The Role of Vacuolar Malate-Transport Capacity in Crassulacean Acid Metabolism and Nitrate Nutrition. Higher Malate-Transport Capacity in Ice Plant after Crassulacean Acid Metabolism-Induction and in Tobacco under Nitrate Nutrition[1]

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Anion uptake by isolated tonoplast vesicles was recorded indirectly via increased H^+-transport by H^+-pumping of the V-ATPase due to dissipation of the electrical component of the electrochemical proton gradient, $\Delta\mu_{H^+}$, across the membrane. ATP hydrolysis by the V-ATPase was measured simultaneously after the Palmgren test. Normalizing for ATP-hydrolysis and effects of chloride, which was added to the assays as a stimulating effector of the V-ATPase, a parameter, J_{mal}^{rel}, of apparent ATP-dependent malate-stimulated H^+-transport was worked out as an indirect measure of malate transport capacity. This allowed comparison of various species and physiological conditions. J_{mal}^{rel} was high in the obligate crassulacean acid metabolism (CAM) species *Kalanchoë daigremontiana* Hamet et Perrier, it increased substantially after CAM induction in ice plant (*Mesembryanthemum crystallinum*), and it was positively correlated with NO_3^- nutrition in tobacco (*Nicotiana tabacum*). For tobacco this was confirmed by measurements of malate transport energized via the V-PPase. In ice plant a new polypeptide of 32-kD apparent molecular mass appeared, and a 33-kD polypeptide showed higher levels after CAM induction under conditions of higher J_{mal}^{rel}. It is concluded that tonoplast malate transport capacity plays an important role in physiological regulation in CAM and NO_3^- nutrition and that a putative malate transporter must be within the 32- to 33-kD polypeptide fraction of tonoplast proteins.

Malate is not only a central metabolite in intermediary flow of carbon in organisms. In higher plants, vacuolar malate accumulation, and hence, transtonoplast malate transport, also plays a paramount role in many physiological functions. These include adjustment of osmotic and turgor potentials in extension growth and movements of stomata and pulvini, pH-regulation, e.g. during nitrate reduction, and others (for review, see Lüttge, 1987). Massive nocturnal accumulation of malic acid in the vacuoles is a characteristic feature of crassulacean acid metabolism (CAM), where the driving force for transport is H^+ pumping by the V-ATPase and the malate^{2-} anion is moving down the electrical gradient thus established (Lüttge et al., 1981). Passive malic acid efflux in the daytime is possibly mediated by specific malate transporters (Fig. 1). Competition and specificity studies have shown that malate and chloride are taken up into tonoplast vesicles by different transporters and do not compete for the same transport sites (White and Smith, 1989; Martinoia et al., 1990).

Many attempts have therefore been made to identify vacuolar malate transporters, carriers and channels, for influx and efflux. A summary of the literature is given in Figure 1. The plants studied include the CAM plants *Kalanchoë daigremontiana* and *Graptopetalum paraguayense*. Most of this work is based on transport studies with isolated tonoplast vesicles, with direct determinations of transport of ^{14}C-labeled malate or with indirect measurements of transport recording relative rates of H^+ transport and membrane potentials using dyes in the absence and presence of organic ions. Channels were studied with the patch-clamp technique (see Fig. 1).

Protein biochemistry has been used to purify malate transporter proteins of plant tonoplasts. A partial purification was achieved with barley (Martinoia and Vogt, 1989; Martinoia et al., 1991; Martinoia and Rentsch, 1992) and with *K. daigremontiana* (Ratajczak et al., 1994a; Steiger et al., 1997). Photoaffinity labeling with photoreactive substrate analogs traced a 37-kD polypeptide in *Catharanthus roseus* that participated in malate transport (Lahjouji et al., 1996; Lahjouji and Canut, 1999). In no case, however, has a definite molecular identification of a tonoplast malate transporter been achieved. The 32-kD polypeptide and putative carrier protein in the malate transport active fraction of tonoplast
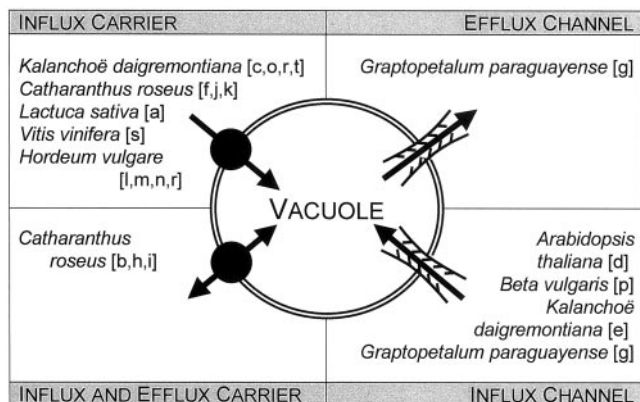
Figure 1. Plant vacuolar malate transporters as described in the literature (c, Buser-Suter et al., 1982; m, Martinoia et al., 1985; o, Nishida and Tominaga, 1987; k, Marigo et al., 1988; i, Lahjouji and Canut, 1999; j, Marigo and Bouyssou, 1989; l, Martinoia and Vogt, 1989; t, White and Smith, 1989; b, Bouyssou et al., 1990; n, Martinoia et al., 1991; r, Rentsch and Martinoia, 1991; f, Dietz et al., 1992; g, Iwasaki et al., 1992; p, Pantoja et al., 1992; a, Blom-Zandstra et al., 1993; q, Ratajczak et al., 1994a; d, Cerana et al., 1995; h, Lahjouji et al., 1996; e, Cheffings et al., 1997; s, Terrier et al., 1998; and i, Lahjouji and Canut, 1999).

polypeptides from *K. daigremontiana* has an N-terminal sequence unknown in protein data banks (Steiger et al., 1997: unpublished sequence of 11 amino acids), which is currently under molecular investigation.

Another approach is physiological comparison of different species under different conditions and in different physiological states. If physiological responses are based on different malate transport capacities and if this may be related to expression of particular polypeptides in the tonoplast membrane, it will allow determination of whether tonoplast malate transporters are involved in physiological regulation in addition or alternative to other functional components, such as the V-ATPase (Lüttge et al., 1995a, 1995b; Lüttge and Ratajczak, 1997). This strategy was chosen in the present work.

We used the constitutive CAM plant *K. daigremontiana*, the C_3/CAM-intermediate ice plant (*Mesembryanthemum crystallinum*) in both states of modes of photosynthesis, and the nitrophilous plant tobacco (*Nicotiana tabacum*) under various nitrogen nutrition regimes for comparisons. Comparisons were made possible by using the combined H^+ transport and ATP hydrolysis test after Palmgren (1990), where malate transport could be assessed by increased H^+ pumping of the V-ATPase due to dissipation of the electrical component of the transmembrane proton-electrochemical gradient, $\Delta\mu_{H+}$, by malate uptake into tonoplast vesicles, and hydrolytic activity of the V-ATPase measured simultaneously. On this basis we can show in the present report that high vacuolar malate transport capacity is related to CAM and excess NO_3^- nutrition.

RESULTS AND DISCUSSION

Effects of Chloride and Malate Anions on the V-ATPase of *K. daigremontiana*

The V-ATPase of higher plants occasionally has been named an "anion-ATPase" (Bennett and Spanswick, 1983; Blumwald and Poole, 1987; Lew and Spanswick, 1985) since it is regulated by various anions. It is inhibited by NO_3^- and stimulated by Cl^-. Chloride may stimulate the V-ATPase by direct effects on the enzyme (Smith et al., 1984; Sze et al., 1992; Ward and Sze, 1992) and by dissipating the electrical component of the electrochemical proton gradient, $\Delta\mu_{H+}$, via Cl^- transport, which allows increased H^+ pumping (Bennett and Spanswick, 1983; Sze, 1985; Pope and Leigh, 1988; Palmgren, 1991). Both effects are difficult to distinguish in experiments with native tonoplast vesicles and cannot be separated in the Palmgren test. Nevertheless, to obtain stimulation of the V-ATPase it was necessary to add chloride to the assays in our tests of relative rates of malate uptake into tonoplast vesicles based on $\Delta\mu_{H+}$ dissipation by malate transport. Therefore, combined anion effects of chloride and malate were first examined, for which tonoplast vesicles of *K. daigremontiana* were chosen with which our laboratory has the most extensive experience.

In the experiments of Figure 2 either anion, chloride, or malate was added first and rates of ATP hydrolysis and acridine orange absorption quenching (i.e. relative rates of H^+ transport) measured until equilibrium of absorption quenching due to vesicle acidification was reached in the Palmgren test. Thereafter, the other anion was added to the assay and rates were determined again in the presence of both anions. Anions were added as potassium salts. Earlier tests comparing potassium chloride with choline chloride checked that K^+ did not interfere and has no effect on anion-dependent H^+ transport mediated by the V-ATPase of tonoplast vesicles (Jochem and Lüttge, 1987).

Increasing the concentrations of chloride and malate from 20 to 50 mM when the respective anions were present alone in the assay (= first anion) increased relative H^+ transport rates significantly ($P < 0.05$) without affecting ATP hydrolysis (Fig. 2, A and B). This must be due to increased anion transport reducing the electrical component of $\Delta\mu_{H+}$ and hence increasing H^+ pumping. The effect of malate increasing H^+ transport was larger than that of chloride ($P = 0.01$ at 50 mM), which confirms earlier observations that vacuolar malate transport is more effective in the CAM plant *K. daigremontiana* than Cl^- transport (Jochem and Lüttge, 1987; White and Smith, 1989; Marquardt-Jarczyk and Lüttge, 1990a). The observations with addition of chloride alone also suggest that chloride stimulation of the V-ATPase (via ATP hydrolysis) must have been already saturated at 20 mM Cl^-. That ATP hydrolysis was similar at 20 and 50 mM concentrations of the respective anions implies that the calculated apparent relative
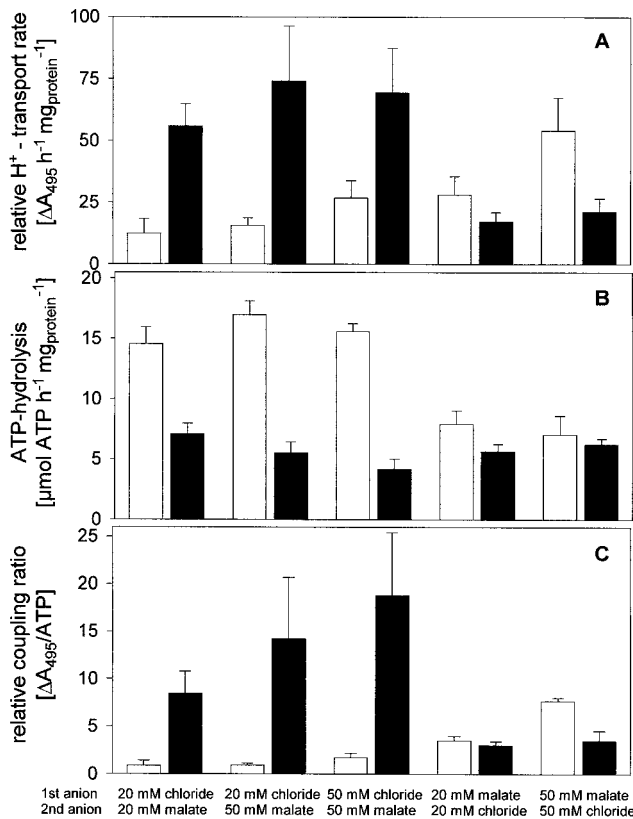
Figure 2. Effects of chloride and malate on proton transport activity (A), Bafilomycin A_1-sensitive ATP-hydrolysis (B), and relative H^+-transport/ATP-hydrolysis coupling ratios (C), i.e. (A):(B), of tonoplast vesicles of *K. daigremontiana* in the presence of Cl^- and malate at two different concentrations (20 and 50 mM, respectively) applied in different sequences, i.e. were the second anion was added after equilibrium was reached in the Palmgren test with the first anion, but the first anion always remained present in the assay when the second anion was given. White columns, first anion; black columns, first and second anion present. Errors are SD, $n = 4$ (two measurements of two independent membrane preparations).

coupling ratios of H^+ transport/ATP hydrolysis increased. ATP hydrolysis was lower and coupling ratios were higher ($P < 0.05$) in the presence of malate alone than with chloride alone in the assays (Fig. 2C).

When malate was added in the presence of chloride relative H^+ transport rates increased substantially ($P < 0.05$). This effect was not significantly ($P = 0.18$) larger with 50 mM than with 20 mM malate, so that the effect must have been already close to saturation at 20 mM malate. Previous studies have also shown that maximum H^+-transport rates in *K. daigremontiana* were obtained with 50 mM malate (Ratajczak et al., 1994a). This is important for the present work, which aims at determining malate transport capacities, so that we must work at saturation. ATP hydrolysis was somewhat decreased ($P < 0.01$) with 50 mM as compared with 20 mM malate in the presence of chloride, and thus apparent coupling ratios increased ($P < 0.01$). The relative rates of H^+ transport obtained with addition of 20 mM malate in the presence of chloride were much larger

($P < 0.01$) than those measured with 20 mM malate alone. Thus stimulation of the V-ATPase by chloride must be a prerequisite for high malate transport activities as indicated by H^+ transport rates. If 50 mM malate was used, however, there was no significant difference in H^+ transport activity as compared with 20 mM malate ($P = 0.16$).

Addition of chloride after malate reduced the relative rates of H^+ transport obtained with malate alone ($P < 0.05$). ATP hydrolysis was also inhibited ($P < 0.01$) so that the coupling ratio was only reduced at 50 mM concentrations of both ions ($P < 0.001$). The relative rates of H^+ transport in the presence of 50 mM chloride and 50 mM malate were less than one-third ($P < 0.01$) when malate was given first and chloride second as compared with the rates obtained with chloride first and malate second. Since chloride and malate do not compete for the same anion transporter (see introduction), one possible explanation for this effect is that they compete for binding at the V-ATPase itself. Chloride and malate can affect the V-ATPase directly (Jochem and Lüttge, 1987). Chloride may be the more potent direct positive effector, but malate may also bind and chloride may not effectively replace malate once it is bound to the enzyme. This putatively better competitive binding of chloride to the enzyme is supported by an experiment where 20 mM chloride and 50 mM malate were added to an assay simultaneously in the beginning of the Palmgren test and this was compared with an assay where malate was added after 20 mM chloride. Relative rates of H^+ transport attained were very similar in both cases, namely 66.7 ± 7.1 and $74.2 \pm 22.3 \ \Delta A_{495} \ h^{-1} \ mg^{-1}$ protein, respectively.

The changes of the apparent coupling ratios under the various conditions described suggest varying transport/ATP use efficiencies. In early studies the V-ATPase of higher plants was considered to be a 2 H^+/1 ATP ATPase (Bennett and Spanswick, 1983), which included the tonoplast ATPase of *Kalanchoë* species (Lüttge et al., 1981; Smith et al., 1982; Lüttge and Smith, 1988). Varying coupling ratios from 1.75 up to 3.28 H^+/ATP were reported later (Davies et al., 1994). An indirect coupling mechanism of the V-ATPase was proposed by Brauer et al. (1993) based on their observation that the two activities of the maize V-ATPase, i.e. H^+ transport and ATP hydrolysis activity, are inhibited by nitrate to different extents. An intriguing possibility for regulation of the V-ATPase was proposed by Moriyama and Nelson (1988), who suggested an intrinsic uncoupling ("slip") of the V-ATPase holoenzyme. Varying coupling ratios might be due to changes of the slip or uncoupling rates of the V-ATPase as it was published by Müller et al. (1997), who observed a 2.0- to 2.4-fold intrinsic uncoupling of the reconstituted lemon epicotyl V-ATPase compared with the fruit V-ATPase. Changes of calculated apparent relative coupling ratios between treatments in Figure 2 are sometimes still much larger, i.e. more than 10-fold in the cases where malate was added after chloride. Such large changes

are not very likely and must be due to non-linearity in the acridine orange absorption quenching test. The exact mechanism of the acridine orange absorption assay is still not clarified (Lee and Forte, 1978; DuPont, 1989; Palmgren, 1991). Moreover, it is important to note that acridine orange is in the bulk solution and not restricted to the membrane surface where the actual H^+ transport is directly effective (Heberle et al., 1994).

In conclusion, these studies with sequential application of anions may bear out interesting new information on the properties of the V-ATPase. This may warrant further investigations where ATP-hydrolysis of detergent-permeabilized tonoplast vesicles and isolated V-ATPase might also be compared. For the purpose of the present work, which aimed at comparing vacuolar malate transport capacities of different plant species under different physiological conditions, the experiments of Figure 2 determine the appropriate conditions of the V-ATPase from the obligate CAM species *K. daigremontiana* used as a basis for these comparisons, given by adding 50 mM chloride and 50 mM malate sequentially. Thus in all comparative measurements shown below in the Palmgren assays 50 mM chloride was added first and after attainment of equilibrium, 50 mM malate was added and apparent relative rates of H^+ transport, as well as ATP-hydrolysis, were determined from the initial slopes of the curves.

Comparison of Relative Anion-Dependent H^+ Uptake Activities by Tonoplast Vesicles from Different Plants under Different Physiological Conditions
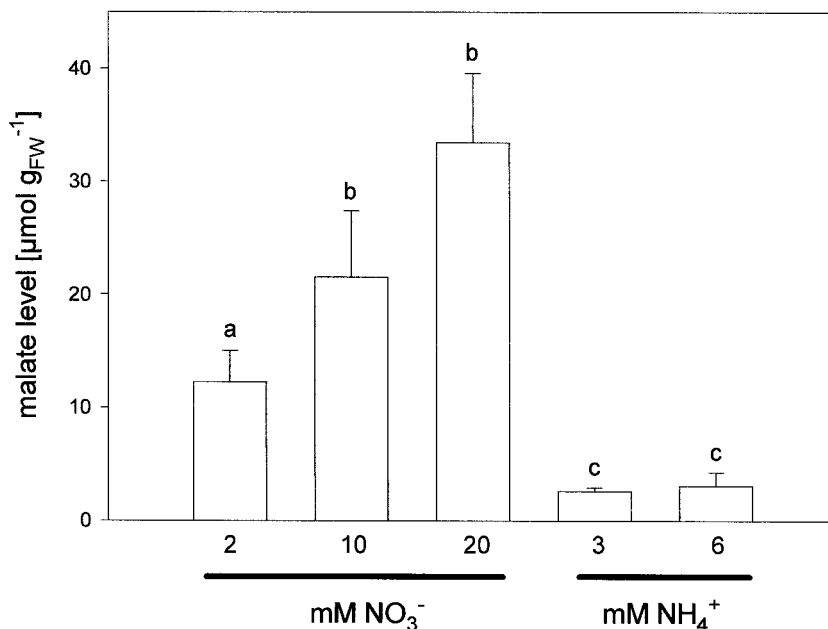
To assess potential vacuolar malate transport capacities of plants under changed physiological conditions *K. daigremontiana*, ice plant, and tobac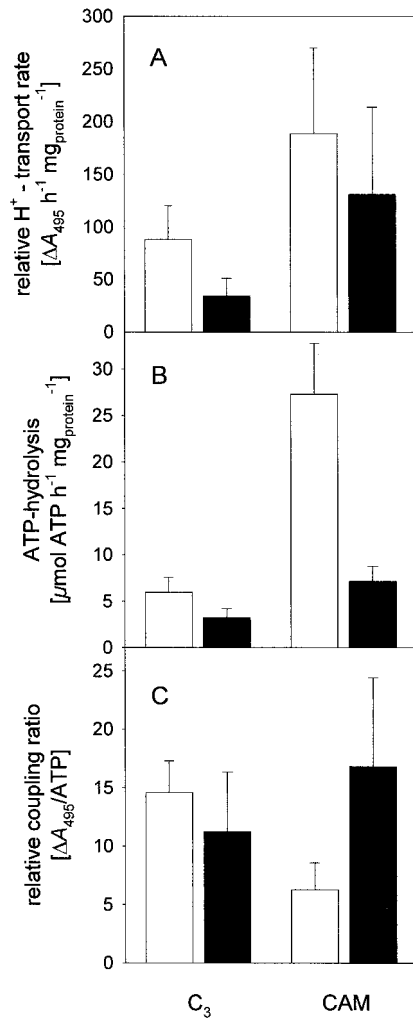co were chosen as experimental plants for the following reasons. The obligate CAM plant *K. daigremontiana* was used as a standard. Among the plants chosen for comparisons in this study, the highest transport capacities were expected due to nocturnal malate accumulation of up to 220 mM during CAM in the vacuoles of this species (Lüttge and Smith, 1988), and malate transport capacities appear to be lower in C_3 plants than in CAM plants (compare, e.g. Jochem and Lüttge, 1987; Kaestner and Sze, 1987; Pope and Leigh, 1987; Struve and Lüttge, 1987; White and Smith, 1989, 1992; Marquardt-Jarczyk and Lüttge, 1990a; Martinoia and Rentsch, 1992). For ice plant with inducible CAM a comparison was made between the C_3 state and the CAM state, where nocturnal vacuolar malate accumulation may be up to 100 mM (Ratajczak et al., 1994b).

Tobacco is known as a nitrophilous plant. This may require particular vacuolar malate transport capacities. Nitrate reduction leads to formation of hydroxyl ions that are neutralized by synthesis of malic acid and accumulation of malate in the vacuoles (Smith and Raven, 1979; Davies, 1986; Allen and Raven, 1987). Leaves of our experimental plants grown in the presence of increased NO_3^- concentrations showed increased vacuolar malate levels, an effect that was not seen with NH_4^+ nutrition (Fig. 3).

Figure 4 shows that the induction of CAM in ice plant greatly increased ATP hydrolysis in the presence of chloride of tonoplast vesicles ($P < 0.001$) as known from previous work (Ratajczak et al., 1994b). It is interesting that when malate was present in addition to chloride, ATP hydrolysis activity was significantly lower ($P < 0.01$) in *K. daigremontiana* (Fig. 2) and in ice plant (Fig. 4) in both states of photosynthesis compared with conditions when only chloride was present. In the presence of both anions, the ATP-hydrolysis activity

Figure 3. Malate levels in leaves of tobacco grown under various nitrogen regimes as indicated. Samples were taken at the start of the light period. Data are mean values ± SD of three independent measurements. Columns marked by different letters (a, b, and c) are statistically significantly different at the $P = 0.05$ value.
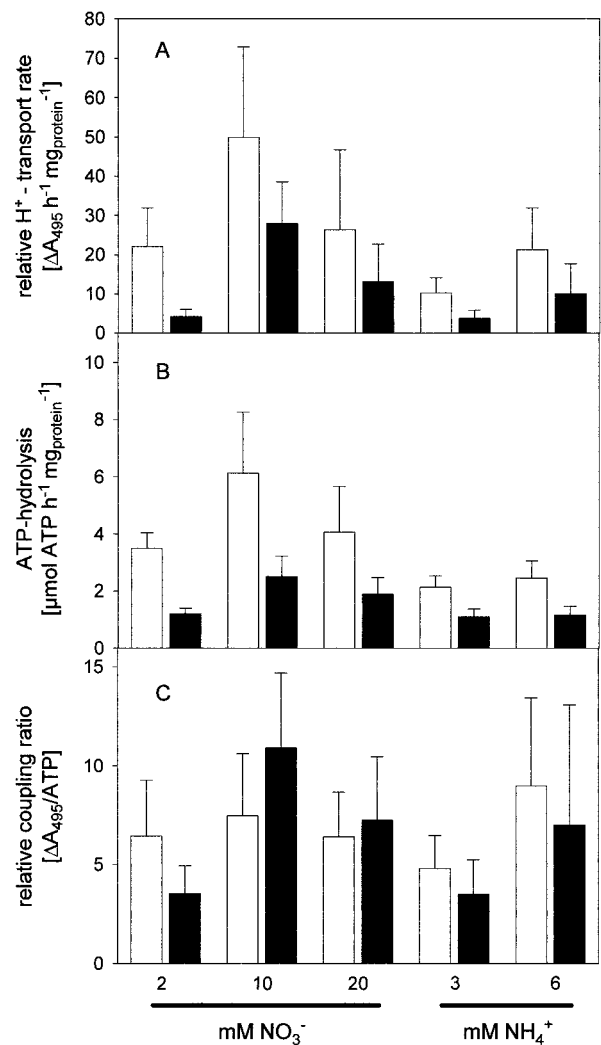
transport. Moreover and most interestingly, relative coupling ratios in the plants with CAM (*K. daigremontiana* and ice plant in the CAM state) increased with malate added in addition to chloride ($P < 0.001$ for both), an effect that was not observed with C_3 ice plant, where the coupling ratio was already high with chloride alone (Fig. 4C).

For tobacco under varied nitrogen nutrition, the patterns obtained are depicted in Figure 5. As expected from the malate levels recorded in the leaves (Fig. 3), high NO_3^- concentrations in the growth medium led to higher rates of ATP hydrolysis and relative rates of H^+ transport. In measurements in the presence of chloride this effect was larger with 10 mM NO_3^- ($P < 0.01$) than with 20 mM NO_3^- as compared with 2 mM NO_3^-. The

Figure 4. Relative H^+ transport rates (A) in the presence of 50 mM Cl^- alone (first anion; white bars) and 50 mM malate added subsequently (second anion; black bars) in the Palmgren assay. B, Concomitantly measured Bafilomycin A_1-sensitive ATP-hydrolysis. C, Calculated relative H^+ transport/ATP hydrolysis ratios for the C_3/CAM intermediate ice plant in the C_3 and CAM state. Data are mean values ± SD: C_3 state nine measurements of four independent preparations, CAM state 10 measurements of two independent preparations.

was higher in ice plant in the CAM state than in the C_3 state ($P < 0.001$), as well as for chloride alone. Chloride-dependent relative H^+ transport activity also increased ($P < 0.01$). A higher relative H^+ transport activity in the presence of chloride with additional supply of malate as observed for *K. daigremontiana* (Fig. 2) was not obtained for ice plant (Fig. 4, and also tobacco, see below: Fig. 5). This suggests that the complex anion effects (see previous section) are different in different species. However, in ice plant in the CAM state also in the presence of malate, relative rates of H^+ transport were much higher than in the C_3 state ($P < 0.01$) and similar to the rates obtained with the obligatory CAM plant *K. daigremontiana*. Increased H^+ transport rates in the CAM state in the presence of the anions indicate increased anion transport capacity including malate

Figure 5. Relative H^+-transport rates (A), ATP hydrolysis (B), and coupling ratios (C) as in Figure 4, but for tobacco grown under various nitrogen regimes as indicated. Data are mean values ± SD: 2 mM NO_3^- 14 measurements of two independent preparations, 10 mM NO_3^- 16 measurements of three independent preparations, 20 mM NO_3^- 20 measurements of two independent preparations, 3 mM NH_4^+ 11 measurements of three independent preparations, and 6 mM NH_4^+ 18 measurements of three independent preparations.

same pattern was found for H$^+$ transport and ATP hydrolysis activity when measured after addition of malate in the presence of chloride, although the values of activity were lower when both anions were present. Presumably the higher NO$_3^-$ concentration, which may have affected the V-ATPase during extraction, was already inhibitory (viz NO$_3^-$ inhibition of the V-ATPase). In a converse manner, no increases were found with NH$_4^+$. Relative rates of H$^+$ transport were even lower with 3 mM NH$_4^+$ than with 2 mM NO$_3^-$ ($P < 0.01$). Relative coupling ratios were higher at the higher NO$_3^-$ concentrations when malate was present in addition to chloride and at 6 mM NH$_4^+$ as compared with 3 mM NH$_4^+$ with chloride alone and malate in addition to chloride ($P < 0.05$; Fig. 5C).

An energization of the tonoplast vesicles avoiding the dual anion effects on the V-ATPase ($\Delta\mu_{H+}$ dissipation and direct interactions with the enzyme) is using the H$^+$ transporting tonoplastic V-PPase, which is not directly sensitive to anions (Marquardt-Jarczyk and Lüttge, 1990b). This poses two problems, however, in relation to the present work. First, it does not allow simultaneous measurement of relative rates of H$^+$ transport and substrate hydrolysis (see "Materials and Methods"). Second, vacuoles of mature leaves of ice plant in the CAM state do not have V-PPase activity (Bremberger and Lüttge, 1992). Nevertheless, we used this approach for comparison determining relative H$^+$ transport activity alone in *K. daigremontiana* and tobacco (Fig. 6). In *K. daigremontiana* relative H$^+$ transport rates in the presence of chloride and malate, respectively, were not significantly different. In a similar manner, in all of the NO$_3^-$ and NH$_4^+$ treatments of tobacco both anions added alone did not cause significantly different relative H$^+$ transport rates. Ten millimolar as compared with 2 mM NO$_3^-$ lead to significantly larger malate- ($P = 0.004$) and chloride- ($P = 0.04$) dependent relative H$^+$ transport rates. Malate- and chloride-

dependent relative H$^+$ transport rates were significantly larger in *K. daigremontiana* than in tobacco grown with 2 mM NO$_3^-$ ($P = 0.007$ and 0.005, respectively). NH$_4^+$-grown plants had comparatively higher rates.

Relative Malate Uptake Capacities of Tonoplast Vesicles from Different Plants under Different Physiological Conditions

Relating different rates of H$^+$ transport to simultaneously measured ATP hydrolysis in the Palmgren test, i.e. calculating the apparent relative coupling ratios presented above, is a first approximation to data normalization. However, in view of the complex anion effects with chloride and malate as revealed by the experiments of Figure 2, a second, more sophisticated normalization to account for the effects of both anions in a better way seems to be important. We approached this by relating the H$^+$ transport rates obtained with chloride and malate added in sequence ($J_{H+}^{(Cl^-+mal)}$) to those measured with chloride alone ($J_{H+}^{(Cl^-)}$) as well as relating both to the respective rates of concomitantly measured ATP hydrolysis ($\sim P^{(Cl^-+mal)}$ and $\sim P^{(Cl^-)}$, respectively), i.e.

$$J_{mal}^{rel} = \frac{J_{H+}^{(Cl^-+mal)}}{J_{H+}^{(Cl^-)}} \cdot \frac{\sim P^{(Cl^-)}}{\sim P^{(Cl^-+mal)}} \tag{1}$$

where the dimension-less parameter J_{mal}^{rel} then gives the ATP-dependent malate-stimulated relative rates of H$^+$ transport as an indirect measure of malate transport capacities. Figure 7 shows the results for some of the experiments of Figure 2, where 20 or 50 mM malate were added to the Palmgren assay after 20 or 50 mM chloride. There is an increased activity of malate-stimulated H$^+$ transport at the higher malate

Figure 6. Relative PP$_i$-dependent H$^+$ transport rates in the presence of either 50 mM chloride (white bars) or 50 mM malate (black bars) alone in the assay for the obligate CAM plant *K. daigremontiana* (*K.d.*) and the tobacco grown under various nitrogen regimes as indicated. Data are mean values ± SD of one to three measurements of membrane vesicle preparations from *K. daigremontiana* (three independent preparations), 2 mM NO$_3^-$ (three independent preparations), 10 mM NO$_3^-$ (three independent preparations), 20 mM NO$_3^-$ (three independent preparations), 3 mM NH$_4^+$ (two independent preparations), and 6 mM NH$_4^+$ (three independent preparations).
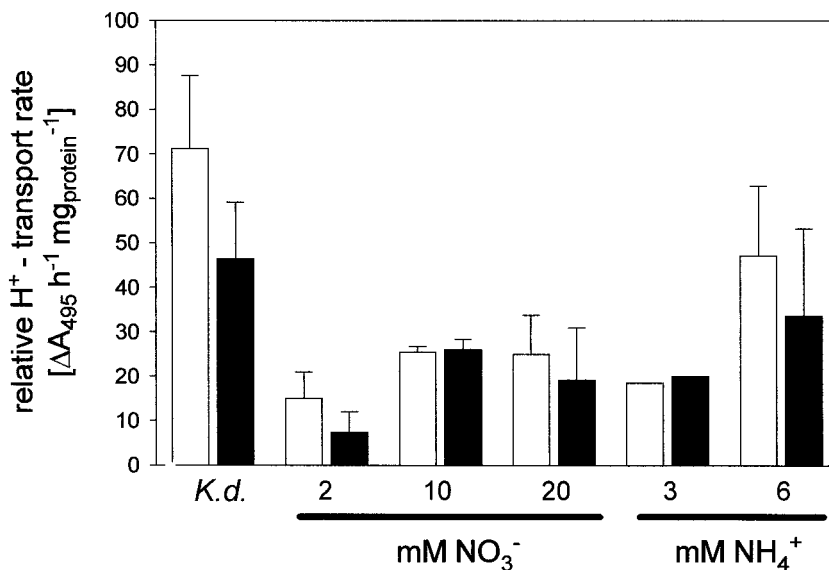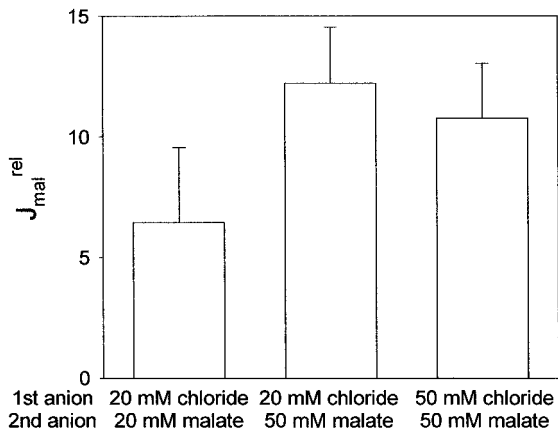
Figure 7. Relative rates (J_{mal}^{rel}) of ATP-dependent malate transport of tonoplast vesicles of *K. daigremontiana* calculated according to Equation 1 when malate was added in the presence of chloride as indicated (same experiments as the respective combinations of chloride and malate in Fig. 2).

concentration that was independent of the chloride concentration ($P < 0.05$).

The comparison of physiological states for ice plant and tobacco is given in Figure 8. CAM induction in ice plant stimulates J_{mal}^{rel} 3.7-fold ($P < 0.01$). This confirms a survey of earlier literature, which suggests that malate uptake activity of tonoplast vesicles is higher in CAM plants than in C_3 plants (see above). The J_{mal}^{rel} rates in ice plant in the CAM state are somewhat lower than in the obligate CAM-plant *K. daigremontiana*, but this is also consistent with expectations, since *K. daigremontiana* generally achieves higher nocturnal malate accumulation than ice plant during CAM.

In tobacco, 10 and 20 mM NO_3^- in the growth medium-stimulated J_{mal}^{rel} as compared with 2 mM NO_3^- ($P < 0.01$). NH_4^+-grown plants also show rather high apparent malate transport capacities that were a little lower than in 10 mM NO_3^-- and 20 mM NO_3^--grown plants. For tobacco, the energization of tonoplast

vesicles via the V-PPase also allowed us to compare directly the malate-dependent (malate alone present in the assays) and the chloride-dependent (chloride alone present in the assays) relative H^+ transport rates (Fig. 6). As compared with 2 mM NO_3^--grown plants at 10 and 20 mM NO_3^-, the ΔA_{495} h^{-1} mg^{-1} protein with chloride was increased by 10.4 and 10.5, respectively, whereas with malate this increase was 18.7 and 12.9, respectively. This supports the conclusion drawn from J_{mal}^{rel} calculations that increased nitrate supply causes a particular demand on malate transport. The pattern of malate dependent relative H^+ transport in PP_i-energized tonoplast vesicles in the NO_3^--grown plants (Fig. 6) is similar to that of J_{mal}^{rel} obtained with the ATP-energized vesicles (Fig. 8).

It is evident that high apparent malate transport capacities are a consequence of CAM and also high NO_3^- supply and most likely a prerequisite for CAM and for NO_3^- reduction by leaf cells. By a very different approach this was also checked by looking for changed polypeptide patterns under the different physiological conditions in the different plants. From *K. daigremontiana* we have previously separated a polypeptide fraction via solubilization with Triton X-100 and hydroxyapatite chromatography, which includes polypeptides of apparent molecular masses of 32, 33, and 34 kD showing malate transport activity after reconstitution in liposomes (Steiger et al., 1997). In this earlier work we also obtained antisera against this whole fraction. In addition we now prepared an affinity-purified antiserum against the 32-kD polypeptide of this fraction (see "Materials and Methods") that we thought was a good candidate for representing a malate transporter. Reconstitution into liposomes of an affinity chromatography-purified 32-kD polypeptide isolated from the hydroxyapatite eluate fraction gave a 127-fold higher malate transport activity compared with native tonoplast vesicles
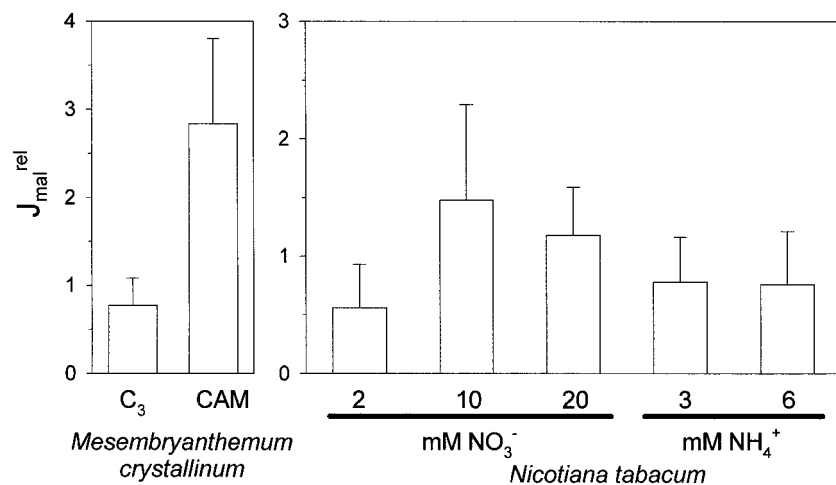
Figure 8. Relative rates of ATP-dependent malate transport of tonoplast vesicles of ice plant in the C_3 and CAM state, and tobacco under different regimes of nitrogen nutrition calculated according to Equation 1 when 50 mM malate was added in the presence of 50 mM chloride (same experiment as in Figs. 4 and 5).

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(Steiger et al., 1997). We have now checked if there are differences in polypeptide patterns of the tonoplast membranes in the range of these molecular masses among the plants used in the present study.

Polypeptide patterns often are difficult to quantify, and no very clear differences were found for tobacco under the different nitrogen nutrition regimes (not shown). The effect of increased malate transport capacity, however, was particularly pronounced in the comparison of ice plant in the C$_3$ with the CAM state (Fig. 8), and in this case there was also a response of polypeptide pattern. Figure 9 shows the 30- to 45-kD range of a silver-stained SDS-PAGE, a western blot immunostained with the antiserum against the hydroxyapatite eluate fraction of *K. daigremontiana* (see above) and a western blot immunostained with the affinity purified antiserum against the 32-kD polypeptide. A 33-kD polypeptide was enhanced and a 32-kD polypeptide newly appeared in the CAM state. This correlation of responses of J$_{mal}$^{rel} and polypeptide patterns underscores the role of malate transporter(s) in CAM. It also shows that proteins in the range of 31 to 34 kD, and in particular the 32-kD polypeptide as suggested by Steiger et al. (1997), must remain strong candidates for a malate transporter.

CONCLUSIONS

Anion transport by isolated tonoplast vesicles can be studied by following increased H$^+$ transport by the V-ATPase elicited by dissipation of the electrical component of $\Delta\mu_{H+}$ during anion transport. As transported anions, chloride and malate act in this way. In addition, however, they may interact with the V-ATPase directly, where Cl$^-$ in particular is a stimulating effector (Sze et al., 1992; Ward and Sze,

1992). Hence anion effects found here with different combinations of chloride and malate were complex. Nevertheless, normalizing malate-dependent relative rates of H$^+$ transport for ATP hydrolysis activity of the V-ATPase determined simultaneously after Palmgren (1990) and for effects of chloride, a parameter of apparent ATP-dependent malate-stimulated H$^+$ transport, J$_{mal}$^{rel}, could be worked out providing an indirect measure of malate transport capacity. This allowed comparisons of species and physiological conditions. It showed clear responses to CAM induction in ice plant and NO$_3$$^-$ nutrition in tobacco. The latter was also confirmed by measuring chloride- and malate-dependent relative H$^+$ transport activity separately by energizing the tonoplast vesicles via the V-PPase. This H$^+$ pumping tonoplast enzyme is not itself anion sensitive and only responds to anions via $\Delta\mu_{H+}$. Thus, we can show for the first time to our knowledge that malate transport capacity in the tonoplast must increase for performance of CAM, as well as under massive supply of NO$_3$$^-$. In ice plant these changes were accompanied by increased levels or newly appearing polypeptides of molecular masses of 32 and 33 kD, which may comprise a vacuolar malate transporter (Steiger et al., 1997).

MATERIALS AND METHODS

Plant Material

Kalanchoë daigremontiana Hamet et Perrier was propagated vegetatively and ice plant (*Mesembryanthemum crystallinum*) was grown from seeds, both from the collections of the Botanical Garden of Darmstadt University. Plants were cultivated in soil (Einheitserde ED-73 DIN 11540–80T) watered with tap water. Seeds of tobacco (*Nicotiana tabacum* L. cv Samsun) were obtained from Dr. Uwe Sonnewald (Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany). Seeds were germinated on moist filter paper and plants were grown on sand. Day length was at least 12 h, supplemented in winter by artificial glasshouse illumination (Philipps SON-T AGRO 400 lamp, Philipps, Wuppertal, Germany; Osram Seatto HQ-TS NDC lamp, Osram, Munich; mercury-vapor lamps, Osram).

Preparation of tonoplast vesicles of the obligate CAM plant *K. daigremontiana* was performed with leaves of the 3rd to 6th node from the apex. Leaves were harvested at the end of the light period in the state of low vacuolar malic acid levels and kept in air-tight bags in a cold room at 4°C overnight until use.

In 4-week-old plants (third leaf pair emerging) of ice plant, CAM was induced by watering with 400 mM NaCl, whereas controls were continued to be watered with tap water. Not earlier than after 12 d of NaCl stress, which fully induces CAM (Ratajczak et al., 1994b), leaves younger than the second leaf pair from the base were harvested in the state of low vacuolar malic acid levels and kept as for the leaves of *K. daigremontiana* until use.

Tobacco plants were watered for 4 weeks in Hoagland nutrient solution number 2 (Hoagland and Arnon, 1950)
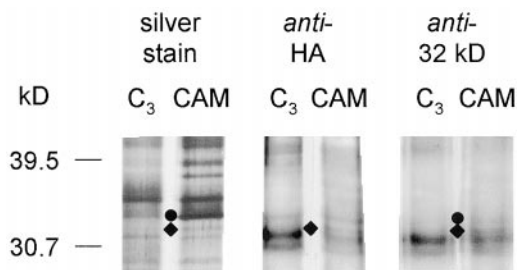
Figure 9. Patterns of polypeptides in the 30- to 45-kD range of vacuolar vesicles obtained from ice plant in the C$_3$ and CAM state after silver staining of the SDS-PAGE electropherogram and western blots immunostained with the antiserum against the hydroxyapatite eluate of *K. daigremontiana* (*anti*-HA) and the affinity-purified antiserum against the 32-kD polypeptide of the hydroxyapatite eluate (*anti*-32 kD). Diamonds and dots indicate a 32-kD polypeptide that was not present in the C$_3$ sample and a 33-kD polypeptide that increased in staining intensity after CAM induction, respectively. Numbers on the left-hand margin indicate molecular masses of standard proteins.

modified after Johnson et al. (1957). The solution contained 10 mM NO_3^-. The plants were then repotted and five groups separated according to nitrogen treatment in the subsequently used nutrient solution, namely 2 mM NO_3^-, 10 mM NO_3^-, 20 mM NO_3^-, 3 mM NH_4^+, and 6 mM NH_4^+. Nitrate was supplied as the Ca^{2+} salt and the pH of the solutions was 5.2 to 5.6. NH_4^+ was applied as NH_3/MES [2-(N-morpholino) ethanesulfonic acid] with the pH adjusted to 5.8. Plants were watered daily with a surplus of solution (2–3 times the pot volume) to avoid concentrating effects of ions in the soil. Leaves of the plants were used after 22 to 26 d.

Membrane Vesicle Isolation

Tonoplast vesicles were prepared by Suc density ultracentrifugation after Bremberger et al. (1988). Homogenization of leaf slices was performed with a medium of 100 mM Tricine (N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine)/ Tris, pH 8.0, 3 mM $MgSO_4$, 3 mM EGTA, 1 mM dithiothreitol (DTT), and 0.5% (w/v) polyvinylpyrrolidone 40 (or 0.5% [w/v] polyvinylpolypyrrolidone in the case of tobacco). Mannitol additions to the homogenization medium were 450 mM for *K. daigremontiana* and ice plant and 300 mM for tobacco. For tobacco 1 μM leupeptin and 1 mM benzamidine were added and also maintained subsequently in dilution and storage media of tonoplast vesicles. After filtration and pre-centrifugation of the homogenate for 10 min at 4,200g the microsomal fraction (supernatant) was subjected to a Suc density ultracentrifugation on a 25% (w/w) Suc cushion in 5 mM HEPES [N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid)]/Tris, pH 7.5, and 2 mM DTT for 90 min at 100,000g. The tonoplast-enriched vesicle fraction was collected from the 25% (w/w) Suc cushion diluted in a medium with 10 mM HEPES/NaOH, pH 7.0, 3 mM $MgSO_4$, and 1 mM DTT, and membranes were collected by centrifugation for 30 min at 300,000g. Membranes were resuspended in storage medium of 10 mM HEPES/NaOH, pH 7.0, 40% (v/v) glycerol, 1 mM DTT, plus 3 mM $MgSO_4$ for *K. daigremontiana* and ice plant, and plus leupeptin/benzamidine for tobacco as indicated above. All steps were performed at 4°C in a cold room. Membrane preparations were frozen in liquid nitrogen immediately after preparation and stored at −75°C until use.

Various Assays

Hot water extracts were obtained from microwave oven-dried leaf material. Malate was measured enzymatically in the extracts after Möllering (1974).

Membrane proteins were determined after Popov et al. (1975) using bovine serum albumin as protein standard. Purity of the membrane preparations was tested by measuring ATP hydrolysis after Ames (1966) in the presence of various effectors of different ATPases specific for different membranes, namely 1 mM azide (inhibitor of mitochondrial F_oF_1-ATPase; O'Neill et al., 1983), 40 nM Bafilomycin A_1 (inhibitor of V-ATPase; Bowman et al., 1988; Dröse et al., 1993), 50 mM chloride (stimulator of V-ATPase; Jochem et al., 1984), 0.1 mM molybdate (inhibitor of non-specific phosphatases; Gallagher and Leonhard, 1982), and 50 mM nitrate (inhibitor of

mitochondrial F_oF_1-ATPase and V-ATPase; O'Neill et al., 1983; Jochem et al., 1984). Tonoplast enrichment given by Bafilomycin A_1-sensitive and NO_3^--sensitive/azide resistant ATP hydrolysis was as follows: *K. daigremontiana*, 54% to 61%; ice plant C_3 state, 73% to 80%; CAM state, 79% to 85%; tobacco 2 mM NO_3^-, 37% to 43%; 10 mM NO_3^-, 50% to 62%; 20 mM NO_3^-, 43% to 55%; 3 mM NH_4^+, 31% to 43%; and 6 mM NH_4^+, 36% to 53% of total ATP hydrolysis activity. Thus the purity was reasonably similar in the plant groups to be compared, i.e. ice plant C_3 and CAM mode of photosynthesis and tobacco varied nitrogen nutrition. Moreover, under the H^+ transport test conditions (see below) only the V-ATPase can transport protons into the membrane vesicles, which assures that only V-ATPase activity is followed. This is different for ATP hydrolysis. However, here too the test conditions were optimized for the V-ATPase. The intraspecific comparisons also should not be much affected by preparation purity as shown above and ATP-hydrolysis rates obtained were corrected for non-Bafilomycin A_1-sensitive contributions (see below).

Simultaneous Measurements of Substrate Hydrolysis and H^+ Transport

For the comparative aims of this study it was important to measure relative rates of H^+ uptake into the native tonoplast vesicles and energization, i.e. ATP hydrolysis by the V-ATPase simultaneously in the same assay under identical conditions. This is possible by the coupled test after Palmgren (1990). Proton transport is measured by recording the quenching of acridine orange absorption at 495 nm. ATP hydrolyzed by the V-ATPase is continuously regenerated via pyruvate kinase from ADP produced and the phospho*enol*pyruvate added, so that ATP concentration in the assay remains constant. The pyruvate produced is reduced to lactate by lactate dehydrogenase. The decrease of NADH concentration in the assay is proportional to ATP hydrolysis and can be recorded by measuring absorption at 340 nm. Measurements at 340 and 495 nm were recorded in 10-s intervals (photometer Uvikon 810, Kontron, Munich). The reaction assay (total volume of 1 mL) contained 37.5 mM MOPS [3-(morpholino) propanesulfonic acid]/1,3-bis-[tris-(hydroxymethyl)-aminomethane]-propane, pH 7.5, 1 mM sodium azide (to inhibit mitochondrial ATPase), 20 μM acridine orange, 1 mM phospho*enol*pyruvate, 2 mM ATP, 300 μM NADH, 20 μL of 660 units/mL pyruvate kinase, 1,050 units/mL lactate dehydrogenase, and 25 to 50 μg protein sample.

An example is shown in Figure 10A, where the test also contained 50 mM KCl (see "Results and Discussion," Fig. 2). Initial slow rates of H^+ transport and ATP hydrolysis after mixing the test components can be explained by minor amounts of Mg^{2+} carried over from the tonoplast preparations into the assay. Addition of saturating $MgSO_4$ (final concentration of 4 mM) to complete the Mg-ATP substrate of the V-ATPase started the actual test. Proton transport activity declined due to attainment of an equilibrium between H^+ influx and efflux of the tonoplast vesicles and a feedback inhibition by the electrochemical gradient, $\Delta\mu_{H^+}$, established.
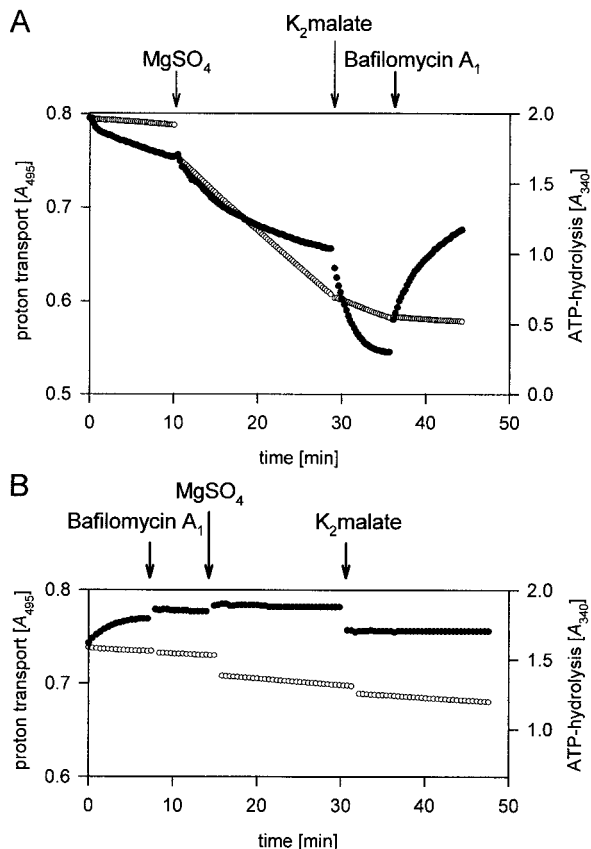
Figure 10. Example of simultaneous measurements of proton transport and ATP-hydrolysis in the Palmgren assay (A). Control experiment inhibiting the V-ATPase by Bafilomycin A_1 at the start (B). Proton transport, ●; left ordinate, acridine-orange absorption quenching (A_{495}). ATP-hydrolysis: ○, right ordinate absorption at 340 nm, A_{340}. Tonoplast vesicles of *K. daigremontiana* (50 μg protein) with 50 mM KCl present at the start. Additions of $MgSO_4$, 50 mM K_2malate, and Bafilomycin A_1 as indicated by arrows on top of the graph.

ATP hydrolysis remained constant, however, which can be explained by the uncoupling of ATP hydrolysis and H^+ transport in the V-ATPase enzyme, i.e. increasing "slip" (Müller et al., 1997) during the test runs. Isolated tonoplast vesicles (and possibly also vacuoles in situ) are never totally tight for protons. Under the conditions given (direction of $\Delta\mu_{H+}$) "leakiness" is H^+ efflux, not H^+ influx. This is indicated by the decline of H^+ transport with time in Figure 10A. Together with the problem of increasing slip, this requires that only the initial slopes of curves as exemplified in Figure 10A are used (Palmgren, 1990). This was of course, done in the present work. Relative H^+ transport activity is given as ΔA_{495} and ATP hydrolysis was quantified in absolute units using Lambert-Beer's law based on oxidation of one NADH per ATP hydrolyzed. H^+ transport and ATP hydrolysis are expressed per unit of time and total sample protein. The values of ATP hydrolysis were corrected for non-Bafilomycin A_1-sensitive conditions determined separately to make sure that only V-ATPase activity is assessed.

After addition of K_2 malate, the electrical component of $\Delta\mu_{H+}$ was dissipated by malate uptake into the vesicles and

H^+ transport was accelerated again (Fig. 10A). At the end the H^+ gradient was dissipated by adding Bafilomycin A_1, which shows again that the test was V-ATPase specific. This was also corroborated by the experiment of Figure 10B where Bafilomycin A_1 was added to the test assay at the beginning. There was a slight continuous Bafilomycin A_1-insensitive ATP hydrolysis, which did not respond to $MgSO_4$ addition (possibly due to contamination of the tonoplast preparation; see above). It is essential that with the V-ATPase being inhibited by Bafilomycin A_1, the addition of K_2 malate did not lead to any H^+ transport. Hence the V-ATPase under the conditions of the Palmgren test also in our hands is the only pathway for H^+ transport (influx) into the tonoplast vesicles. Passive H^+ diffusion with the $\Delta\mu_{H+}$ direction given in the test (Fig. 10A) is an efflux.

As detailed in "Results and Discussion" (with refs. therein), a problem of studying anion transport in tonoplast vesicles energized by the V-ATPase arises from the fact that the V-ATPase is not only affected by anions indirectly via $\Delta\mu_{H+}$ dissipation through anion transport, but also directly via anion/V-ATPase-protein interactions. This problem can be circumvented by energizing the tonoplast vesicles by the H^+ transporting V-PPase (Marquardt-Jarczyk and Lüttge, 1990b). This precludes the essential advantage of the Palmgren test measuring energization (ATP hydrolysis) simultaneously with H^+ transport because, unlike ATP, there is no way of enzymatic regeneration of PP_i in the test assay. However, we used V-PPase energization of tonoplast vesicles for some comparisons. The H^+ transport activity of the V-PPase was measured by recording acridine orange absorption at 495 nm. The reaction assay (total volume 1 mL) contained 37.5 mM MOPS/1,3-bis-[tris-(hydroxymethyl)-aminomethane]-propane, pH 7.5, 20 μM acridine orange, 2 mM $MgSO_4$, 25 to 50 μg protein sample, and either 50 mM KCl or 50 mM K_2 malate. Because the V-PPase activity has been demonstrated to be saturated at less than 50 mM potassium (Marquardt and Lüttge, 1987), at both potassium concentrations used, i.e. 50 and 100 mM in the presence of KCl and K_2 malate, respectively, optimal V-PPase activities are attained. Reaction was started by addition of 0.2 mM Na_4-PP_i.

SDS-PAGE, Western-Blot Analysis, and Immunostaining

SDS-PAGE was performed after Laemmli (1970) on Mini-Protean II (1-mm spacers, Bio-Rad, Munich) gels containing 17% (w/v) total acrylamide concentration (weight ratio of acrylamide : N,N'-methylene bisacrylamide = 37.5:1) at 20 mA per gel at room temperature. After electrophoresis, proteins separated in the gel matrix were stained with silver according to Merril et al. (1981). The LMW Marker kit and the prestained Kaleidoskope Marker kit (Bio-Rad) were used for determination of apparent molecular masses of proteins in gels subjected to silver staining and western blot, respectively.

Proteins were transferred from SDS-PAGs to Immobilon P membranes (Millipore, Dassel, Germany) following the western-blot method of Towbin et al. (1979) in a Semi-Dry

transblot chamber (Bio-Rad). Immunostaining was performed using alkaline phosphatase-coupled secondary goat-anti-rabbit IgG-antibodies (Sigma, Deisenhofen, Germany) and the western light immunodetection kit (Serva, Heidelberg) as described in Fischer-Schliebs et al. (1997). Primary antisera used were a polyspecific polyclonal antiserum against a fraction of Triton X-100 solubilized and hydroxyapatite purified tonoplast proteins of *K. daigremontiana*, which was active in malate transport when reconstituted in liposomes (Ratajczak et al., 1994a; Steiger et al., 1997) and an affinity-purified antiserum against a 32-kD peptide from this hydroxyapatite fraction. Affinity purification of antibodies was performed according to Betz and Dietz (1991). Proteins of the hydroxyapatite eluate were separated by SDS-PAGE (see above), and western blotted. Some traces of the Immobilon P membrane were immunostained with the polyspecific polyclonal antiserum against the hydroxyapatite fraction using the p-nitrobluetetrazolium/5-bromo-4-chloro-3-indolylphosphate-p-toluidine method (Blake et al., 1984). Other traces remained unstained and the region of the 32-kD polypeptide, which is part of a cluster of prominent polypeptides exhibiting molecular masses between 30 and 34 kD in the hydroxyapatite fraction, identified in the stained traces was cut out. The cut out sections were stripped with 100 mM Gly/HCl, pH 2.5, and the stripping solution was brought to pH 7.5 with Tris. This solution contained affinity-purified antibodies against the 32-kD polypeptide. The immobilon membrane was carefully washed with phosphate-buffered 150 mM NaCl (PBS), blocked with 1% (w/v) ovalbumin in PBS, incubated over night at 4°C with the polyspecific polyclonal antiserum, washed in PBS, stripped (the supernatant was again adjusted to pH 7.5 with Tris), washed in PBS, and subjected to repeated purification procedure starting with blocking. This was done up to 11 times. Optimum enrichment of the affinity-purified 32-kD antiserum was obtained after six steps, although purification was not complete.

Statistics

For the Palmgren assays, the numbers of different preparations in the comparisons were 2 to 4 and the number of measurements were 7 to 20. Data shown in the figures are mean values of all measurements performed with the preparations available for different plants or plants grown under different conditions. Differences between species and physiological states discussed were checked by the *t* test of COSTAT (Cohort Software, Berkeley, CA) after a test for equal variances was performed with the *F* test of COSTAT. Significance levels (*P*) are given for comparison in the text.

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