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Regulation of the Lemon-Fruit V-ATPase by Variable Stoichiometry and Organic Acids

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Abstract. The lemon-fruit V-ATPase can exist in two forms: nitrate-sensitive and nitrate-insensitive. Here we report the results of measurements of H^+/ATP stoichiometries using two kinetic methods: one based on steady-state ΔpH and one based on initial rates of H^+-pumping. Our findings indicate that the nitrate-insensitive fruit V-ATPase has an H^+/ATP stoichiometry of ~1, while both the nitrate-sensitive fruit V-ATPase and the epicotyl V-ATPase have stoichiometries of 2, under zero-load conditions. As ΔpH increases, the stoichiometry of the nitrate-sensitive fruit V-ATPase decreases to 1. Under similar conditions, the stoichiometry of the epicotyl enzyme remains 2. Thus, the pH-dependent variable stoichiometry of the lemon-fruit V-ATPase may represent a key factor in juice sac vacuolar hyperacidification. On the other hand, the H^+/ATP stoichiometry of the epicotyl V-ATPase can decrease from 2 to 1 in the presence of a membrane potential. The low pH of the fruit vacuole is not due solely to the lower H^+/ATP stoichiometry of its pump. We show that lumenal citrate and malate improve the coupling of both the epicotyl and fruit V-ATPases and enhance their ability to generate a pH gradient. Since citrate accumulation is restricted to fruit vacuoles, it may be another important determinant of vacuolar pH.

Key words: *Citrus lemon* — Organic acids — Stoichiometry — Transmembrane electrical potential — V-ATPase

Introduction

The vacuolar H^+-ATPases (V-ATPases) are responsible for the acidification of various endomembrane compartments in eukaryotic cells, including the large central vacuoles of plant cells (Sze, 1985; Forgac, 1989; Nelson, 1991; Nelson & Taiz, 1991; Taiz, 1992; Barkla & Pantoja, 1996; Bowman & Bowman, 1996; Taiz & Nelson, 1996; Finbow & Harrison, 1997). Structurally related to the ATP synthases, or F-ATPases, of mitochondria and chloroplasts, V-ATPases are organized into two large domains: a hydrophilic catalytic complex, V_1, made up of 8 different subunits, and a hydrophobic transmembrane H^+ channel, V_o, composed of 5 different subunits (Gluck et al., 1996; Luttge & Ratajczak, 1997; Forgac, 1999). The mechanochemistry of the V-ATPase proton pumps is thought to be similar to that of the F-ATPases. According to the current model, the hydrolysis of ATP by the V_1 complex generates a rotary torque in the V_o complex that is used to drive proton transport across the membrane (Elston, Wang & Oster, 1998; Grabe, Wang & Oster, 2000).

Since V-ATPases have three ATP-hydrolyzing catalytic sites and six proton-binding proteolipid subunits, the theoretical H^+/ATP stoichiometry of V-ATPases is 2 (Cross & Taiz, 1990), which is in good agreement with experimental measurements (Bennett & Spanswick, 1984; Schmidt & Briskin, 1993). On the other hand, intracellular compartments are rarely acidified to the theoretical limit attainable by their proton pumps. For a V-ATPase with a stoichiometry of 2, the thermodynamic maximum corresponds to a trans-tonoplast ΔpH of ~4.6, equivalent to a lumenal pH of ~2.4 (Rea & Sanders, 1987; Davies, Poole & Sanders, 1993). Yet, the typical lumenal pH for acidic intracellular compartments is around 5.0. The lack of correlation between theoretical maximum and actual pH gradient across vacuolar membranes has led to the hypothesis that the V-ATPase is under kinetic regulation (Bennett & Spanswick, 1984). Several mechanisms have been proposed to be involved in the regulation of V-ATPases, including counter ion conductance, oxidation, V_1 dissociation, and slip (Moriyama & Nelson, 1989; Tu, Nagahashi & Brouillette, 1987; Müller et al., 1996).

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Plant vacuoles provide useful experimental material for studying V-ATPase regulation because of the great diversity of their lumenal pHs. Although typical plant vacuoles have an internal pH of 5.0 – 5.5, some vacuoles, such as those of *Begonia lucerna*, can reach as low as pH 0.9 (Taiz & Zeiger, 1998). The pH gradient across the *Begonia* leaf tonoplast exceeds the theoretical limit for a pump with an H^+/ATP stoichiometry of 2, raising the possibility that some plant V-ATPases may have an H^+/ATP stoichiometry of 1.

Organic acids may also play a role in regulating vacuolar pH. Virtually all hyperacidifying plant cells accumulate organic acids instead of chloride as counter ions, and their vacuolar pH usually corresponds to the lowest pK_a of the accumulated acid. However, since organic acids are thought to enter the vacuole in the dissociated form, their role would be to buffer the vacuolar lumen rather than to acidify it.

In lemon fruits, the vacuolar pH can reach as low as 2.2. Thus, the trans-tonoplast pH gradient of juice sac cells is close to the calculated maximum attainable by a V-ATPase with an H^+/ATP stoichiometry of 2 (Müller et al., 1996). ATP-driven H^+-transport in tonoplast-enriched juice sac vesicles is only marginally sensitive to the V-ATPase inhibitor bafilomycin, and is partially sensitive to high vanadate concentrations (50 – 300 μM) (Müller et al. 1996; Müller et al., 1997). The lemon-fruit V-ATPase is also less sensitive than typical V-ATPases to inactivation by oxidation and *N*-ethylmaleimide (NEM) (Müller et al., 1996). Furthermore, cold dissociation of V_1 subunits in the presence of nitrate completely inhibits proton transport in lemon epicotyl membranes, but has little effect on the H^+-pumping activity of most juice-sac vesicles, even though similar amounts of catalytic subunits are released. A side effect of the V_1 dissociation treatment is that the sensitivity of the juice-sac H^+-pumping activity to vanadate is increased. In partially nitrate-sensitive fruit V-ATPases, we found that the H^+/ATP coupling ratio was strongly reduced by the cold inactivation treatment (Müller et al., 1999).

We hypothesized that lemon fruit vacuoles contain two interchangeable forms of the V-ATPase: a nitrate-sensitive, vanadate-insensitive form and a nitrate-insensitive, vanadate-sensitive form. Evidence for seasonal variation in the nitrate-sensitivity of the fruit V-ATPase was obtained. The minimum nitrate sensitivity occurs during the winter, and the maximum nitrate-sensitivity occurs during the spring and early summer. The lower nitrate-sensitivity in the fall and winter was correlated with a lower H^+/ATP coupling ratio during these months (Müller et al., 1999).

What is the significance of the variable H^+/ATP coupling ratio in lemon fruits? We have already shown that a decrease in the initial rate coupling ratio, which is determined in the absence of a pH gradient, is not an indicator of slip, which represents pH-dependent intrinsic uncoupling of the pump (Müller et al., 1999). However, if the measured H^+/ATP coupling ratio were to correspond to the H^+/ATP stoichiometry of the enzyme, it would provide evidence for regulation of vacuolar pH in lemon and other plant vacuoles, by variable H^+/ATP stoichiometry, as previously proposed by Davies, Hunt & Sanders (1994).

Here we present evidence, based on two independent methods, that differences in the H^+/ATP coupling ratios of nitrate-sensitive and nitrate-insensitive lemon fruit V-ATPases do, indeed, correspond to differences in their H^+/ATP stoichiometries. However, the nitrate-sensitive fruit and epicotyl V-ATPases both have the capacity to shift to an H^+/ATP stoichiometry of 1, either in the presence of a membrane potential (epicotyl), or when a pH gradient forms (fruit). While pH-dependent variable stoichiometry undoubtedly represents a distinct advantage in the hyperacidification of juice-sac vacuoles, it is not the sole determining factor. We have found that organic acids on the inside of the vesicles improve the coupling of proton transport to ATP hydrolysis and allow the generation of a steeper pH gradient. Thus, organic-acid accumulation represents another key factor in regulating vacuolar pH.

Materials and Methods

Materials

Lemon seeds (*Citrus limon* L. var. Schaub Rough Lemon) for growing seedling epicotyls were generously supplied by Willits & Newcomb, Inc., Arvin, CA 93203. Lemon fruits (var. Eureka) were harvested from a tree on the campus of the University of California, Santa Cruz. Reduced nicotinamide-adenine dinucleotide (NADH) was from Boehringer (Mannheim, Germany). All other chemicals were purchased from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA).

Membrane Preparation

Tonoplast-enriched membranes from lemon fruit juice sacs and epicotyls were prepared as described previously (Müller et al., 1999). To avoid dissociation of the V-ATPase, fruit juice sacs were homogenized in highly buffered HB_f (1.5 M MOPS-KOH, pH 8.5, 2.25% polyvinylpyrrolidone-40, 0.75% bovine serum albumin, 7.5 mM EDTA, 2 mM DTT, and 0.1 mM PMSF) and epicotyls were homogenized in HB_e (0.5 M MOPS-KOH, pH 8.5, 1.5% polyvinylpyrrolidone-40, 0.5% bovine serum albumin, 5 mM EDTA, 2 mM DTT, and 0.1 mM PMSF). The homogenates were centrifuged for 20 min at 12,000 × g. The recovered supernatants were subjected to ultracentrifugation for 60 min at 132,000 × g, and the microsomal pellets obtained were purified on a 10%/35% sucrose step gradient for 60 min at 132,000 × g. The 10%/35% interface containing tonoplast-enriched membranes was recovered, diluted with a buffer suitable for proton pumping experiments, RB (in mM: 10BTP-Mes pH 7.0, 20 KCl, 1 EDTA, 2 DTT, 0.1 PMSF) and pelleted for 20 min at 174,000 × g. The tonoplast-enriched mem-

branes were resuspended in RB at a final concentration of ~5 μg membrane protein/μl.

Stoichiometry Measurements

Two kinetic methods were used to determine the H^+/ATP stoichiometries of lemon fruit and epicotyl V-ATPases. The first was based on a steady-state model describing the establishment of a proton gradient across membrane vesicles (Brauer et al., 1989; Schmidt & Briskin, 1993). The second was derived from the initial-rate continuous spectrophotometric assay of Palmgren (1990). In both cases, proton pumping and ATP hydrolysis were measured simultaneously (Müller et al., 1999). Proton transport was monitored by following the absorbance decrease of acridine orange at 495 nm. ATP hydrolysis was coupled to the oxidation of NADH and followed as the NADH absorbance decrease at 340 nm. The assay medium consisted of (in mM) 10 MOPS-BTP, pH 7.0, 2 ATP, 150 KCl, 1 sodium azide, 1 phospho*enol*pyruvate, and 50 μM sodium vanadate, 0.5 μM valinomycin, 0.25 mM of freshly prepared NADH, 25 μl/ml of a mixture of pyruvate kinase and lactate dehydrogenase (Sigma), and, unless stated differently, 20 μM acridine orange. The reaction was started with 4 mM $MgCl_2$ and the absorbance values at 340 nm and 495 nm were recorded in 15-sec intervals with a Spectronic Genesis 5 spectrophotometer (Spectronic, Rochester, NY) interfaced with a PC running the MultiWL computer program (M. L. Müller and A. Murphy). The results were plotted after correction for mixing artefacts. All experiments were done in the presence of 20–25 μg membrane protein, unless stated differently.

Steady-State Model of H^+-Transport Time Course

In this model, a time course of the pH gradient formation is extrapolated from the absorbance quenching of acridine orange at steady state, as described by Schmidt and Briskin (1993). ΔpH at steady state is multiplied by the internal buffering capacity of the membrane vesicles (B_i) and so converted to μmoles H^+/mg protein (Maloney, 1979; Brauer et al. 1989). The resulting value is multiplied by the leakage and slip rate constant (k_i), which results in an estimate of the net amount of protons transported per unit time.

pH Gradient at Steady State

To calibrate the pH gradient, membrane vesicles were resuspended in RB adjusted with citrate to pH values between 4.4 and 6.9. The vesicles were centrifuged for 15 min at 174,000 × g, resuspended in the same buffer and "slow-frozen" by cooling to −70°C without previous immersion in liquid nitrogen. "Slow-freezing" was aimed at loading the vesicles with buffer while they were induced to go through inversion cycles. Thawed, preloaded membrane vesicles were added to complete but unbuffered reaction mix (containing KCl, valinomycin and acridine orange) and the absorbance at 495 nm was continuously monitored. After one minute, 100 μl of 0.5 M MOPS-BTP, pH 6.9 or 7.9, containing 17.5 μM acridine orange, was added to the reaction mix to generate pH gradients ranging from 0 to 3.5 pH units. The absorbance at 495 nm decreased, as acridine orange was drawn into the acidic vesicular lumen. After one further minute, the pH gradient was collapsed with 10 mM NH_4Cl buffered to pH 6.9 or pH 7.9. The resulting absorbance jump was used for the calibration of the pH gradient. Concentrations of acridine orange inside and outside the vesicles were estimated assuming a vesicular volume of 10 μl/mg protein (Poole, Melhorn & Racker, 1985). Because calibration curves and proton-pumping experiments were performed under similar conditions with the same amount of membrane protein, the vesicular volume does not affect the calculations and is therefore arbitrary.

Intra-Vesicular Buffering Capacity

The method described by Maloney (1979) was adapted to measure the vesicles' internal buffering capacity. The aim of the method is to obtain a value specific to the vesicular membrane, rather than to the buffer contained within. Therefore, the membrane vesicles were washed three times with 100 mM KCl and resuspended in 150 mM KCl, 10 μM valinomycin to a final concentration of 0.65 ± 0.05 mg/ml. Washed vesicles (0.7 ± 0.1 mg) were degassed for 10 min with nitrogen gas. Then, pH jumps were induced by adding 25, 50, or 100 nmol H^+ (HCl), under constant stirring and a flow of nitrogen. The change in pH was monitored with a standard pH meter connected to a plotter. Outer buffering capacity (B_o), total buffering capacity (B_t) and internal buffering capacity ($B_i = B_o - B_t$) were calculated according to Schmidt and Briskin (1993) in the presence and absence of gramicidin D. The internal buffering capacity values found for fruit and epicotyl tonoplast vesicles were 0.198 ± 0.027 μmol $H^+ \cdot (\text{pH unit})^{-1} \cdot mg^{-1}$ and 0.203 ± 0.023 μmol $H^+ \cdot (\text{pH unit})^{-1} \cdot mg^{-1}$, respectively. These values were found to be relatively constant over the pH range used for their determination (5.15 to 6.49). At low pH and in the absence of gramicidin, B_t tended to be underestimated, leading to an overestimated value for B_i. However, when B_t was determined in the presence of gramicidin, B_i remained pH-independent.

Slip and Leakage Rates

Membrane leakage and slip rate constants (k_i) at any time during the formation of a pH gradient were calculated according to the model described by Tu et al. (1987) with the following equation:

$$k_i = -\frac{\ln\left(1 - \frac{\delta}{\delta_s}\right)}{t}$$

where δ and δ_s represent the net value of protons transported at time t and at steady state, respectively (Müller et al., 1997). The rate constant k_i was determined independently for each proton pumping experiment used in the stoichiometry calculations.

Continuous Spectrophotometric Assay

In the continuous spectrophotometric assay, the proton gradient at steady state, as measured by acridine orange absorbance quenching, was calibrated under dye-limiting conditions. When the pump reached steady state, all dye molecules accumulated inside the tonoplast vesicles and were quenched. A curve was constructed where the quenching of acridine orange (ΔA_{495}) was correlated to the number of dye molecules transported (Fig. 4*B*). The slope of the (ΔA_{495}) calibration curve at any of its points, described by its first derivative, was used to measure the initial rate of proton pumping. Provided the concentration of acridine orange was *not* limiting *under initial rate conditions,* it can be assumed that a direct relationship exists between protons and acridine orange transported (H^+:acridine orange (1:1)) (Palmgren, 1990).

ATP Hydrolysis Rate

Under all conditions tested, the rate of ATP hydrolysis, coupled to the oxidation of NADH, was found to be linear. ATPase activity (in $\mu\text{mol Pi} \cdot mg^{-1} \cdot min^{-1}$) was calculated according to the following formula (Nørby, 1988):

$$\frac{\Delta A_{340}/t \cdot 10^3}{(\varepsilon(\text{NADH}) - \varepsilon(\text{NAD})) \cdot \text{Protein}}$$

where $\Delta A340/t$ is the decrease in absorbance at 340 nm/min. [ε(NADH)-ε(NAD)] equals $6.22 \cdot 10^3$ cm^{-1} M^{-1} and represents the difference in absorbance coefficients of NADH and NAD^+ and "Protein" is the amount of protein in the reaction mix in mg.

Electrical Transmembrane Potential

The electrical potential gradient built up across the tonoplast membrane was measured according to Bennett and Spanswick (1983) in conditions similar to the conditions used for measuring proton pumping, but with 2.5 μM oxonol VI instead of acridine orange in the reaction mix. The absorbance values of oxonol VI at 610 nm and 580 nm were measured every two seconds after starting the H^+-pumping reaction with Mg^{2+}. An electrical potential across the membrane results in a shift in A_{610}-A_{580}. Forty micrograms of fruit or epicotyl tonoplast-enriched membranes were found to be optimal for membrane potential measurements.

Organic Acids and Anions

To test the effect of organic acids and other anions on the V-ATPase-driven pH-gradient formation, fruit and epicotyl vesicles were preloaded with 50 or 75 mM K_3-citrate, 100 mM K_2-malate, 100 mM K_2SO_4, or 150 mM KCl. Membrane vesicles were diluted in RB containing the potassium salt of the anion to be loaded and centrifuged for 15 min at 174,000 × g. The membrane pellet was resuspended in the same buffer and slow-frozen to –70°C. Proton pumping and ATP hydrolysis measurements were performed as described above, except that the reactions were started by adding the preloaded vesicles to a complete reaction mix. The preloading concentrations were chosen so as to maintain a constant osmolarity of 300 mosmol.

In experiments balanced for cationic and anionic charges, sorbitol was used to balance osmolarity.

Protein Concentration

Estimates of protein concentrations were done routinely with Amido Black (Popov, Schmitt & Matthies, 1975).

Results

As described in Material and Methods, H^+/ATP stoichiometries of the lemon fruit and epicotyl V-ATPases were determined by two kinetic methods in which the proton transport rate was either extrapolated from the pH gradient at steady state (steady-state method), or was assessed directly from the calibrated rate of acridine orange accumulation (initial-rate method).

Steady-State Method

In the steady-state model, to avoid underestimating the pH gradient built up across the vesicular membrane, it is critical to perform H^+-pumping experiments under dye-saturating conditions. With low

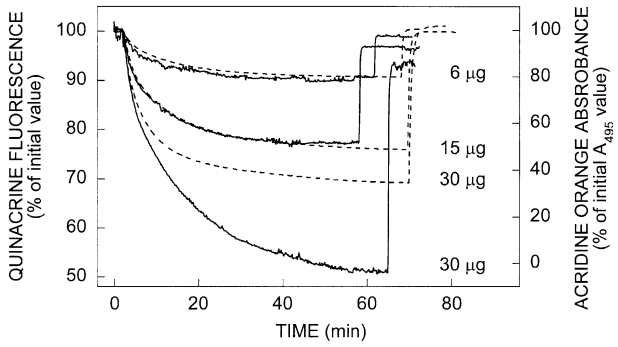


Fig. 1. Determination of the optimal amount of tonoplast-enriched vesicles from lemon fruits to be used for proton-pumping experiments. H^+-transport was measured by acridine orange absorbance (- - -) and quinacrine fluorescence (——) quenching. The indicated amount of membrane protein was added to a reaction mix containing 10 μM quinacrine or 17.5 μM acridine orange. Once established, the pH gradient was collapsed with 0.25 μM gramicidin.

concentrations of membrane protein in the reaction mix, proton pumping, as measured by acridine orange-absorbance quenching or quinacrine-fluorescence quenching, exhibited identical curves (Fig. 1). With 17.5 μM acridine orange in the reaction mix, dye became limiting when more than 15–20 μg fruit tonoplast vesicles were used. Under the same conditions, 10 μM quinacrine did not become limiting until the amount of membrane protein was >40 μg (*data not shown*). All subsequent experiments were performed with acridine orange, under dye-saturating conditions.

Examples of the pH-jump experiments performed to calibrate acridine orange-absorbance curves are shown in Fig. 2 *A*. The addition of vesicles, preloaded with buffer adjusted to pH values ranging from 4.4 to 6.9, into the unbuffered reaction mix resulted in a spike in absorbance reflecting the turbidity introduced with the vesicles. As acridine orange equilibrated inside and outside the vesicles according to vesicular pH, a new equilibrium was reached. The addition of concentrated MOPS-BTP buffer (pH 6.9 or 7.9) generated the pH gradients used for calibration, and the concomitant acridine-orange-quenching curves. At this point, the final concentrations of buffer, KCl, valinomycin and acridine orange were 50 mM, 150 mM, 0.5 μM and 17.5 μM, respectively. The pH gradients were collapsed with ammonium chloride (last portion of the curves in Fig. 2*A*) and the measured change in absorbance (ΔA_{495}) was correlated to the established ΔpH (Fig. 2*B*). The nonlinearity of the pH calibration curves may be due to proton leakage during the establishment of the pH jump, or alternatively, to limited dimerization of the dye. While decay of the pH gradient used for constructing the calibration curves would lead to an overestimation of the pH gradient established during the proton-pumping experiments, and therefore to an

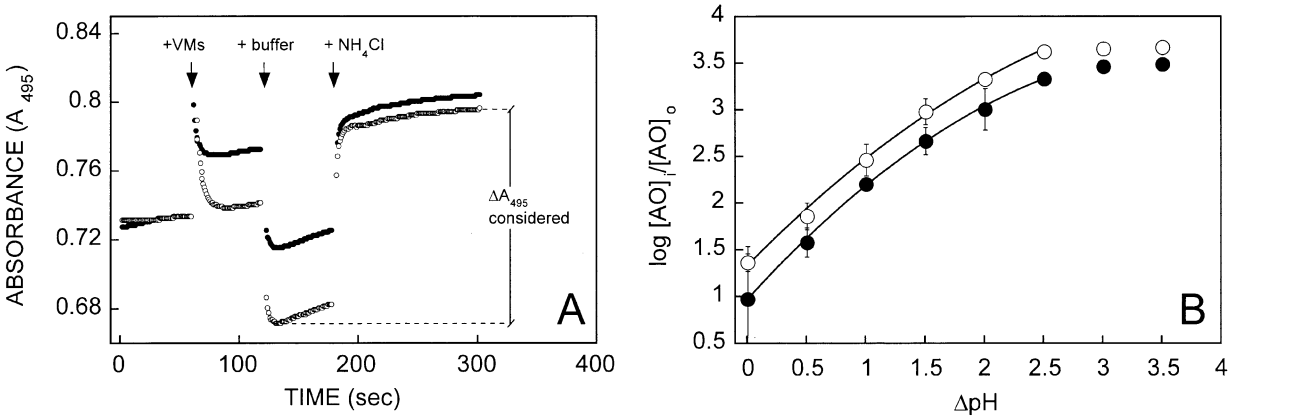


Fig. 2. Calibration of acridine orange accumulation in tonoplast-enriched vesicles from lemon fruits and epicotyls, in response to pre-imposed pH gradients. (*A*): Vesicles preloaded with buffer at pH 5.4 (○) and 5.9 (●) were diluted into unbuffered H^+ pumping reaction mix containing 17.5 μM acridine orange (+*VMs*). Concentrated MOPS-BTP buffer, pH 7.9, containing 17.5 μM acridine orange, was added to a final concentration of 50 mM (+*buffer*). 10 mM ammonium chloride was used to collapse the membrane potential ($+NH_4Cl$). (*B*): Calibration curves obtained for artificially imposed pH gradients in tonoplast-enriched vesicles from fruits (○) and epicotyls (●). Results obtained for the fruit vesicles represent the average of six independent experiments. The epicotyl results are the average of three independent experiments. The calibration curves are described by the following equations: $y = 0.212x^2 + 0.2360x - 0.2957$ (Epicotyl, *r*: 0.999) and $y = 0.1818x^2 + 0.1562x - 0.5044$ (Fruit, *r*: 0.998).

overestimation of the stoichiometry, dimerization of the dye would affect both the calibration and the experiments similarly, and thus would not affect the stoichiometry. The method fails at $\Delta pH > 2.5$, presumably because the vesicles do not maintain their integrity when resuspended in buffer with a pH significantly lower than pH 5. This suggests that the fruit tonoplast is asymmetric with respect to its pH stability, since in vivo it is able to function at pH 2.2 on its lumenal face. The offset of the fruit calibration curve relative to the epicotyl's amounts to 0.35 units of the $\log[AO]_i/[AO]_o$ scale. This indicates that, at any ΔpH, 2.2 times as many acridine orange molecules accumulate inside fruit vesicles than in epicotyl vesicles. Since the calibration curves were normalized for protein concentration, this suggests that epicotyl vesicles bear about twice as much protein per unit volume than fruit tonoplast vesicles.

Based on our findings, the internal buffering capacity of the vesicles was constant over a range of pH going from 5.15 to 6.49. Briskin & Reynolds-Niesman (1990) reported a constant value from pH 4.5 to 6.5 for red beet plasma membrane vesicles. Brauer et al. (1989) found a value that was relatively constant from pH 4.8 to 6.5 in maize root vesicles. Based on these combined results, we decided to use a constant value of B_i for the calculation of the proton transport rate.

The steady-state ΔpH, internal buffering capacity, and slip-plus-leakage rate were combined as previously described (Brauer et al., 1989; Schmidt and Briskin, 1993):

$$H^+ \text{ transport rate} = \Delta pH \cdot B_i \cdot k_i;$$

and the lemon fruit and epicotyl pump stoichiometries were calculated (Table 1). Note that, even though Table 1 presents averaged data, H^+/ATP stoichiometry values were determined independently for each proton pumping/ATP hydrolysis experiment, after extracting ΔpH, k_i and ATP-hydrolysis rate from individual proton-pumping and ATP-hydrolysis curves. The values shown for the fruit were obtained with 70 to 90% bafilomycin-sensitive preparations and in the calculation of the H^+-transport rate, it was thus assumed that 70 to 90% of the pH gradient built up in the fruit was due to the bafilomycin-sensitive pump. Proton transport by the epicotyl preparations was 100% bafilomycin-sensitive. For both, fruit and epicotyl vesicles, only the bafilomycin-sensitive ATP hydrolysis rates are reported. Under these conditions, the calculated H^+/ATP stoichiometries of the epicotyl and fruit V-ATPases were 2.10 ± 0.26 and 0.87 ± 0.16, respectively. Under the same conditions, the calculated H^+/ATP stoichiometry for 30 to 50% bafilomycin-sensitive fruit preparations was 0.43 ± 0.03. The meaning of this result is, however, questionable, because the pH gradient attributed to the bafilomycin-sensitive activity is only marginal. By assuming that the bafilomycin-sensitive pump is capable of establishing 100% of the pH gradient by itself, the calculated stoichiometry amounts to 1.01 ± 0.14.

To determine the possible effects of the electrical transmembrane potential, similar experiments were performed with epicotyl vesicles in the absence of valinomycin. Under these conditions, the initial rate of H^+-pumping was about half the rate found with the membrane potential collapsed by valinomycin (*data not shown*). However, the ΔpH at steady state remained unchanged (Table 1). The apparent stoichiometry of the epicotyl V-ATPase in the absence of

Table 1. H^+/ATP stoichiometries of the V-ATPases from lemon fruits and epicotyls, as determined by the steady-state ΔpH method

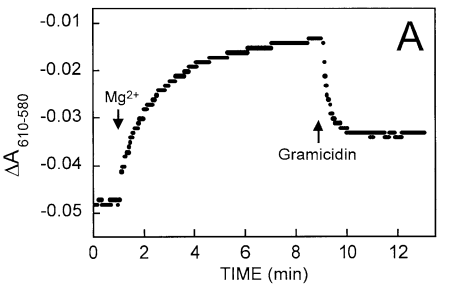
	Epicotyl		Fruit
	+Valinomycin	−Valinomycin	+Valinomycin
ΔpH	2.86 ± 0.10	2.97 ± 0.31	2.48 ± 0.10
B_i (μmol H^+ · (pH unit)$^{-1}$ · mg^{-1})	0.203 ± 0.023	0.203 ± 0.023	0.198 ± 0.027
k_i (min^{-1})	0.424 ± 0.113	0.199 ± 0.078	0.111 ± 0.025
H^+ transport (μmol · mg^{-1} · min^{-1})	0.247 ± 0.072	0.120 ± 0.052	0.055 ± 0.012
ATP hydrolysis (μmol · mg^{-1}min$^{-1)}$	0.116 ± 0.025	0.112 ± 0.033	0.063 ± 0.009
Stoichiometry	2.10 ± 0.26	1.11 ± 0.32	0.87 ± 0.16

The H^+/ATP stoichiometries of the bafilomycin-sensitive H^+-ATPases of lemon were determined based on the steady-state ΔpH established in tonoplast-enriched vesicles. ΔpH, internal buffering capacity (B_i), slip-plus-leakage rate (k_i), proton transport and ATP hydrolysis were calculated as described in Material and Methods. For epicotyl vesicles, measurements were performed in the presence or not of 0.5 μM valinomycin. ΔpH, k_i, ATP hydrolysis and H^+/ATP stoichiometries were determined independently for each experiment performed. Average values are shown.

valinomycin was 1.11 ± 0.32, about half the value found in the presence of valinomycin. In summary, based on steady-state measurements, the H^+/ATP stoichiometry of the epicotyl V-ATPase is 2 in the presence of valinomycin and 1 in the absence of valinomycin. The H^+/ATP stoichiometry of the fruit V-ATPase is 1 even in the presence of valinomycin.

Electrical Transmembrane Potential

Since the apparent H^+/ATP stoichiometry for the epicotyl V-ATPase was reduced in the absence of valinomycin, presumably due to the build-up of a membrane potential, we used oxonol VI absorbance to measure the electrical potential difference across tonoplast-enriched membranes from lemon fruits and epicotyls (Fig. 3). Assays were conducted with or without KCl and/or valinomycin. In the absence of valinomycin and KCl in the reaction mix, an electrical potential was built up within seconds across both fruit and epicotyl membranes (Fig. 3*A*). In the presence of 150 mM KCl, this membrane potential appeared to be only transient in epicotyl vesicles (Fig. 3*B*), as no recovery could be measured after addition of gramicidin (Fig. 3*C*). In general, the electrical transmembrane potential across fruit tonoplast membranes was three to seven times higher than that found in epicotyl vesicles, and was hardly affected by chloride. In the presence of both KCl and valinomycin, neither the fruit, nor the epicotyl tonoplast showed an electrical potential. These results indicate that the membrane potential of the fruit tonoplast is relatively insensitive to chloride, but can be abolished by valinomycin in the presence of potassium. Chloride has a much greater effect on the membrane potential of the epicotyl tonoplast, reducing it by about 60%. Moreover, in the presence of chloride, the membrane potential of the epicotyl tonoplast is only transient. As in the case of the fruit, valinomycin plus potassium completely abolishes the membrane potential across the epicotyl tonoplast.



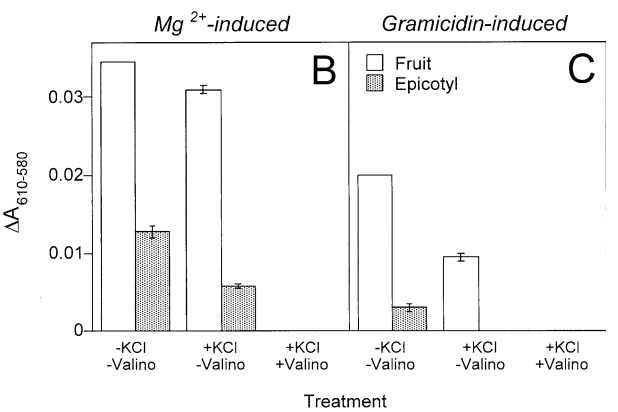


Fig. 3. Measurement of the electrical membrane potential across tonoplast-enriched vesicles from lemon fruits and epicotyls. 40 μg of vesicles were used in H^+-pumping reactions containing or not containing 150 mM KCl and 0.5 μM valinomycin. 2.5 μM of oxonol VI was used to monitor the membrane potential established during proton pumping. The reaction was started with magnesium and collapsed with 6 μM gramicidin. Absorbance changes of oxonol VI at 580 and 610 nm were measured simultaneously and the difference ($\Delta A_{610-580}$) is reported. (*A*): Typical curve obtained with fruit tonoplast vesicles. (*B* and *C*): Magnesium- and gramicidin-induced changes in $\Delta A_{610-580}$ for fruit and epicotyl vesicles under described conditions.

Initial-Rate Method

When steady-state pH gradients were investigated in the presence of 25 μg epicotyl vesicles or 35 μg fruit membranes, acridine orange remained limiting up to a

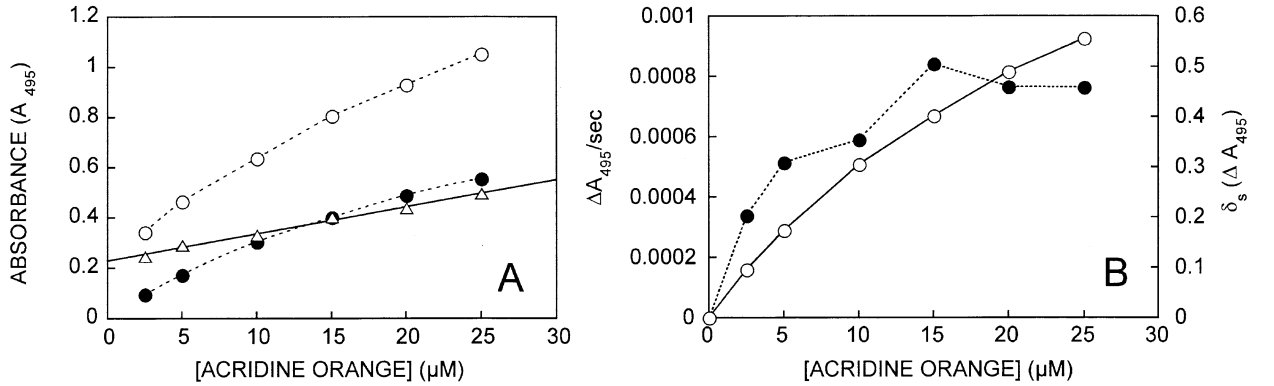


Fig. 4. Calibration of acridine orange accumulation in tonoplast-enriched vesicles from lemon fruits. 35 μg of fruit vesicles were added to an ATP-regenerating proton pumping-reaction mix containing 2.5 to 25 μM of acridine orange. The H^+ pumping reaction was started with magnesium, and the decrease in acridine orange absorbance at 495 nm was measured. (*A*) Initial acridine orange absorbance before the start of the H^+ pumping reaction (○); absorbance of quenched acridine orange at steady state (Δ) and difference between the previous two (vesicular acridine orange content, δ_s) (●). (*B*) Initial rate of acridine orange quenching (●) and total acridine orange quenching at steady state (δ_s) (○).

Table 2. H^+/ATP stoichiometries of the V-ATPases from lemon fruits and epicotyls, as determined by the initial-rate method

	Epicotyl		Fruit	
	+Valinomycin	−Valinomycin	Low-coupling prep.	High-coupling prep.
H^+ pumping (μmol · mg^{-1} · min^{-1})	0.325 ± 0.055	0.215 ± 0.076	0.215 ± 0.076	0.169 ± 0.050
ATP hydrolysis (μmol · mg^{-1} · min^{-1})	0.161 ± 0.016	0.157 ± 0.041	0.068 ± 0.005	0.086 ± 0.027
Stoichiometry	2.02 ± 0.36	1.35 ± 0.13	0.94 ± 0.19	2.00 ± 0.22

20 μg and 35 μg of tonoplast-enriched vesicles from epicotyls and fruits, respectively were used in all measurements.
Measurements for epicotyl vesicles were done in the presence or not of 0.5 μM valinomycin, and fruit preparations with high and low initial-rate coupling ratios (IRCR) were compared.

concentration of ~25 μM. On the other hand, as little as 15 μM were saturating when measuring initial rates of proton pumping (Fig. 4*B*). Therefore, a concentration of 17.5 μM acridine orange was chosen to determine the stoichiometries of the lemon fruit and epicotyl V-ATPases by the initial rate method. Under these conditions, the acridine orange quenching rate, as determined from the δ_s curve in Fig. 4*B*, was found to be 0.55 $\Delta A_{495} \cdot \mu M^{-1} \cdot$ mg protein^{-1} and 0.49 $\Delta A_{495} \cdot \mu M \cdot$ mg protein^{-1} for epicotyl and fruit vesicles respectively. The nonlinearity of the δ_s curve is described in Fig. 4*A*. Note that the A_{495} curve at time zero follows the same nonlinearity as the curve describing ΔpH at steady state (δ_s curve). In contrast, the quenched acridine orange curve (A_{495} at steady state) is linear with respect to the concentration of acridine orange used. The linearity of the steady-state curve indicates that all of the acridine orange is quenched inside the vesicles at steady state, whereas the nonlinearity of the δ_s curve is merely a consequence of the nonlinearity of the A_{495} curve at time zero. The latter curve departs from linearity due to partial quenching of acridine orange in the reaction mix at high concentrations, presumably as a consequence of dimerization of the dye. It is H^+ pumping-independent.

Table 2 shows the results of H^+/ATP-stoichiometry calculations based on the initial-rate method. Epicotyl tonoplast vesicles were compared with two different types of lemon-fruit preparations: nitrate-insensitive preparations with a low coupling ratio (harvested between September and December) and nitrate-sensitive preparations with a high coupling ratio (harvested between January and April) (Müller et al., 1999). The nitrate-sensitive fruit preparations exhibited ~70% inhibition of proton pumping by bafilomycin. The results show that both the epicotyl and the high-coupling-ratio fruit H^+/ATPases have an H^+/ATP stoichiometry of 2, while the nitrate-insensitive, low-coupling-ratio fruit preparations have an H^+/ATP stoichiometry of ~1. When epicotyl membranes were assayed in the absence of valinomycin in the reaction mix, the resulting stoichiometry was decreased to nearly half its value in the presence of valinomycin. Thus, the absence of valinomycin lowers the H^+/ATP stoichiometry of the epicotyl V-ATPase from 2 to 1, whether measured by the steady-state or initial-rate methods. Using the initial-rate method, the calculated H^+/ATP stoichiometry of the nitrate-sensitive, high-coupling-ratio fruit V-ATPase was 2, while that

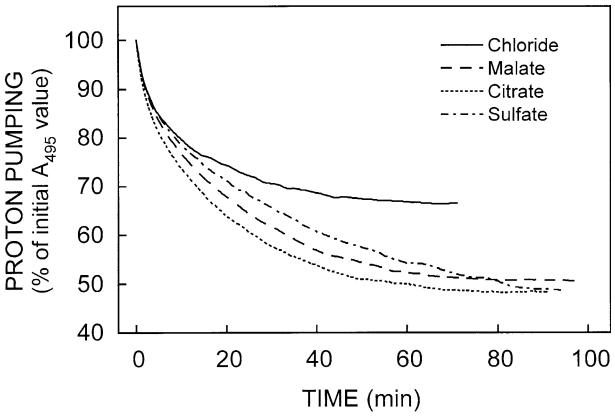


Fig. 5. Effect of intra-vesicular organic acids on the pH gradient established across the tonoplast of lemon-fruit vesicles. Vesicles were preloaded with 150 mM potassium chloride (———), 100 mM potassium sulfate (–·–·) 100 mM potassium malate (— — —), or 75 mM potassium citrate (· · · ·). Osmolarity of the added salt was constant at 300 mosmol. 11 μg vesicles were used in proton pumping reactions monitored by the absorbance quenching of acridine orange as described in Material and Methods.

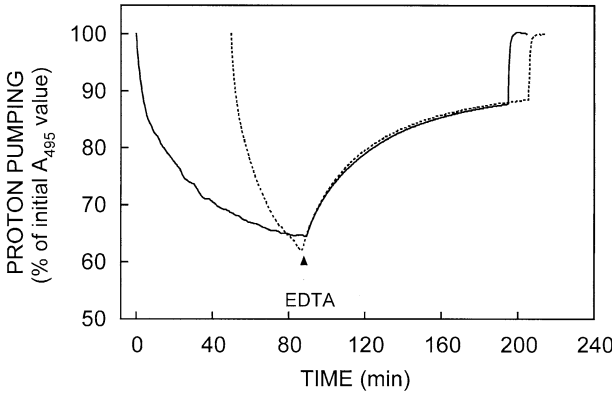


Fig. 6. Effect of intra-vesicular citrate on the proton leakage rate across the membrane of tonoplast-enriched lemon fruit vesicles. Conditions were as described in the legend to Fig. 5, with vesicles preloaded with potassium chloride (——), and potassium citrate (· · · ·). Once a pH gradient was established, the reaction was stopped by chelating the magnesium required for catalysis with 50 mM EDTA, and the passive proton leakage across the vesicular membrane was monitored. Eventually, the remaining proton gradient was collapsed with nigericin.

of the nitrate-insensitive, low-coupling-ratio fruit V-ATPase was 1.

Effect of Organic Acids

In preliminary experiments it was established that substituting K_3-citrate or K_2-malate for KCl in the H^+-pumping reaction mix, thereby supplying an organic anion on the *outside* of the vesicles, affected neither the rate of proton pumping, nor the pH gradient at steady state (Müller and Taiz, unpublished data). To determine the effect of organic anions supplied on the *lumenal face* of the vacuolar membrane, thus mimicking the in vivo condition in organic acid-accumulating vacuoles, tonoplast-enriched vesicles were preloaded with potassium malate, potassium citrate, potassium sulfate, or potassium chloride. To avoid an artifactual osmotic pressure effect, the concentration of the salts were all adjusted to 300 mosmolar. The ability of the preloaded membranes to generate a MgATP-dependent pH gradient was measured by recording the absorbance quenching of acridine orange. In the presence of equal amounts of protein (~11 mg), malate-, citrate- and sulfate-loaded vesicles all generated a higher ΔpH than chloride-loaded vesicles (Fig. 5). In these experiments, the H^+-pumping reaction was started with the vesicles, with MgATP already present in the reaction mix. Therefore the initial rate of proton pumping can be determined less reliably than with membranes pre-equilibrated in the reaction mix. Nevertheless, the order of effectiveness of the tested anions appeared to be citrate > malate > sulfate > chloride, based on the H^+-pumping rates after the first 5 minutes.

To test whether the higher pH gradient built up in citrate-loaded vesicles was due to reduced proton leakage rather than to increased pumping, EDTA was used to chelate Mg^{2+} and inhibit the V-ATPase after a pH gradient was established (Fig. 6). It was found that the stimulatory effect of citrate was not due to proton leakage.

To explore the mechanism of the stimulation of H^+ pumping by organic anions, the effect of lumenal citrate on the slip rate was determined in both fruit and epicotyl membranes (Tu et al., 1987, Müller et al. 1997). The vesicles were loaded with 150 mM KCl, or with 50 mM K_3-citrate plus 100 mM sorbitol, in order to balance both, cationic and anionic charges, and the osmolarity. In both the fruit and the epicotyl vesicles, the rate of ATP hydrolysis was unchanged by the presence of citrate in the lumen (Fig. 7*C–D*). Note the biphasic ATP-hydrolysis rate in fruit vesicles, a consequence of the progressive coupling of the enzyme. Although the initial rate of proton pumping in citrate-loaded fruit vesicles was found to be ~14% lower than in chloride-loaded vesicles (Fig. 7*A*), the *initial* slip rate was drastically lower in citrate-loaded than in chloride-loaded fruit vesicles, resulting in more efficient coupling of the pump when catalysis proceeded (Fig. 8). In citrate-loaded epicotyl vesicles, the rate of proton pumping was slightly increased (~15%) and the slip-plus-leakage rate slightly decreased (less that 3% over the initial 10 minutes of pumping) (Fig. 7*B,* Fig. 8). Lumenal citrate increased the total quench by ~25% in both epicotyl and fruit vesicles (Fig. 7*A–B*). Therefore, in both the fruit and the epicotyl vesicles, citrate on the inside of the vesicles resulted in tighter coupling of the V-ATPase and allowed the generation of a steeper pH gradient.

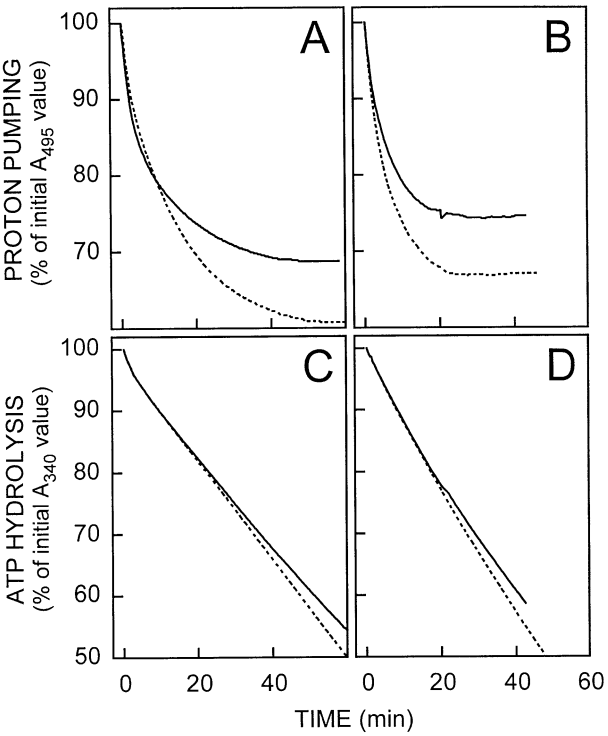


Fig. 7. Effect of intra-vesicular citrate on the proton-pumping and ATP-hydrolysis activities of tonoplast-enriched vesicles from lemon fruits and epicotyls. Vesicles were preloaded with 150 mM potassium chloride (——) or 50 mM potassium citrate plus 100 mM sorbitol (– – – –), in order to maintain osmolarity and potassium concentration constant at 300 mosmol and 150 mM respectively. (*A* and *B*) Proton-pumping curves; (*C* and *D*) ATP-hydrolysis curves. Results for fruit vesicles are reported in (*A*) and (*C*) and results for epicotyl vesicles are reported in (*B*) and (*D*). 11 μg membrane protein was used in all cases.

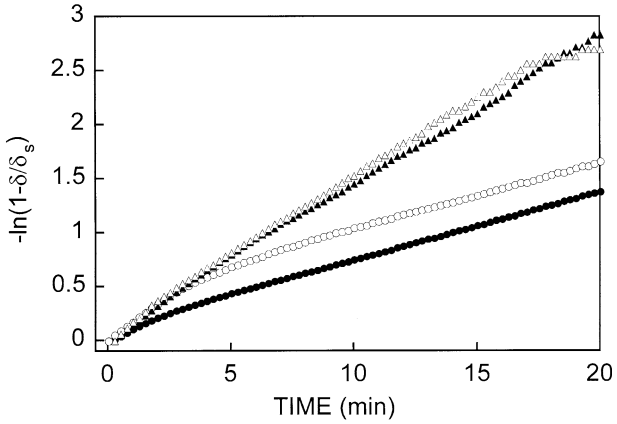


Fig. 8. Effect of intra-vesicular citrate on the slip-plus-leakage rates of tonoplast-enriched vesicles from lemon fruits and epicotyls. The slip-plus-leakage curves corresponding to the reactions described in Fig. 7 were calculated according to Tu et al. (1987) for epicotyl vesicles preloaded with chloride (Δ) or with citrate (▲); or for fruit vesicles preloaded with chloride (○) or citrate (●).

Discussion

Briskin & Reynolds-Niesman (1990) proposed that H^+/ATP stoichiometries of proton pumps should be determined by more than one technique in order to overcome the sources of error inherent to any given method. We used two kinetic methods, one based on initial rates of H^+-pumping and ATP hydrolysis and one based on the mathematical analysis of the steady-state ΔpH, to determine the H^+/ATP stoichiometries of the lemon-fruit and epicotyl V-ATPases. The steady-state ΔpH method has previously been successfully applied to stoichiometry determinations (Brauer et al., 1989; Briskin & Reynolds-Niesman, 1990; Schmidt & Briskin, 1993), and its limitations extensively described (Briskin & Reynolds-Niesman, 1990). The initial-rate method was developed by Palmgren (1990) for the simultaneous measurement of proton-pumping and ATP-hydrolysis rates for studies on the plasma membrane H^+-ATPase of *Avena sativa*. Since in his experimental system the coupling of proton pumping to ATP hydrolysis was lower than predicted, he concluded that the intravesicular accumulation of anions artificially amplified the quenching of acridine orange, thus making the method unsuitable for quantifying proton transport (Palmgren, 1990; 1991). In the present report, we demonstrated that the method can be used successfully to determine the stoichiometry of the vacuolar H^+/ATPase, provided the influence of anions is reduced by the presence of valinomycin and excess potassium, and that the hydrolytic activity of the V-ATPase is isolated from other contaminating ATPases by considering only the bafilomycin A_1-sensitive activity present on the vesicles.

We previously established that hyperacidification of lemon juice-sac vacuoles is correlated with several unique properties of the fruit tonoplast membrane and V-ATPase. The tonoplast of fruit vacuoles is less permeable to protons than that of the epicotyl, allowing the vacuole to maintain a steeper pH gradient (Müller et al., 1996). The native fruit V-ATPase is also less sensitive to a variety of V-ATPase inhibitors than the epicotyl V-ATPase, including nitrate, bafilomycin A_1, and oxidation (Müller et al., 1996). Insensitivity to inhibitors suggests that kinetic regulation of the enzyme may be minimal, enabling the V-ATPase to approach thermodynamic equilibrium. Finally, we found that the fruit tonoplast V-ATPase can exist in two states making up two sub-populations of V-ATPases, a nitrate-sensitive (normal) and a nitrate-insensitive (altered) population (Müller et al., 1999). The two populations differ in their initial-rate H^+/ATP coupling ratios (IRCRs). Since low IRCR and high IRCR fruit V-ATPases established equilibrium pH gradients of the same magnitude, we hypothesized that the two sub-populations of V-ATPases were probably characterized by equal H^+/ATP stoichiometries (Müller et al., 1999). While the

two kinetic methods used in the present report resulted in a stoichiometry of 2 for the epicotyl V-ATPase, the findings were more ambiguous for the fruit enzyme. Fruit preparations with a low IRCR were found to have a stoichiometry of ~1 as determined by either method. In contrast, the stoichiometry of fruit preparations with a high IRCR was found to be 2 when measured by the initial rate method, and ~1 when calculated by the steady-state model. This apparent contradiction can be explained if one considers how the stoichiometries were determined. In the initial-rate method, the pump operates in the absence of an electrical potential (in the presence of valinomycin and KCl) and in the presence of a limited pH gradient. In contrast, in the steady-state method, the proton transport rate is extrapolated from the pH gradient at steady state, taking into account the pH-dependent slip-plus-leakage rate. Combined, the stoichiometry results derived from the two models suggest that the H^+/ATP coupling ratio of the high IRCR fruit V-ATPase is progressively reduced during the establishment of a pH gradient. Rather than undergo slip (the slip-plus-leakage rate remains low), the fruit V-ATPase appears to be capable of variable stoichiometry. Since the maximum steady-state ΔpH is inversely related to the pump's stoichiometry, a variable stoichiometry mechanism may prove beneficial to the fruit V-ATPase, allowing it to attain the extreme pH gradients that are present across the tonoplast of ripe fruits. This confirms and extends the findings of Davies et al. (1994) who, based on patch-clamp studies, proposed a ΔpH-induced variable stoichiometry, with values comprised between 1.74 and 3.28, for the V-ATPase of red beet. Recently, the results of Davies et al. have received partial support from the patch-clamp studies of Yabe et al. (1999) who found an H^+/ATP stoichiometry of 3.5 for the V-ATPase of yeast.

Since ΔpH induced a decrease in the stoichiometry of the fruit V-ATPase, we examined whether the presence of an electrical transmembrane potential would also result in a reduction in H^+/ATP stoichiometry. The epicotyl V-ATPase is more sensitive to electrical membrane potentials than the fruit V-ATPase, which is hardly affected by the absence of valinomycin (*data not shown*). In the absence of valinomycin (presence of a limited membrane potential), the apparent stoichiometry of the epicotyl V-ATPase was found to be 1.11 ± 0.32 when calculated by the steady-state ΔpH method, and 1.35 ± 0.13 when calculated by the initial-rate method. Thus, in contrast to the ΔpH-induced variable stoichiometry measured in the fruit V-ATPase, the reduction in stoichiometry measured for the epicotyl V-ATPase in the presence of a limited transmembrane potential occurs as soon as catalysis proceeds. This can easily be explained by the fact that the membrane potential is built up during the initial seconds of proton pumping, in contrast to the pH gradient, which forms over several minutes.

A surprising finding was that the steady-state ΔpH generated by the epicotyl V-ATPase was the same in the presence or the absence of valinomycin, and under no conditions was the calculated ΔpH attributed to the bafilomycin-sensitive fruit V-ATPase larger than that built up in epicotyl vesicles. This was particularly intriguing because, for a fixed protein concentration, acridine orange-absorbance quenching in fruit vesicles was usually double that observed with epicotyl vesicles. From the ΔpH calibration curves, however, it is evident that for a given pH gradient and protein concentration, twice as many protons accumulate in fruit tonoplast vesicles than in epicotyl vesicles. If the fruit and the epicotyl vesicles have the same size, this would indicate that fruit vesicles bear two times less protein than epicotyl vesicles. Moreover, it can be assumed, based on western blotting, that fruit tonoplast vesicles bear fewer V-ATPases/mg protein than epicotyl vesicles (Müller et al., 1996). Nevertheless, based on the lower slip rate and the higher ΔpH that was attained with purified V-ATPases reconstituted in artificial vesicles (Müller et al., 1997), the maximum ΔpH established by the fruit V-ATPase was still predicted to be higher than that built up by the epicotyl enzyme. As this was not verified with the native tonoplast vesicles used in the present study, we conclude that the limiting factor in proton-pumping experiments with isolated tonoplast vesicles is not the proton pump itself, but other factors such as proton leakage and secondary transport systems present on the native membrane.

In vivo, hyperacidification of plant vacuoles has been correlated with organic acid accumulation, with the pK_a of the accumulated acid determining the pH of the vacuolar lumen. Thus, increasingly acidic vacuoles accumulate anions in the following order: Cl^--malate^{2-}citrate^{3-} oxalate^{2-}. Acidic limes accumulate much more citrate than sweet limes, further suggesting that citrate accumulation plays a role in acidification (Brune et al., 1998). We have shown that, when compared to chloride, citrate on the lumenal side of the vacuolar membrane decreases the slip rate of the fruit V-ATPase. The rate of catalysis is not significantly affected. From a mechanochemical perspective, the resulting increase in the rate of proton pumping can be thought of as an increase in the rate of rotation of the V_o complex (Grabe et al., 2000). Grabe et al. (2000) postulated the requirement for a "brake" subunit that prevents reverse rotation of the rotor (and consequent proton leakage) when the V_1 complex is dissociated from V_o. It is possible that such a brake complex would exert drag on the rotor even when V_1 is attached, inducing slip in the enzyme. The presence of organic acids on the lumenal face of the V-ATPase may reduce that drag, resulting in higher pump efficiency.

Although organic acids may be required for hyperacidification, V-ATPascs could not lower the lumenal pH to 0.9, as in *Begonia lucerna,* unless the stoichiometry of the pump is < 2. Rastogi and Girvin (1999) have shown that the proteolipid (c subunit) has a different conformation at acidic pH than under basic conditions, with the protonatable rotor site buried within the rotor at pH ~5, but exposed at the rotor periphery at pH ~8. Thus, at acidic pH, proton-binding sites could be withdrawn from participation in proton exchange. This would, in effect, lower the H^+/ATP stoichiometry of the pump (Grabe et al., 2000). According to our findings with the lemon epicotyl and fruit enzymes, V-ATPases may differ in their ability to modify the proteolipid conformation and thus their ability to use variable H^+/ATP stoichiometry.

In summary, our studies to date have established the following factors as contributors to the phenomenon of hyperacidification in lemon vacuoles: 1) a proton-impermeant membrane; 2) a V-ATPase that is refractory to various types of kinetic inhibition; 3) a V-ATPase with a low slip rate; 4) a V-ATPase with the ability to reduce its stoichiometry from 2 to 1 in the presence of a pH gradient; 5) organic acids in the lumen to reduce slip at low internal pH.

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References

Barkla, B.J., Pantoja, O. 1996. Physiology of ion transport across the tonoplast of higher plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **47:**159–184

Bennett, A.B. Spanswick, R.M. 1983. Optical measurements of ΔpH and $\Delta\psi$ in corn root membrane vesicles: kinetic analysis of cl^- effects on a proton-translocating ATPase. *J. Membrane Biol.* **71:**95–107

Bennett, A.B. Spanswick, R.M. 1984. H^+-ATPase activity from storage tissue of *Beta vulgaris. Plant Physiol.* **74:**545–548

Bowman, B.J., Bowman, E.J. 1996. Mitochondrial and vacuolar ATPases. *In*: The Mycota III. Biochemistry and Molecular Biology. R. Brambl and G.A. Marzluf, editors, pp. 57–83. Springer-Verlag, Berlin

Brauer, D., Tu, S.-L, Hsu, A.-F., Thomas, C.E. 1989. Kinetic analysis of proton transport by the vanadate-sensitive ATPase from maize root microsomes. *Plant Physiol.* **89:**464–471

Briskin, D.P., Reynolds-Niesman, I. 1990. Determination of H^+/ATP stoichiometry for the plasma membrane H^+-ATPase from red beet (*Beta vulgaris L.*) storage tissue. *Plant Physiol.* **95:**242–250

Brune, A., Gonzalez, P.C., Goren, U., Zehavi, R., Echeverria, E. 1998. Citrate uptake into tonoplast vesicles from acid lime (*Citrus aurantifolia*) juice cells. *J. Membrane Biol.* **166:**197–203

Cross, R., Taiz., L. 1990. Gene duplication as a means for altering H^+/ATP ratios during the evolution of F_0F_1 ATPases and synthases. *FEBS Lett.* **259:**227–229

Davies, J.M., Hunt, I., Sanders, D. 1994. Vacuolar H^+-pumping ATPase variable transport coupling ratio controlled by pH. *Proc. Natl. Acad. Sci. USA* **91:**8547–8551

Davies, J.M., Poole, R.J., and Sanders, D. 1993. The computed free energy change of hydrolysis of inorganic pyrophosphate and ATP: apparent significance for inorganic pyrophosphate-driven reactions of intermediary metabolism. *Biochim. Biophys. Acta* 1**141:**29–36

Elston, T., Wang, H., Oster, G. 1998. Energy transduction in ATP synthase. *Nature* **91:**510–514

Finbow, M.E., Harrison, M.A. 1997. The vacuolar H^+-ATPase: a universal proton pump of eukaryotes. *Biochem. J.* **324:**697–712

Forgac, M. 1989. Structure and function of the vacuolar class of ATP driven pumps. *Physiol. Rev.* **69:**765–796

Forgac, M. 1999. Structure and properties of the vacuolar H^+-ATPases. *J. Biol. Chem.* **274:**12951–12954

Gluck, S.L., Underfill, D.M., Iyori, M., Holliday, L.S., Kostrominova, T.Y. 1996. Physiology and biochemistry of the kidney vacuolar H^+-ATPase. *Ann. Rev. Physiol.* **58:**427–445

Grabe, M., Wang, H., Oster, G. 2000. The mechanochemistry of V-ATPase proton pumps. *Biophys. J.* **78:**2796–2813

Lüttge, U., Ratajczak, R. 1997. The physiology, biochemistry and molecular biology of the plant vacuolar ATPase. *In:* Advances in Botanical Research Vol. 25. The Plant Vacuole. R.A. Leigh and D. Sanders editors, pp. 253–296. Academic Press, Oxford

Maloney, P.C. 1979. Membrane conductance *of Streptacoccu Iactis. J. Bacteriol.* **140:**197–205

Moriyama, Y., Nelson, N. 1989. Cold inactivation of vacuolar H^+-ATPases. *J. Biol. Chem.* **264:**3577–3582

Müller, M.L., Irkens-Kiesecker, U., Kramer, D., Taiz, L. 1997. Purification and reconstitution of the vacuolar H^+-ATPases from lemon fruits and epicotyls. *J. Biol. Chem.* **272:**12762–12770

Müller, M.L., Irkens-Kiesecker, U., Rubinstein, B., Taiz, L. 1996. On the mechanism of hyperacidification in lemon. *J. Biol. Chem.* **271:**1916–1924

Müller, M.L, Jensen, M., Taiz, L. 1999. The vacuolar H^+-ATPase of lemon fruit is regulated by variable H^+/ATP coupling and slip. *J. Biol. Chem.* **274:**10706–10716

Nelson, N. 1991. Structure and pharmacology of the proton-ATPases. *Trends Pharmacol. Sci.* **12:**71–75

Nelson, N., Taiz, L. 1991. The evolution of H^+-ATPases. *Trends Biochem. Sci.* **14:**113–116

Nørby, J.G. 1988. Coupled assay of Na^+, K^+-ATPase activity. *Methods Enzymol.* **156:**116–119

Palmgren, M.G. 1990. An H^+-ATPase assay: proton pumping and ATPase activity determined simultaneously in the same sample. *Plant Physiol.* **94:**882–886

Palmgren, M.G. 1991. Acridine orange as a probe for measuring pH gradients across membranes: mechanism and limitations. *Anal. Biochem.* **192:**316-321

Poole, R.J., Melhorn, R.J., Racker, L. 1985. *In:* Biochemistry and Function of Vacuolar Adenosine-Triphosphatase in Fungi and Plants. B. Marin editor pp.114–118. Springer-Verlag, Berlin

Popov, N., Schmitt, M., Matthies, H. 1975. Eine störungsfreie Mikromethode zur Bestimmung des Proteingehaltes in Gewebehomogenaten. *Acta Biol. Med. Ger.* **34:**1441–1446

Rastogi, V., Girvin, M. 1999. Structural changes linked to proton translocation by subunit c of the ATP synthase. *Nature* **402:**263–268

Rea, P.A., Sanders, D. 1987. Tonoplast energization: two H^+ pumps, one membrane. *Physiol. Plant* **71:**131–141

Schmidt, A.L., Briskin, D.P. 1993. Energy transduction in tonoplast vesicles from red beet (*Beta vulgaris L.*) storage tissue: H^+ substrate stoichiometries for the H^+-ATPase and H^+-PPase. *Arch. Biochem. Biophys.* **301:**165–173

Sze, H. 1985. H^+-translocating ATPases: advances using membrane vesicles. *Annu. Rev. Plant. Physiol.* **36:**175–208

Taiz, L. 1992. The plant vacuole. *J. Exp. Biol.* **172:**113–122

Taiz, L., Nelson, N. 1996. Evolution of V- and F-ATPases. *In:* Origin and Evolution of Biological Energy Conversion. H. Baltscheffsky, editor, pp. 291–305. VCH Verlagsgesellschaft, Germany

Taiz. L., Zeiger, E. 1998. Solute transport. *In:* Plant Physiology. L. Taiz and E. Zeiger, editors, pp. 125–152. Sinauer Associates, Sunderland

Tu, S.-L, Nagahashi, G., Brouillette, J.N. 1987. Proton pumping kinetics and origin of nitrate inhibition of tonoplast-type H^+-ATPase. *Arch. Biochem. Biophys.* **256:**625–637

Yabe, L, Horiuchi, K., Nakahara, K., Hiyama, T., Yamanaka, T., Wang, P.-C., Toda, K., Hirata, A., Ohsumi, Y., Hirata, R., Anraku, Y., Kusaka, L 1999. Patch clamp studies on V-type ATPase of vacuolar membrane of haploid *Saccharomyces cerevisiae*. *J. Biol. Chem.* **274:**34903–34910