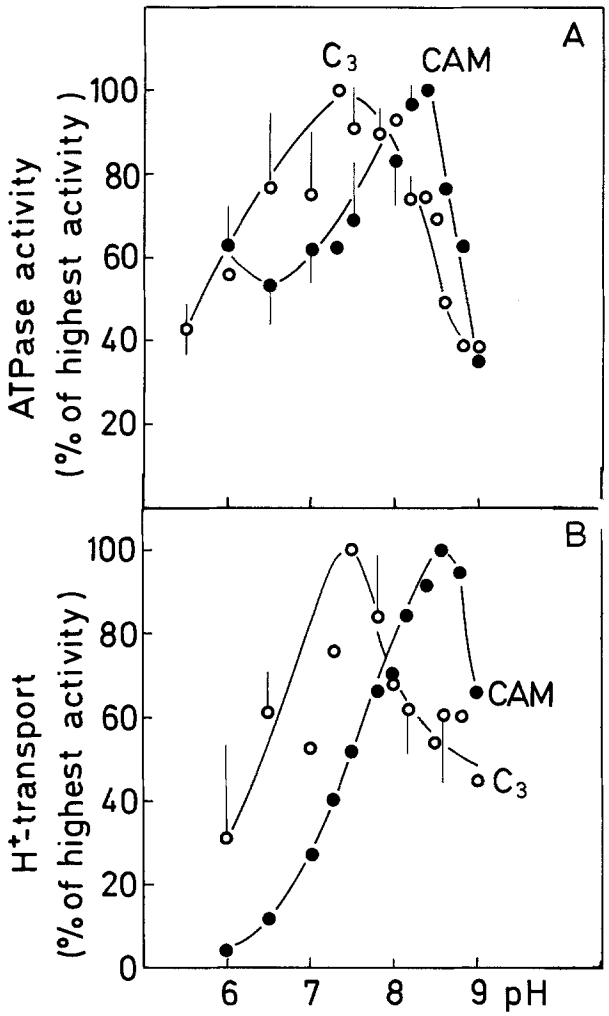

Fig. 7A, B. The ATP-hydrolysis activity (**A**) and H^+transport activity (**B**) of tonoplast vesicles (fraction P_A) from *M. crystallinum* in the CAM and C_3 states at various pH of the test medium (pH adjusted with Bistris-Propane-Mes-buffer) and test temperature of 25° C. Proton-transport activity was measured by using 9-aminoacridine (30 µM). Vertical bars = 2 × SD

dending on pH. Nevertheless, the same results were obtained with quinacrine and 9-aminoacridine and in Fig. 7B the results with 9-aminoacridine are presented. Perhaps the difference in the pH optimum between vesicles from CAM-*M. crystallinum* and from C_3-*M. crystallinum* indicates that the characteristics of the tonoplast ATPases in the CAM and the C_3 states are different in relation to induction or regulation of CAM.

Membrane-potential measurements

Oxonol dyes are a class of permeant anions with have been shown to respond rapidly and quantitatively to the positive transmembrane potentials inside vesicles (Bashford et al. 1979; Scherman and

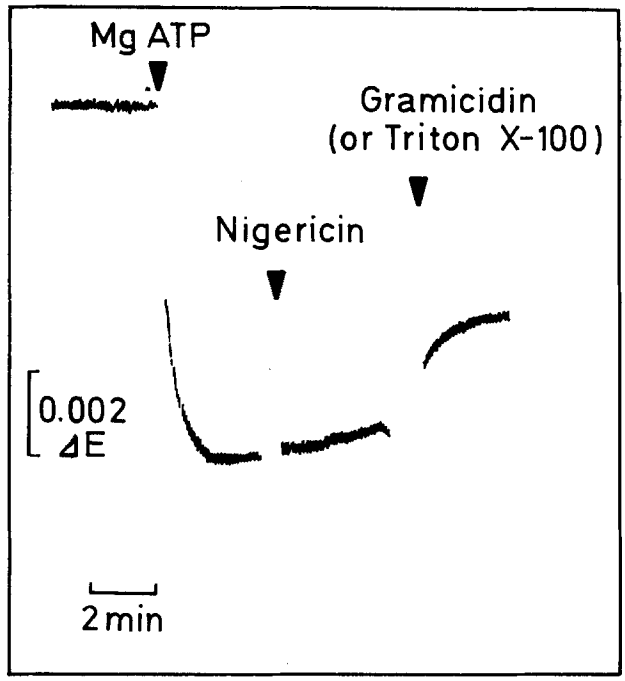

Fig. 8. Absorption change of Oxonol-VI after addition of 3 mM MgATP in the absence of permeant anions at pH 8.0 and 25° C. Tonoplast vesicles (fraction P_A) from C_3-*M. crystallinum* were used, but vesicles from CAM-*M. crystallinum* showed the same effect. Final concentration of Nigericin = 0.5 µM, of Gramicidin = 2 µM, of Triton X-100 = 0.01%

Henry 1980), OX-VI being the most sensitive dye (Bashford and Thayer 1977). The response of oxonol dyes apparently depends on shifts in absorption between the free and membrane-bound dye.

Figure 8 shows the response of OX-VI measured as a shift in $A_{590nm-610nm}$ upon the addition of 3 mM MgATP. The maximal degree of reaction was reached after some seconds and subsequently the polarization of the membrane decreased.

Both, C_3- and CAM-derived vesicles showed the generation of a membrane potential ($\Delta\Psi$). Nigericin, which exchanges K^+ against H^+ had no influence on $\Delta\Psi$, whereas Gramicidin, a channel-forming ionophore and Triton X-100, a detergent, caused the dissipation of $\Delta\Psi$. Permeant anions like NO_3^-, Cl^-, malate^{2-} reduced $\Delta\Psi$.

Conclusions

The presence of a vacuolar ATPase and its increase in activity during induction of CAM in *M. crystallinum* is well established (Struve et al. 1985). Using isopycnic centrifugation for membrane purification, this communication presents a comparative physiological analysis pointing out similarities and

differences between tonoplast ATPases derived from *M. crystallinum* in the C_3 and CAM states.

The characteristics of tonoplast ATP-hydrolysis and H^+transport activities in the C_3 and CAM states are similar regarding chloride-stimulation, nitrate-inhibition, Mg-ATP-dependence, detergent and ionophore influence, substrate specifity and DIDS-inhibition. These properties are also shared with vacuolar ATPases from other sources including non-green plants (see references in Sze 1985).

There are, however, two differences in the characteristics shown in the two metabolic states. First, in the case of C_3-*M. crystallinum*, malate stimulation of ATP hydrolysis can only be observed in the presence of Triton X-100, whereas the tonoplast ATPase from CAM-*M. crystallinum* shows malate stimulation in the absence and the presence of Triton X-100. Second, the pH optimum of ATP hydrolysis and H^+ transport is more alkaline in the CAM state than in the C_3 state.

These differences may be pertinent to a putative role of the tonoplast ATPase in the regulation of CAM. It is still not clear, however, whether the increase in tonoplast ATPase activity during induction of CAM is caused by the activation of preexisting enzyme protein or by de-novo synthesis. If there is a de-novo synthesis, the question is still open as to whether de-novo synthesis would give rise to a new isoenzyme or simply to increased amounts of the same isoenzyme that is already present in the C_3 state. In order to elucidate this problem the ATPase will have to be solubilized and examined electrophoretically and by immunological methods.

In conclusion, besides ATP-hydrolysis activity in tonoplast vesicles from *M. crystallinum* in the C_3 and the CAM states, this work demonstrates H^+ transport across the tonoplast by using the method of quinacrine and 9-aminoacridine fluorescence quench. In addition to this, the use of the optical probe Oxonol-VI shows the formation of an electric-potential gradient ($\Delta\Psi$) which is reduced by the addition of Cl^-, malate^{2-} and NO_3^- (data not shown) and totally dissipated in the presence of Triton X-100 or Gramicidin. Thus, all the conditions required to demonstrate the existence of an electrogenic, ion-translocating ATPase generating an electrochemical gradient across membranes of tightly sealed vesicles are fulfilled according to Sze (1985). Finally, an increase in ATP-hydrolysis and H^+transport activities after induction of CAM has been demonstrated. It should be emphasized that similar results were obtained by using different methods: differential inhibition of protoplast homogenates, vacuoles (Struve et al. 1985) and tonoplast vesicles (this communication). These results and physiological considerations of the energy budget of CAM cells (Lüttge et al. 1981; Smith et al. 1982; Jochem et al. 1984; Struve et al. 1985) underline the essential role of vacuolar ATPases in the physiological function of CAM.

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