The vacuolar $H^+\text{-ATPase}$ of lemon fruits is regulated by variable $H^+/ATP$ coupling and slip*

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Lemon fruit tonoplasts, unlike those of seedling epicotyls, contain nitrate-insensitive $H^+\text{-ATPase}$ activity (Müller, M. L., Irkens-Kieseker, U., Rubinstein, B., and Taiz, L. (1996) J. Biol. Chem. 271, 1916–1924). However, the degree of nitrate-insensitivity fluctuates during the course of the year with a seasonal frequency. Nitrate uncouples $H^+$ pumping from ATP hydrolysis both in epicotyls and in nitrate-sensitive fruit V-ATPases. Neither bafilomycin nor oxidation cause uncoupling. The initial rate $H^+\text{/ATP}$ coupling ratios of epicotyl and the nitrate-sensitive fruit proton pumping activities are the same. However, the $H^+\text{/ATP}$ coupling ratio of the nitrate-insensitive fruit $H^+$ pumping activity is lower than that of nitrate-sensitive and epicotyl $V$-ATPases. Several properties of the nitrate-insensitive $H^+\text{-ATPase}$ of the fruit indicate that it is a modified $V$-ATPase rather than a P-ATPase: 1) insensitivity to low concentrations of vanadate; 2) it is initially strongly uncoupled by nitrate, but regains coupling as catalysis proceeds; 3) both the nitrate-sensitive and nitrate-insensitive fruit $H^+$-pumps have identical $K_m$ values for MgATP, and show similar pH-dependent slip and proton leakage rates. We conclude that the ability of the juice sac $V$-ATPase to build up steep pH gradients involves three factors: variable coupling, i.e. the ability to regain coupling under conditions that initially induce uncoupling; a low pH-dependent slip rate; the low proton permeability of the membrane.

The vacuolar $H^+\text{-ATPases}$ (V-ATPases) of eukaryotic cells are large, multimeric proton pumps composed of 10 to 13 different subunits organized into a hydrophilic catalytic complex, $V_1$, and a hydrophobic transmembrane $H^+$ channel, $V_o$ (1). V-ATPases are structurally related to the ATP synthases, or F-ATPases, of mitochondria and chloroplasts. F- and V-ATPases exhibit many structural and functional similarities at the molecular level, including several homologous subunits which play central roles in catalysis and proton transport (2, 3). Because of these similarities in structure and catalytic mechanism, F- and V-ATPases are also thought to exhibit similar mechanoochemical properties (4). When operating in ATP synthesis mode, F-ATPases convert the proton-motive force present across the internal membrane of chloroplasts and mitochondria into a rotary torque used to drive the synthesis of ATP by the catalytic $F_o$ sector (5, 6). Conversely, when F-ATPases operate in proton pumping mode, they hydrolyze ATP at their catalytic site and convert the liberated energy into a rotary torque utilized to drive proton transport across the membrane (7). V-ATPases are also thought to operate via a rotary mechanism, even though direct experimental evidence for rotation is lacking (1).

In V- and F-ATPases, the efficiency of coupling between ATP hydrolysis and proton transport represents a critical, rate-limiting factor. Both pumps have been proposed to undergo slip, or intrinsic uncoupling, under specific conditions (8–10). During slip, ATP is hydrolyzed at the catalytic site without being coupled to $H^+$ transport. Since V-ATPases are known to operate far from thermodynamic equilibrium, slip may represent one of the enzyme’s regulatory mechanisms.

In lemon fruit juice sac cells, the vacuolar pH can reach as low as 2.2, about 3 pH units lower than in typical plant vacuoles. In fact, the trans-tonoplast pH gradient in lemon fruits is close to the calculated maximum for a V-ATPase operating at thermodynamic equilibrium (4 to 5 pH units) assuming an $H^+/ATP$ stoichiometry of 2 (11, 12). We have previously shown that proton pumping by tonoplast-enriched juice sac vesicles is largely insensitive to the V-ATPase inhibitors nitrate, bafilomycin $A_1$, and partially sensitive to high vanadate concentrations (50 to 300 $\mu$M) (13). The proton pumping activity of juice sac vesicles is also less sensitive to oxidation and N-ethylmaleimide (NEM) than that of tonoplast-enriched vesicles from seedling epicotyls. In addition, cold inactivation in the presence of nitrate, which completely inhibits proton transport in epicotyl vesicles, has little effect on the $H^+$ pumping activity of juice sac vesicles, even though the treatment induces the release of similar proportions of catalytic subunits from both types of vesicles. Since $V_1$ dissociation treatment increased the sensitivity of the juice sac $H^+$ pumping activity to vanadate, we hypothesized that a second, vanadate-sensitive proton pump, possibly possessing a lower $H^+/ATP$ stoichiometry that enables it to generate a steeper pH gradient than the V-ATPase, may be present on the tonoplasts of juice sac cells (13).

After purification and reconstitution into artificial proteoliposomes, both the epicotyl and juice sac proton pumps exhibited equal sensitivities to nitrate, bafilomycin $A_1$, NEM, and oxidation. Thus, the insensitivity of the juice sac $H^+$ pumping activity to these inhibitors appears to depend on some component(s) of the native membrane. However, purified and reconstituted fruit V-ATPases remained partially sensitive to vanadate and exhibited only half as much slip as the epicotyl V-ATPase (14). Slip was calculated from proton pumping curves, in the absence of an electric potential gradient, by the method of Tu et al. (15). Under these conditions, slip reflects...
intrinsic uncoupling induced by the build-up of a pH gradient across the membrane. This method gives no indication on the H⁺/ATP coupling ratio of the pump in the absence of a pH gradient.

H⁺/ATP coupling ratios provide a measure of pump efficiency (16). If two pumps have the same H⁺/ATP stoichiometry, but differ in their coupling ratios, the pump with the higher H⁺/ATP coupling ratio would be expected to generate a steeper ΔpH than the pump with the lower coupling ratio. Thus the coupling ratio of the lemon fruit V-ATPase might be higher than that of the epicotyl V-ATPase even if the two pumps had the same stoichiometry. To test this hypothesis, we have carried out experiments to characterize the coupling ratios of the fruit and epicotyl V-ATPases under initial rate conditions. Moreover, since the H⁺ pumping activity of the fruit is relatively insensitive to V₁ dissociation, we also determined the effect of V₁ dissociation treatment on the coupling ratio of the juice sac proton pump(s). Our results show that, in contrast to pH-dependent slip, the H⁺/ATP coupling ratios of the juice sac and epicotyl proton pumps, determined under initial rate conditions, are the same. Whereas V₁ dissociation with nitrate induced the complete uncoupling of the epicotyl V-ATPase, only a fraction of the fruit H⁺ pumping activity was uncoupled under identical conditions. However, the kinetic properties of the juice sac proton pumps after V₁ dissociation treatment were identical to those in the control vesicles. This strongly suggests that in juice sac vesicles, the residual proton pumping activity after V₁ dissociation treatment belongs to a nitrate-resistant subpopulation of V-ATPases, rather than to a different type of proton pump.

A 20 month analysis of the nitrate sensitivity of tonoplast-enriched vesicles from juice sacs showed that the proportion of nitrate resistant activity varied during the course of the year. As the proportion of uncoupled enzymes varies, the overall H⁺/ATP coupling ratio measured in juice sac vesicles changes. The significance of this type of variable H⁺/ATP coupling in lemon fruits is unclear, but the nitrate resistant activity may represent a specialized group of V-ATPases which, under extreme conditions, is able to maintain, if not build up, the large ΔpH across the tonoplast of juice sacs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lemon seeds (Citrus limon L. var. Schaub Rough Lemon) for growing seedling epicotyls were generously supplied by Willits & Newcomb, Inc., Arvin, CA. 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. All other chemicals were purchased from Sigma or Fisher.

**Membrane Preparation**—Tonoplast-enriched membranes from lemon fruit juice sacs and epicotyls were prepared as described previously (14). Fruit juice sacs were homogenized in fruit homogenization buffer (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 phenylmethylsulfonyl fluoride) and epicotyls were homogenized in epicotyl homogenization buffer (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 phenylmethylsulfonyl fluoride). After a first centrifugation of the homogenates for 20 min at 12,000 × g, the supernatants were subjected to ultracentrifugation for 60 min at 132,000 × g, and the microsomal pellets obtained were further 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 RB (10 mM BTP-Mes, pH 7.0, 20 mM KCl, 1 mM EDTA, 2 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride), and pelleted for 20 min at 174,000 × g. The tonoplast-enriched membranes were resuspended in RB at a final concentration of ~5 μg of membrane protein/μl.

**V₁ Dissociation**—Membrane vesicles were made up to 0.3 mg of protein/ml in 2.5 ml of RB containing 5 mM ATP, 7 mM MgSO₄, and 0 or 500 mM KNO₃. They were incubated on ice for 1 h and centrifuged 15 min at 412,000 × g. The membrane pellet was resuspended in 750 μl of RB and used for activity measurements and immunoblotting. The proteins in 50 μl of the resuspended pellet and 167 μl of the supernatant were used for immunoblotting after being precipitated with 10% trichloroacetic acid, washed with cold acetone, lyophilized, and separated by SDS-PAGE.

**Activity Assay**—Proton pumping and ATP hydrolysis by tonoplast-enriched vesicles were measured simultaneously by using the continuous spectrophotometric assay of Palmgren (17). In this assay, proton transport into the vesicles was followed by measuring the absorbance decrease of acridine orange at 495 nm. Simultaneously, ATP hydrolysis was measured by coupling the appearance of ADP to the oxidation of NADH and by following the NADH absorbance decrease at 340 nm.

**FIG. 1.** Simultaneous measurement of ATP hydrolysis and H⁺ pumping by tonoplast-enriched vesicles from juice sacs. ATP hydrolysis was measured by coupling the oxidation of NADH, measured at 340 nm (——), to the appearance of ADP, and proton pumping was measured as the absorbance quenching of acridine orange at 495 nm (——). Once a steady state ΔpH was reached, the proton gradient was collapsed with 4 μg gramicidin or 0.25 μg nigericin. 20–25 μg of juice sac or epicotyl membrane protein were typically used in all experiments.

**FIG. 2.** Inhibition of the proton pumping activity of tonoplast-enriched juice sac vesicles by nitrate. The effect of increasing concentrations of potassium nitrate on the maximum rate of proton pumping by three different preparations of tonoplast-enriched membranes from juice sacs was measured by quinacrine fluorescence quenching. ~80 μg of membrane protein were used in each reaction. ○ represents a so called nitrate-insensitive preparation; □ represents a nitrate-sensitive preparation; △ a partially nitrate-sensitive preparation.

**TABLE I**

| Treatment          | Epicotyl | Fruit |
|--------------------|----------|-------|
| 100 mM KNO₃       | 100      | 72 ± 10 |
| 1 mM Bafilomycin A₁ | 26 ± 1   | 92 ± 9  |

**Sensitivity of the Citrus limon V-ATPases to nitrate and bafilomycin A₁**

The proton pumping activities of tonoplast-enriched vesicles from juice sacs and seedling epicotyls were measured in the presence of the typical V-ATPase inhibitors nitrate and bafilomycin A₁. Quinacrine fluorescence quenching was here used to measure proton transport.
assay medium consisted of 10 mM MOPS-BTP, pH 7.0, 2 mM ATP, 150 mM KCl, 1 mM sodium azide, 0.5 μM valinomycin, 0.25 mM very freshly prepared NADH, 1 mM phosphoenolpyruvate, 20 μM acidine orange, and 25 μM of a mixture of pyruvate kinase and lactate dehydrogenase (Sigma). 50 μM sodium vanadate was also present in the mixture unless stated differently. The reaction was started with 4 mM MgCl₂ and the absorbance values at 340 and 495 nm were recorded in 15-s intervals with a Spectronic Genesis 5 spectrophotometer (Spectral, Rochester, NY) interfaced with a PC running the MultiWL computer program (M. L. Müller and A. Murphy). After correction for mixing artifacts, the results were loaded into a graphics program and plotted out (Fig. 1). When activity rates are reported, the slopes of the initial rates of proton pumping and ATP hydrolysis were calculated and expressed in arbitrary units (a.u.). The calculated H⁺/ATP coupling ratios were independent of the amount of membrane protein used in the assay (data not shown). We nevertheless chose to do all experiments in the presence of 20–25 μg of membrane protein, unless stated differently. When proton pumping was measured in the presence of nitrate in the reaction mixture, quinacrine fluorescence quenching was substituted for acridine orange absorbance quenching. If the effect of nitrate was to be assessed on proton pumping alone, the conditions were as described previously (13). If H⁺/ATP coupling ratios were to be determined, quinacrine fluorescence quenching was measured in the assay mixture described above, so that H⁺ pumping and ATP hydrolysis activities were measured in the same conditions. Because the initial rate of H⁺ pumping differed from the maximum activity rate in the presence of nitrate, the latter was chosen for all activity measurements in the presence of nitrate.

Immunoblotting—The proteins of tonoplast-enriched vesicles from epicotyls and juice sacs were separated by SDS-PAGE according to Laemmli (18) in 12% polyacrylamide gels and the transfer to nitrocellulose was done as described previously (13). The blots were incubated with a primary antibody to the 70-kDa subunit of the corn V-ATPase and visualized by a peroxidase-coupled secondary antibody reaction (Vectorstain ABC kit, Vector Laboratories, Burlingame, CA) (13).

Protein Concentration—Estimates of protein concentrations were done routinely with Amido Black (19).

RESULTS

Sensitivity to Nitrate—Although we had previously found that proton pumping by fruit juice sac vesicles was largely insensitive to inhibition by nitrate and bafilomycin A₁ (13) (Table I), we observed that there was some variability in the nitrate insensitivity of the lemon fruit V-ATPase from one membrane preparation to another (Fig. 2). Fig. 3A shows the nitrate sensitivity of tonoplast-enriched fruit vesicles from preparations made over a period of 20 months. All fruits were harvested at the same state of development from a single tree situated on the campus of the University of California, Santa Cruz. We found that, over the duration of the experiment, the inhibition of the proton pumping activity by 100 mM KNO₃ followed a sigmoidal pattern, ranging from 0 to 20% inhibition in the fall to 70–80% in the spring.

In addition to the seasonal variation in nitrate sensitivity noted above, a temporal component was also observed in the kinetic study of nitrate inhibition. As shown in Fig. 3B, even in a “nitrate-insensitive” juice sac preparation, H⁺ pumping appeared to be initially inhibited by nitrate. However, after 1–2 min, the activity underwent a progressive “recovery” to reach a maximum rate comparable to that of the control. This temporal delay in the attainment of the maximum rate of proton pumping was observed under all experimental conditions used (including experiments done in the presence of acidine orange) and whether an ATP regeneration system was present or not. However, we observed that the ATP regeneration system tended to reduce the magnitude of the delay. In contrast to H⁺ pumping, ATP hydrolysis did not show a similar lag phase (data not shown). This suggests that in the presence of KNO₃, the fruit proton pumps were initially uncoupled, but became progressively coupled as catalysis progressed.

Sensitivity to Vanadate—The lemon fruit V-ATPase was previously found to be partially sensitive to high concentrations (>200 μM) of vanadate (13, 14). We therefore tested the effects of vanadate on the initial rates of proton pumping (Fig. 4, A and B) and ATP hydrolysis (Fig. 4, C and D), and on the deduced coupling ratio (Fig. 4, E and F) of tonoplast vesicles from juice sacs and epicotyls. Curves representing two types of juice sac preparations are shown, a “nitrate sensitive” one (open circles), which was ~70% inhibited by 100 mM KNO₃ and a “nitrate insensitive” preparation (open triangles), which was inhibited only ~30% by the same concentration of nitrate.

As shown in Fig. 4, A and C, respectively, proton pumping and ATP hydrolysis by tonoplast vesicles of epicotyls exhibited little sensitivity to vanadate in the 0–50 μM range. Thus, the deduced coupling ratio of the epicotyl V-ATPase increased only slightly between 10 and 50 μM vanadate. The lack of effect of vanadate suggests that contaminating P-ATPase activity is not a significant factor in our measurements of the coupling ratio of the epicotyl V-ATPase (Fig. 4E). High concentrations of vanadate inhibited proton pumping to some extent, while ATP hydrolysis appeared to be stimulated under the same conditions. However, this latter effect appeared to be an artifact due to the oxidative properties of vanadate which, at high concentration, oxidized the NADH used in the coupled assay (data not shown).

In contrast to the results with tonoplast vesicles from epicotyls, ATP hydrolysis by lemon fruit membranes was inhibited...
by \(-50\%\) by 50 \(\mu\text{M}\) vanadate (Fig. 4D). However, proton pumping was only slightly inhibited by low concentrations of vanadate in both the nitrate-sensitive and the nitrate-insensitive preparations. This suggests that a considerable amount of contaminating plasma membrane ATPase activity, presumably in the form of leaky vesicles incapable of generating a \(\Delta\text{pH}\), may be present in the fruit tonoplast-enriched preparations. Both ATP hydrolysis and proton pumping were inhibited by 400 \(\mu\text{M}\) vanadate to \(60\%\) of their activity in the presence of 50 \(\mu\text{M}\) vanadate. The nitrate-sensitive fruit preparation was about twice as active in proton pumping as the nitrate-insensitive activity. As a result, the coupling ratio calculated for the nitrate-sensitive preparation is about 70\% higher than that of the nitrate-insensitive preparation, and is comparable to the coupling ratio of the epicotyl V-ATPase (Fig. 4F).

\(V_1\) Dissociation—We previously reported that cold dissociation in the presence of KNO\(_3\) caused the release of catalytic subunits from fruit vesicles without affecting the total ATP-dependent \(H^+\) pumping activity relative to controls (13). The only effect of nitrate treatment on the fruit \(H^+\) pumping activity was to increase the sensitivity to high concentrations of vanadate (200 \(\mu\text{M}\) from 30\% inhibition in control vesicles (treated with cold an 5 \(\mu\text{M}\) MgATP alone) to 60–70\% in vesicles treated with cold 5 \(\mu\text{M}\) MgATP and 500 \(\mu\text{M}\) KNO\(_3\). Fig. 5 confirms that the amount of catalytic subunit released from fruit membranes by nitrate was comparable to the amount dissociated from epicotyl vesicles, as determined by immunoblotting. The ATP-dependent proton pumping activity, measured in the absence of vanadate, is given below the immunoblots.

To determine whether the \(H^+\)/ATP coupling ratio of the fruit V-ATPase had been altered by the treatment with 500 \(\mu\text{M}\) KNO\(_3\), nitrate-insensitive and nitrate-sensitive fruit vesicle preparations were subjected to cold inactivation in the presence of 500 \(\mu\text{M}\) KNO\(_3\). \(H^+\) pumping and ATP hydrolysis were measured simultaneously after washing the cold-released vesicles with buffer. The results are shown in Fig. 6 (A to D). For a comparison, the activity of similarly treated epicotyl vesicles is also presented (Fig. 6, E and F). In the nitrate-
insensitive fruit preparation, KNO₃ treatment had little or no effect on either the H⁺ pumping or ATP hydrolysis activities (Fig. 6A) and thus, the coupling ratio of the nitrate-insensitive preparation was unaffected. However, the nitrate-treated sample was strongly inhibited by 500 mM vanadate (Fig. 6B). The results obtained with the nitrate-sensitive fruit preparation confirmed these findings (Fig. 6C and D) and showed that 50 μM vanadate was sufficient to inhibit most of the proton pumping activity after KNO₃ treatment. Table II shows a comparison of the H⁺ pumping and hydrolytic activities, and the coupling ratios of the "partially nitrate-sensitive" fruit preparation used in Fig. 6C and D. Under control conditions (non-dissociated), the vanadate-sensitive ATPase activity was largely independent of H⁺ pumping, since 50 μM vanadate inhibited ATP hydrolysis by 58% and H⁺ pumping by only 16%. However, after V₁ dissociation, 50 μM vanadate inhibited ATP hydrolysis and H⁺ pumping by 71 and 74%, respectively, indicating that the vanadate-sensitive ATP hydrolytic activity is coupled to proton transport. Since the amount of vanadate-sensitive H⁺ pumping activity was doubled in absolute value after V₁ dissociation (0.27 to 0.54 arbitrary units), the dissociation procedure appears to have partially transformed the vanadate-insensitive H⁺ pumping activity into a vanadate-sensitive activity. Overall, the H⁺/ATP coupling ratio of the partially nitrate-sensitive preparation, which in the control was 2.66 a.u. in the presence of 50 μM vanadate, dropped to 0.61 ± 0.04 a.u. after V₁ dissociation, whether vanadate was present or not (Table II). Thus V₁ dissociation has a major uncoupling effect on the nitrate-sensitive H⁺ pumping activity of juice sac vesicles.

A detailed analysis of vanadate sensitivity and coupling ratios was also performed under control and V₁ dissociation conditions for a nitrate-sensitive fruit preparation (Fig. 7). In the control vesicles, 50 μM vanadate inhibited proton pumping by only 27% (Fig. 7A), whereas ATP hydrolysis was inhibited by...
The proton pumping and hydrolytic activities of a partially nitrate-sensitive juice sac preparation were determined under the conditions described in the legend to Fig. 6. Activities and coupling ratios are given in arbitrary units and the inhibition by 50 μM vanadate is given in arbitrary units and as a percentage of the activity of the sample not treated with vanadate.

### Table II

Activities and coupling ratios of fruit tonoplast vesicles treated for V₁ dissociation

| Treatment          | ATP hydrolysis | H⁺ pumping |
|--------------------|----------------|------------|
|                    | Activity | VO₄ | inhibition | Activity | VO₄ | inhibition | Coupling ratio |
| Controls:         |          |     |           |          |     |           |               |
| -VO₄              | 1.24     | 0.72| 58        | 1.67     | 0.27| 16        | 1.34          |
| +50 μM VO₄        | 0.53     |     |           | 1.40     | 0.54| 74        | 2.66          |
| V₁-dissociated    | 1.12     | 0.80| 71        | 0.73     | 0.54| 74        | 0.65          |
| +50 μM VO₄        | 0.32     |     |           | 0.19     | 0.54| 74        | 0.58          |

53% by the same concentration (Fig. 7B). After V₁ dissociation, 50 and 100 μM vanadate inhibited the proton pumping activity by 69 and 89%, respectively (Fig. 7A). The H⁺/ATP coupling ratio, which in the control was ~1.8 a.u. in the presence of 50 μM vanadate, dropped to ~0.62 a.u. after V₁ dissociation (Fig. 7C). At 400 μM vanadate the coupling ratio of the control was ~2.3, while that of the V₁ dissociated membranes was ~0.1. Thus, high concentrations of vanadate have opposite effects on the coupling ratios of control versus V₁ dissociated vesicles, increasing the former while decreasing the latter.

**ATP Kinetics**—Since the residual H⁺-ATPase activity after V₁ dissociation treatment clearly differed from the control activity in terms of vanadate sensitivity, both activities were further characterized with respect to Kₘ and Vₘₐₓ in a fruit preparation exhibiting ~40% sensitivity to 100 mM KNO₃ (Fig. 8). From the curves in Fig. 8B, it is clear that the ATP hydrolytic activity was largely unaffected by nitrate up to a substrate concentration of 2 mM ATP. Beyond 2 mM, the first-order kinetics of the control vesicles diverged from strict Michaelis-Menten kinetics, suggesting the presence of some contaminating activity (Fig. 8B). From the Hanes-Woolf linearizations of the first-order kinetics between 0 and 2 mM ATP, we calculated an identical Kₘ of 0.16 mM ATP and an identical Vₘₐₓ for the vesicles treated in the presence or absence of KNO₃ (Fig. SD). In contrast to ATP hydrolysis, the Vₘₐₓ of the proton pumping activity was reduced 50% by nitrate treatment (Fig. 8, A and C). However, the Kₘ of 0.20 mM ATP for the nitrate-treated sample was identical to that of the control, suggesting that the same type of enzyme was active after V₁ dissociation as before.

In order to determine whether the treatment with nitrate might have left intact a population of V-ATPases with a different coupling ratio than the normal V-ATPase, we normalized the initial rates of pumping of control and nitrate-treated vesicles and compared their pH gradient at steady state (Fig. 9A). The ΔpH built up by nitrate-treated vesicles did not appear to differ significantly from that of control vesicles. The pH-dependent slip and leakage rate constants of control and nitrate-treated vesicles were calculated and normalized for the apparent proton pumping rate at any time during the ΔpH build up. As shown in Fig. 9B, the pH-dependent slip and leakage were identical in control and nitrate-treated vesicles.

**Uncoupling by Nitrate**—Experiments were carried out to determine whether the epicotyl V-ATPase and the nitrate-sensitive component of the fruit V-ATPase exhibited the same pattern of uncoupling by nitrate. Because acridine orange was reported to dissipate pH gradients in the presence of KNO₃ (20), we used quinacrine fluorescence quenching to measure proton transport in the experiments where nitrate was present.

As shown in Fig. 10, A and B, nitrate inhibited the H⁺ pumping and ATP hydrolysis activities of both the epicotyl and the fruit tonoplast vesicle preparations, although the total in-

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**Fig. 7.** Effect of V₁ dissociation treatment on the vanadate sensitivity of a partially nitrate-sensitive juice sac preparation. Tonoplast-enriched vesicles were treated for V₁ dissociation in the presence or absence of 500 mM KNO₃. After washing in buffer, the membrane-bound enzymes were assayed for H⁺ pumping and ATP hydrolysis. Initial activity rates and coupling ratios in the presence of increasing concentrations of vanadate are reported. A, H⁺ pumping activities; B, ATPase activities; and C, H⁺/ATP coupling ratios. ○, vesicles treated in the absence of nitrate; ●, vesicles treated in the presence of 500 mM KNO₃.
hibition was greater for epicotyl vesicles. The average coupling ratios of two fruit and two epicotyl preparations in the presence of increasing nitrate concentrations are shown in Fig. 10C. Note that only the nitrate sensitive activity was considered. The progressive decrease in the $H^+$/ATP coupling ratio between 0 and 50 mM KNO$_3$ clearly indicates that in fruit and epicotyl vesicles, nitrate inhibits proton pumping to a greater extent than ATP hydrolysis, and thus uncouples the V-ATPase. If only the nitrate-sensitive portion of the total activity is considered, uncoupling of the fruit V-ATPase occurs more readily at very low concentrations of KNO$_3$, at concentrations >20 mM, however, the epicotyl is more strongly uncoupled by nitrate than the fruit. In the presence of 50–100 mM KNO$_3$, the $H^+$/ATP coupling ratio of the fruit tonoplast vesicles was consistently about twice that of the epicotyl vesicles.

Bafilomycin A$_1$—Similar experiments were carried out to measure the effect of bafilomycin A$_1$ on the coupling ratios of fruit and epicotyl tonoplast membrane vesicles (Fig. 11, A and B). In preliminary experiments it was found that fruit preparations that were more nitrate-sensitive exhibited increased sensitivity to bafilomycin as well. Fig. 11, A and B, show the effects of bafilomycin on the $H^+$ pumping and ATP hydrolysis activities of an epicotyl preparation and a bafilomycin-sensitive fruit tonoplast preparation, respectively. The $H^+$/ATP coupling ratios between 0 and 1 µM bafilomycin are shown in Fig. 11C. Bafilomycin had no effect on the coupling ratios of either the epicotyl or the fruit V-ATPases.

Oxidation—We previously demonstrated that oxidation inhibits the proton pumping activity of the epicotyl V-ATPase, and that the oxidative inactivation is partially reversible by DTT (13). Accordingly, the effect of oxidation on the $H^+$/ATP coupling ratios of epicotyl tonoplast vesicles was examined. To avoid artifacts due to contaminating ATPases, only the bafilomycin- and NEM-sensitive ATP hydrolysis and proton pumping activities were used for the determination. Exposing epicotyl tonoplast vesicles to air at 22 °C in the absence of reductant reduced both $H^+$ pumping and ATP hydrolysis activities in parallel (Fig. 12A). Moreover, DTT reversal was the same in both cases. The calculated coupling ratios are shown in Fig. 12B. Oxidation had no effect on the $H^+$/ATP coupling ratios of epicotyl V-ATPases.

**DISCUSSION**

V-ATPases normally operate far from thermodynamic equilibrium and are therefore considered to be under kinetic regulation (e.g. 11). Kinetic regulation of the V-ATPase may involve inhibitors (21, 22), $V_1$ dissociation from the membrane (23), slip induced by $\Delta\mu_{\text{H}^+}$ (10), and variable $H^+$/ATP stoichiometry than the V-ATPase is present on the membrane. For the V-ATPase to reach thermodynamic equilibrium it would have to become refractory to kinetic regulation.

We previously reported that ATP-dependent proton pumping by juice sac vesicles was unusually insensitive to nitrate and other V-ATPase inhibitors (13). Although the juice sac V-ATPase became sensitive to inhibitors after being solubilized, purified, and reconstituted into liposomes, its pH-de-
A comparison of nitrate-sensitive versus nitrate-insensitive fruit preparations indicates that nitrate-sensitive vesicles have higher H+/ATP coupling ratios than nitrate-insensitive preparations. Since the coupling ratio of the nitrate-sensitive vesicles is comparable to that of epicotyl preparations, and assuming that the epicotyl tonoplast is energized by a V-ATPase alone, the nitrate-sensitive juice sac vesicles are energized by only one type of proton pumping ATPase: the V-ATPase. If it contained a mixture of P- and V-type ATPases, with H+/ATP stoichiometries of 1 and 2, respectively (11, 25–27), one would expect the coupling ratio of the fruit preparation to be lower than that of the epicotyl.

Even though the coupling ratio of the so-called nitrate-insensitive juice sac preparations was lower than that of tonoplast vesicles from epicotyls, two observations suggest that these vesicles also bear a V-ATPase rather than another type of H+-ATPase: 1) during the initial seconds of H+ pumping in the presence of nitrate, the nitrate-insensitive fruit preparations are temporarily uncoupled. The subsequent recovery of coupling is dependent on the presence of MgATP and may involve some type of subunit rearrangement or possibly a phosphorylation reaction. 2) Nitrate-insensitive juice sac preparations show kinetic properties and vanadate sensitivities similar to the more nitrate-sensitive juice sac preparations after V1 dissociation treatment. In the latter case, as shown below, the residual H+ pumping activity is thought to be due to a V-ATPase, based on its $K_m$ and the simultaneously measured ATP hydrolysis kinetics, even though the calculated coupling ratio is lower than in epicotyl V-ATPases.

Moriyama and Nelson (10) have proposed that nitrate induces uncoupling of proton transport from ATP hydrolysis in V-ATPases. In tonoplast-enriched vesicles from lemon juice sacs and epicotyls, nitrate indeed had such an effect. When only the nitrate-sensitive components of H+ pumping and ATP hydrolysis were considered (defined as the portions of the activities inhibited by 400 mM KNO3), juice sac and epicotyl preparations exhibited significant uncoupling under treatment with 1 to 100 mM KNO3. At 50 mM KNO3, the H+/ATP coupling ratio of epicotyl V-ATPases had dropped an average 89% from its initial value, while the fruit enzymes' uncoupling averaged ~72% of their initial coupling ratio. Accordingly, a subpopulation of nitrate-sensitive V-ATPases requires 400 mM KNO3 for inhibition while remaining resistant to uncoupling by 50 to 100 mM KNO3.

Although the H+ pumping activity of fruit vesicles becomes more sensitive to vanadate after nitrate treatment, in untreated vesicles vanadate is most effective in the 50 to 300 μM range. Since P-type H+-ATPases are generally inhibited by 1.0 to 10 μM vanadate, it is unlikely that the vanadate sensitivity of the lemon fruit H+ pumping activity involves the inhibition of a P-type enzyme (14). However, an ATP hydrolytic activity that is sensitive to low concentrations of vanadate is clearly present in fruit vesicle preparations. This is best illustrated by the H+/ATP coupling ratios in the presence of increasing concentrations of vanadate. For nitrate-sensitive and nitrate-insensitive juice sac preparations, the coupling ratios rise sharply in the presence of 0 to 50 μM vanadate, and then

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2 M. Jensen, unpublished data.
remain stable up to 500 μM VO₄. This indicates that the "contaminating" activity is mainly hydrolytic, and as it can be eliminated by low concentrations of vanadate (≤50 μM), it could be due to a P-type ATPase. Since concentrations of vanadate between 50 and 500 μM inhibited the hydrolytic and pumping activities to the same extent, i.e. the H⁺/ATP coupling ratio remains stable, high concentrations of vanadate appear to inhibit a single H⁺-ATPase activity which is distinct from the activity inhibited by low concentrations of vanadate.

V₁ dissociation treatments in the presence of high nitrate did not significantly modify the H⁺/ATP coupling ratio in nitrate-insensitive juice sac preparations. However, V₁ dissociation did increase the sensitivity of the proton pump to high concentrations of vanadate. In nitrate-sensitive preparations, H⁺/ATP coupling ratios were strongly reduced by the V₁ dissociation treatment, and concomitantly, proton pumping became highly sensitive to both high and low concentrations of vanadate. In the preparations analyzed, which were initially ~20% sensitive to vanadate, KNO₃ treatment increased the vanadate sensitivity to 50% of the initial activity. Simultaneously, the coupling ratio dropped to 1/2 or 1/4 of its value before KNO₃ treatment. Three explanations are possible. 1) KNO₃ treatment
uncouples the V-ATPases present on the membranes while simultaneously coupling a population of P-type H⁺-ATPases which were initially uncoupled. 2) KNO₃ treatment uncouples one of two populations of V-ATPases, the remaining population being vanadate-sensitive. 3) KNO₃ uncouples one of two populations of V-ATPases and modifies the remaining V-ATPases, inducing them to become vanadate-sensitive.

While the first explanation would yield a result similar to what was observed, it would imply that nitrate treatment induces the sealing of a leaky membrane fraction associated with the putative P-type hydrolytic activity. To our knowledge, no such effect of nitrate on the formation of sealed vesicles has been reported. Thus, we consider this explanation unlikely.

In the second explanation, there are two populations of V-ATPases: classical, nitrate-sensitive V-ATPases and nitrate-insensitive, vanadate-sensitive V-ATPases. However, this explanation does not account for the overall increased vanadate sensitivity of proton pumping after V₁ dissociation treatment.

The third explanation differs from the second in that a population of V-ATPases becomes vanadate-sensitive as a result of nitrate treatment. The increase in vanadate sensitivity could be a consequence of a molecular rearrangement that allows the V₁ sector to become vanadate-sensitive.

It has been argued that inhibition of the V-ATPase by nitrate was the result of two distinct phenomena (28, 29). At high concentrations, the chaotropic properties of nitrate promote the dissociation of the V₁ sector from Vₒ (28), while below 50 mM, nitrate was proposed to involve a different mode of action (29, 30). Our present results indicate that there is no distinct low- and high-nitrate effects on the lemon V-ATPases, as from 0 to 400 mM KNO₃, the effect of nitrate corresponds to the progressive uncoupling of V₁ from Vₒ which ends with the physical dissociation of the V₁ sector from the membrane. Note that even after treatment of epicotyl vesicles with 500 mM KNO₃, ~50% of the V₁ sectors remain attached to the membrane, even though proton pumping is completely inhibited. Thus, physical release of the V₁ sector from the membrane is not required for inhibition of proton pumping, but may be required for inhibition of ATP hydrolysis.

Dschida and Bowman (31) proposed that the low concentration effect of nitrate was due to its oxidizing properties and induced the formation of disulfide cross-links in V₁ subunits, confomational changes, and subsequent release of V₁ sectors from the membrane. Accordingly, the oxidative inactivation of proton pumping that we had observed in epicotyl vesicles may not have been due exclusively to disulfide bonding at the catalytic site (13, 14, 32, 33), but could also have involved some oxidation-induced uncoupling of the enzyme (34). Furthermore, sulphydryl groups of the γ subunit of the lettuce chloroplast F-ATPase have been shown to be involved in proton slipp (35). Our results, obtained by simultaneously measuring bafilomycin- and NEM-sensitive H⁺ pumping and ATP hydrolysis at different time points during oxidative inactivation show that no H⁺/ATP uncoupling was taking place during oxidative inactivation. Even though this does not preclude nitrate from having a specific oxidizing effect, it indicates that oxidative inactivation, as we were measuring it previously, does not uncouple the enzyme and probably takes place exclusively at the catalytic site.

Bafilomycin A₁ is thought to inhibit V-ATPases by binding to the 100-kDa subunit and/or the proteolipid of the V₁ sector, but its mode of action, as well as that of other macrolide antibiotics, is still unknown (36–38). We found that, like oxidation, bafilomycin A₁ did not uncouple H⁺ pumping from ATP hydrolysis.

It is important to note that in the present study H⁺/ATP
coupling was measured in the absence of an electrical potential gradient and in the presence of a limited pH gradient developing during the initial few minutes of H⁺ pumping. H⁺/ATP coupling ratios under these initial rate conditions might differ drastically from those encountered under the conditions prevailing in vivo, e.g. \( \Delta \varphi = +20 \text{ mV; } \Delta \text{pH} = 2 \text{ to } 4.5 \text{ pH units.} \) According to Tu et al. (15), and our own findings (14), intrinsic uncoupling, or slip, is proportional to the pH gradient built up across the membrane. When slip is determined during the attainment of a steady state pH gradient, the fruit V-ATPase was shown to exhibit half as much slip as the epicotyl V-ATPase (14). Thus, a lower rate of slip in the presence of a pH gradient remains the most likely factor allowing the generation of a steeper pH gradient by the fruit V-ATPase. Although the coupling ratios of the fruit and epicotyl V-ATPases measured in the absence of a pH gradient were equal, the fact that the V-ATPase of juice sacs retained some coupling in the presence of nitrate, whereas the epicotyl V-ATPase did not, may be significant biologically. Nitrate may be mimicking the effect of another stress, such as low cytosolic pH, which would tend to inactivate a normal V-ATPase. The ability of the fruit V-ATPase to adjust to adverse conditions may be a key factor in the overall regulation of vacuolar pH in lemon.

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