The vacuolar H\textsuperscript{+}-ATPases (V-ATPases) of lemon fruits and epicotyls were detergent-solubilized, purified by column chromatography, and reconstituted into artificial proteoliposomes. During purification, a vanadate- and nitrate-sensitive ATPase activity, consisting of partially disassembled V-ATPase complexes, was resolved from the V-ATPase peak. ATPase and H\textsuperscript{+}-transport activities of the purified, reconstituted V-ATPases of both fruit and epicotyl exhibited similar inhibitor profiles, except that the fruit V-ATPase retained partial vanadate sensitivity. Since the V-ATPase activity of native fruit tonoplast vesicles is insensitive to inhibitors (Müller, M. L., Irkens-Kiesecker, U., Rubinstein, B., and Taiz, L. (1996) J. Biol. Chem. 271, 1916-1924), membrane lipids or other factors may protect the fruit V-ATPase from inactivation in vivo. A kinetic analysis of H\textsuperscript{+}-pumping and H\textsuperscript{+}-leakage indicated that the reconstituted epicotyl V-ATPase exhibited twice as much intrinsic uncoupling or slip as the reconstituted fruit V-ATPase. Comparison of their subunit compositions by SDS-polyacrylamide gel electrophoresis indicated that the reconstituted fruit V-ATPase is enriched in two polyepitides of 33/34 and 16 kDa. Moreover, the stalks of negatively stained juice sac V-ATPases appeared thicker than those of epicotyl V-ATPases in electron micrographs.

The juice sacs of lemon fruits contain cells that can acidify their vacuoles to as low as pH 2.2 (1). In contrast, the vacuoles of the surrounding fruit tissues as well as those of vegetative tissues are maintained in the typical vacuolar pH range, 5.0-6.0. The occurrence in lemon of two types of vacuoles with vastly different luminal pH values provides a convenient experimental system to probe the mechanisms underlying the control of steady state vacuolar pH. One hypothesis to explain the extreme acidity of the juice sac vacuoles is that their H\textsuperscript{+}-ATPase (V-ATPase)\footnote{This research was supported in part by the United States Department of Energy Grant DE-FG03-84ER13245 (to L. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.} is a functionally specialized isoform capable of generating a greater pH gradient than vegetative V-ATPases. In an earlier report (2), we compared the ATP-driven H\textsuperscript{+}-pumping activities of tonoplast-enriched membrane vesicles isolated from juice sacs and seedling epicotyls. In native vesicles, the juice sac V-ATPase generated a steeper proton gradient than the V-ATPase of epicotyls. However, since the epicotyl tonoplast was more permeable to protons than the juice sac tonoplast, the steeper ΔpH generated by the juice sac V-ATPase may have resulted from differences in membrane permeability rather than from intrinsic properties of the pumps. On the other hand, the two H\textsuperscript{+}-pumping activities differed with respect to several kinetic parameters. The epicotyl activity showed a typical V-ATPase profile with respect to ions and inhibitors (i.e. stimulation by chloride, inhibition by nitrate, bafilomycin A\textsubscript{1}, and N-ethylmaleimide (NEM), and insensitivity to vanadate). In contrast, the proton pumping activity of juice sac tonoplasts was insensitive to nitrate, bafilomycin, and NEM, and was partially inhibited by vanadate. Sensitivity of the juice sac ATPase activity to nitrate and NEM increased following detergent treatment, consistent with the juice sac proton pump's identity as a V-ATPase. However, evidence for the possible existence of a second H\textsuperscript{+}-ATPase on the juice sac tonoplast was also obtained. In nitrate-induced V\textsubscript{1}-dissociation experiments, the epicotyl vacuolar H\textsuperscript{+}-pumping activity became inactivated with the release of the catalytic subunit from the membrane. Despite the loss of a major portion of the catalytic subunit, the juice sac membranes retained 100% of their H\textsuperscript{+}-pumping activity following nitrate treatment, although vanadate sensitivity increased (2). Solubilization of the fruit membranes with n-dodecyl-β-D-maltoside and centrifugation on a glycerol gradient resulted in the resolution of two peaks of ATPase activity. The denser of the two peaks was strongly inhibited by nitrate, partially inhibited by vanadate, and exhibited a typical V-ATPase subunit composition on SDS-PAGE gels. The second peak was a vanadate- and nitrate-sensitive ATPase of unknown identity (2). It appears that the V-ATPases of lemon fruits and epicotyls may be strongly influenced by their native membranes. To compare the two proton pumps in the same membrane environment we have characterized the properties of purified and reconstituted V-ATPases from fruits and epicotyls. In addition, we have compared negatively stained juice sac and epicotyl tonoplast vesicles by electron microscopy. Our results suggest that native tonoplast lipids of the juice sacs play important roles not only in reducing proton permeability, but in protecting the V-ATPase from inactivation by inhibitors. However, the ability to generate a steeper pH gradient appears to be an intrinsic property of the juice sac V-ATPase. The nature of the vanadate-sensitive “second H\textsuperscript{+}-ATPase” remains unresolved, but may represent partially disassembled V-ATPase complexes.

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*This research was supported in part by the United States Department of Energy Grant DE-FG03-84ER13245 (to L. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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\footnote{The abbreviations used are: V-ATPase, vacuolar H\textsuperscript{+}-ATPase; ACMA, 9-amino-6-chloro-2-methoxy-acridine; BCA, bicinchoninic acid; BTP, 1,3-bis[(trishydroxymethyl)methylamino]propane; DTT, dithiothreitol; Mes, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; n, H\textsuperscript{+}/ATP stoichiometry; NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; QB, Econo-Q running buffer; RB, resuspension buffer; PAGE, polyacrylamide gel electrophoresis.}
</ref>
Purified and Reconstituted V-ATPases of Lemon

EXPERIMENTAL PROCEDURES

Materials—Lemon seeds (Citrus limon L. var. Schaub Rough Lemon) were generously supplied by Willis & Newcomb, Inc., Arvin, CA. Lemon fruits (var. Eureka) were harvested from trees on the campus of the University of California, Santa Cruz. Bafilomycin A1 was from Sigma, BCA protein assay reagents were from Pierce, and n-dodecyl-β-D-maltoside was from Calbiochem. Escherichia coli polar lipid extract was purchased from Avanti Polar Lipids; the NanoOrange® protein quantitation kit and 9-amino-6-chloro-2-methoxy-acridine (ACMA) were from Molecular Probes. All bulk chemicals were purchased from Sigma and Fisher.

Juice Sac and Epitolyte Membrane Preparation—Tonoplast-enriched membranes from lemon fruit juice sacs and epitolytes were prepared as described previously (2). All steps were carried out at 4 °C, and the membranes were maintained on ice. Briefly, juice sacs of three lemons were released into 100 ml of cold fruit homogenization buffer (1.5 M MOPS-KOH, pH 8.5, 2.25% polyvinylpyrrolidone-40, 0.75% bovine serum albumin, 5.7 mM EDTA, 2 mM DTT, and 0.1 mM PMSF). They were ground using a mortar and pestle or, alternatively, homogenized with a Waring blender in a 250-ml flask filled to the top with juice sacs and homogenization buffer, and hermetically sealed to avoid oxidation. Epitolytes (40 g, fresh weight) were homogenized with a razor blade and homogenized in 150 ml of cold epitolyte 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 PMSF) using a mortar and pestle. Homogenates were filtered through a 0.28-mm nylon mesh and centrifuged at 12,000 × g for 15 min (Sorvall SS-34 rotor) to eliminate cellular debris, nuclei, and plastids. The supernatant was subsequently centrifuged at 132,000 × g in a Beckman SW-28 rotor for 60 min at 4 °C in a Beckman SW-28 rotor. The microsomal pellet obtained was resuspended in 15 ml of resuspension buffer (RB; 10 mM BTP-Mes, pH 7.0, 20 mM KCl, 1 mM EDTA, 2 mM DTT, and 0.1 mM PMSF) and further purified on a 10%/35% sucrose step gradient made up in 10 mM BTP-Mes, pH 7.0, 250 mM sorbitol, 100 mM KCl, 1 mM azide, 250 mM valinomycin, 2.5 mM ATP, and either quinacrine or ACMA fluorescence quenching. The reaction mix contained 10 mM BTP-Mes, pH 7.0, 250 mM sorbitol, 100 mM KCl, 1 mM azide, 250 mM valinomycin, 2.5 mM ATP, and either quinacrine (10 μM) or ACMA (1.5 μM). For tonoplast-enriched vesicles, 50 μM vanadate was included in the mix. 100 μg of tonoplast-enriched membrane protein or 0.4–1.0 mg of reconstituted proteoliposomes were typically used, and the reaction was started with 4.5 mM MgSO4. Fluorescence quenching (quinacrine: 423 nm excitation, 502 nm emission wave lengths; ACMA: 430 nm excitation, 500 nm emission) was measured in a Perkin-Elmer LS-5 fluorescence spectrophotometer (Perkin-Elmer Corp.).

Proton Pumping Assays—Proton pumping by tonoplast vesicles and reconstituted proteoliposomes was monitored by quinacrine or ACMA fluorescence quenching. The reaction mix contained 10 mM BTP-Mes, pH 7.0, 250 mM sorbitol, 100 mM KCl, 1 mM azide, 250 mM valinomycin, 2.5 mM ATP, and either quinacrine (10 μM) or ACMA (1.5 μM). For tonoplast-enriched vesicles, 50 μM vanadate was included in the mix. 100 μg of tonoplast-enriched membrane protein or 0.4–1.0 mg of reconstituted proteoliposomes were typically used, and the reaction was started with 4.5 mM MgSO4. Fluorescence quenching (quinacrine: 423 nm excitation, 502 nm emission wave lengths; ACMA: 430 nm excitation, 500 nm emission) was measured in a Perkin-Elmer LS-5 fluorescence spectrophotometer (Perkin-Elmer Corp.).

Calculation of Slip Rate Constants—Uncoupling or “slip” rates were estimated based on a kinetic model described by Tu et al. (7). According to this model, the proton pumping rate at any given time point during the formation of the gradient can be represented by the following equation,

\[ \frac{dS}{dt} = -nR \left( k_1 + k_2 \delta \right) \]

where \( \delta \) and \( \delta \) represent the proton pumping rate and the net amount of proton transport, respectively; \( n \) is the coupling ratio or stoichiometry of the pump; \( R \) is the ATP hydrolysis rate, and \( k_1 \) and \( k_2 \) are first order rate constants representing membrane leakage and slip, respectively. The sum of \( k_1 \) and \( k_2 \) can be represented by \( k_s \), \( nR \) is constant, and at steady state, \( d\delta / dt = 0 \). It follows that

\[ k_s \delta = nR \]

where \( \delta \) is the steady state value of \( \delta \). After substituting and integrating

\[ \ln \left( \frac{1}{\delta} \right) = -n/kt \]

Equation 3 allows one to estimate \( k_s \), the combined leakage and slip rate constant, from proton pumping traces. By analogy, the proton leakage rate can be estimated by linearizing the curve obtained after a pH gradient has been generated, and \( H^- \)-pumping has been inhibited with ETDA. Again, proton leakage is assumed to obey first order kinetics

\[ \frac{d\delta}{dt} = -k_s \delta \]

where \( k_s \) represents the leakage rate constant for this portion of the curve. After integration,
and separated on a 1 \times 100$-cm Sephacryl S-400 HR column eluted at 4.5 m l/h. $A_{280}$ absorbance curves (top panels) and ATPase activity profiles (bottom panels) are shown. ○○○○, nitrate-sensitive activity; ⋅⋅⋅⋅, vanadate-sensitive activity. F, T, A, β, and B indicate the elution volumes of the molecular mass markers blue dextran 2,000,000, thyroglobulin, alcohol dehydrogenase, β-amylase, and bovine serum albumin, respectively.

\[ \Delta \delta / \Delta t = - k_{f}. \]  

(Eq. 5)

The slip rate constant, $k_{f}$, is estimated by subtracting $k_{b}$ from $k_{f}$.

**ATPase Assays**—ATP hydrolysis measurements were carried out in a reaction mix containing 2.5 mM ATP, 4.5 mM MgSO$_4$, 100 mM KCl, 1 mM azide, 1 mM molybdate, 2 μM gramicidin, and 1 mg/ml sonicated L-α-phosphatidylcholine liposomes in 25 mM BTP-Mes buffer, pH 7.0. The total reaction volume was 500 μl, and the reaction was started by adding the enzyme to the mix. After 30 min at 37 °C, the reaction was stopped by adding 1.25 ml of Fiske and Subbarow (5) reagent. After 30 min at room temperature, absorbance of the samples at 660 nm was measured in a Spectronic Genesis 5 spectrophotometer (Milton Roy, Rochester, NY). Boiled membranes were used for background estimation. Where nitrate-sensitive or vanadate-sensitive activity is reported, the results are expressed as the difference in activity in the presence or absence of 400 mM KNO$_3$ or 400 μM Na$_3$VO$_4$, respectively.

**Protein Concentration**—Protein concentrations were measured routinely by a modified BCA protein assay (6) or with the NanoOrange™ protein quantitation kit after precipitation of the proteins with cold acetone and delipidation with diethyl ether.

**Gel Electrophoresis**—SDS-PAGE was according to Laemmli (8) in 12 or 13.5% polyacrylamide gels. The samples were made up in sample buffer to a final concentration of 60 mM Tris-HCl, pH 6.8, 4% SDS, 5% DTT, 10% glycerol, and 0.0125% bromphenol blue. The gels were developed with silver.

**Electron Microscopy**—The electron microscopy experiments were conducted at the laboratory of Prof. Ulrich Lütting in Darmstadt, Germany. Tonoplast-enriched membranes were pelleted and resuspended at room temperature to a final concentration of ~1 mg/ml protein in 10 mM potassium phosphate buffer, pH 7.0, containing 5 mM ATP. Negative staining was performed with a solution of 2% methylamine tungstate according to the successive droplet method (9). A 5-μl droplet of membrane suspension was applied to a Formvar-coated 700-mesh/hexagonal grid. After 2 min, the droplet was wicked off with filter paper and replaced with a 5-μl droplet of 2% methylamine tungstate. After 15–20 s the stain was also wicked off, and the grid was allowed to dry. Specimens were examined and photographed with a Zeiss EM902 electron microscope (Carl Zeiss, Oberkochen, Germany) operated at 80 kV in the electron filter mode.

**RESULTS**

As a first step in the purification, detergent-solubilized tonoplast-enriched membranes from epicotyls and fruits were layered onto a Sephacryl S-400 HR column, and the protein and ATPase activities were monitored. The protein distribution and ATPase activity profiles are shown in Fig. 1. An octyl-β-glucoside solubilization resulted in a single peak of ATPase activity corresponding to a molecular mass of about 4,500 kDa for both fruit and epicotyl membranes (Fig. 1, A and B). Analysis of the fractions by SDS-PAGE indicated that the peak fractions were enriched in subunits for the V-ATPase (data not shown). The high molecular mass of the complex indicated that the V-ATPase was migrating as an aggregate. The epicotyl peak was inhibited by nitrate only, whereas the fruit peak was sensitive to both nitrate and vanadate.

If n-dodecyl-β-D-maltoside was used to solubilize the membranes, two peaks of ATPase activity were obtained for both fruit and epicotyl membranes, a nitrate-sensitive V-ATPase activity peak, which migrated either as a 4,500-kDa aggregate or as a lower molecular mass aggregate of about 1,500 kDa (Fig. 1, C and D). The second peak was both nitrate- and vanadate-sensitive. Treatment of the octyl-β-glucoside peak fractions with n-dodecyl-β-D-maltoside did not induce the appearance of the second peak (data not shown). Hence the second peak is not a degradation product of the first peak, but appears to be specifically solubilized from the membrane by n-dodecyl-β-D-maltoside.

The Sephacryl S-400 HR V-ATPase peak fractions from the n-dodecyl-β-D-maltoside solubilization were further purified on two successive Econo-Q anion exchange columns. After a first passage over the column, both the fruit and the epicotyl V-ATPases showed an activity peak eluting at 0.1 M KCl. This
peak was nitrate-sensitive and vanadate insensitive in the electrolytic preparation, and nitrate-sensitive and partially vanadate-sensitive in the case of the fruit. In addition, the fruit preparation exhibited a second peak of activity at 0.065 M KCl that was inhibited equally by nitrate and vanadate (data not shown).

Both the single electrolytic ATPase activity peak and the fruit peak eluting at 0.1 M KCl contained typical V-ATPase subunits when analyzed by SDS-PAGE. The nitrate- and vanadate-sensitive enzyme activity peak eluting at 0.065 M KCl appeared to co-purify with selected V-ATPase subunits rather than with any specific polypeptides. However, the presence of a low abundance contaminant with high ATP hydrolytic activity cannot be ruled out.

When the fractions making up the more nitrate-sensitive activity peak were pooled and further purified on a second Econo-Q column, further separation of the vanadate-sensitive from the nitrate-sensitive activities was achieved (Fig. 2). The peak of maximum nitrate-sensitive activity was further enriched in the complete set of V-ATPase subunits and was depleted in contaminating bands, mainly a 100 kDa polypeptide (Fig. 2B, fraction 40). Bands at 97, 66, 55/56, 52, 42/43, 36, 33, 31, 17, 14, and 13 kDa co-migrated with the peak of activity. Most notably, the doublet at 33/34 kDa was present only in the fruit preparation, and only the 33-kDa component of the doublet co-migrated with the more nitrate-sensitive activity peak. In most experiments, a 16-kDa band also co-migrated with the nitrate-sensitive activity peak, although it appears to be shifted to fraction 42 in the gel of Fig. 2B.

The nitrate- and vanadate-sensitive ATPase activity peak eluting at 0.065 M KCl was enriched in the 97- and 36-kDa bands, and in the 55/56-kDa doublet. The 33/34-kDa doublet was present, but only the 34-kDa component of the doublet co-migrated with the peak of nitrate- and vanadate-sensitive activity. The 66-kDa polypeptide (V-ATPase catalytic or A subunit) was also present, although in reduced amounts compared with the 55/56-kDa doublet (V-ATPase “regulatory” or B subunit). The strong doublet at 25/26 kDa, present in all fractions eluting from both Econo-Q columns, did not show a consistent pattern of co-migration with any of the two activities and is therefore thought to represent a contaminant.

The specific activities of the purified V-ATPases (average ± S.D. of four purifications) were 9.5 ± 1.5 μmol of P_i mg^-1 min^-1 and 6.9 ± 2.2 μmol P_i mg^-1 min^-1 for the epicotyl and fruit, respectively. The sensitivities of the two purified V-ATPases to various inhibitors is shown in Fig. 3. Both V-ATPases were about equally inhibited by nitrate (Fig. 3A), bafilomycin (Fig. 3B), and NEM (Fig. 3C). In contrast, only the fruit V-ATPase showed partial inhibition by vanadate (Fig. 3D). Fig. 3D also shows that the fractions making up the V-ATPase peak after S-400 HR chromatography progressively lost their vanadate sensitivity with subsequent Econo-Q column purifications. This indicates either that the vanadate sensitivity is associated with a contaminating ATPase, or that a subpopulation of V-ATPases, perhaps partial V_1 complexes, exhibit nitrate- and vanadate-sensitive ATPase activity. The latter hypothesis is supported by the Econo-Q activity profiles and gels in which the main vanadate-sensitive peak exhibited a subunit composition compatible with that of a V_1 complex partially depleted of its catalytic subunit (Fig. 2).

Partially purified fruit and epicotyl V-ATPases from a step elution of the second Econo-Q column (see “Experimental Procedures”) were reconstituted into artificial proteoliposomes, and their proton pumping activities were compared. When the proton gradients had stabilized, the reactions were stopped by adding EDTA, allowing the pH gradients to collapse due to proton leakage.

In Fig. 4, the upper panel shows six different comparisons based on five different experiments (an epicotyl trace is shown twice in B and E, and a fruit trace is shown twice in A and B). Panels A and C represent equal protein concentrations. The proteoliposome concentrations in Fig. 4, B and C, were chosen to give equal initial rates of proton pumping. In Fig. 4, D and E, the protein concentrations were normalized to generate equal fluorescence quenching at equilibrium. In Fig. 4E the proteoliposomes also displayed equal proton leakage rates. Fig. 4F represents aged proteoliposomes with leaky membranes.

When equal protein concentrations of freshly prepared proteoliposomes were used (Fig. 4, A and C) or when the concentrations of reconstituted fruit and epicotyl proteoliposomes were adjusted to yield equal initial rates of proton pumping (Fig. 4, B and C), the reconstituted fruit V-ATPase consistently generated a steeper pH gradient than the reconstituted electrolytic enzyme. When the proteoliposome concentrations were adjusted so as to build up equal pH gradients at equilibrium, the initial rate of pumping by the fruit V-ATPase was lower than that by the electrolytic enzyme (Fig. 4D). This latter result was obtained even when the proteoliposomes exhibited equal leakage rates (Fig. 4F).

Leakage and intrinsic uncoupling or “slip” rates were estimated according to Tu et al. (7) as detailed under “Experimental Procedures.” For each of the curves shown in Fig. 4, A–F, the rate constants k_1, k_3, and k_2 are given in the table in the lower
If all five fruit and epicotyl traces are considered, the average leakage rate constants for the fruit and epicotyl proteoliposomes are approximately equal, 0.047 ± 0.029 and 0.045 ± 0.011, respectively. In contrast, the slip rate constants average 0.341 ± 0.121 for the fruit V-ATPase, and 0.687 ± 0.212 for the epicotyl enzyme. The average epicotyl/fruit slip ratio is 2.0 ± 0.3. These values were obtained by considering the initial third of the proton pumping curves and the second half of the leakage curves. If the entire curves were included in the calculations, the epicotyl/fruit slip ratio averaged 2.4 ± 0.4.

Note that the slip rate of the reconstituted epicotyl V-ATPase was higher than that of the fruit enzyme under every condition tested.

The reconstituted proteoliposomes containing purified fruit and epicotyl V-ATPases exhibited similar polypeptide profiles, as shown in Fig. 5. Both had bands at 66, 55/56, 52, 42, 36, 31, 17, 16, and 13 kDa. In addition, the fruit V-ATPase contained bands at 100 and 78 kDa, as well as a doublet at 33/34 kDa. The 100- and 78-kDa bands by themselves had no ATPase activity as shown by the second Econo-Q profile (Fig. 2). Quantitative differences were also observed. For example, the fruit enzyme was strongly enriched in a 16-kDa polypeptide, which was slightly depleted in the catalytic subunit (66 kDa), compared with the epicotyl, and had a more pronounced doublet at 55/56 kDa.

Table I shows the sensitivities of the two reconstituted proton pumps to nitrate, bafilomycin, and NEM. Proton pumping by the reconstituted fruit V-ATPase was slightly less sensitive to nitrate and NEM than the epicotyl V-ATPase, especially at low concentrations, but it was as sensitive as the epicotyl V-ATPase to bafilomycin. The fruit V-ATPase also retained its partial sensitivity to vanadate (Fig. 6A). Because the fruit proteoliposomes were 100% sensitive to low concentrations of bafilomycin, the vanadate sensitivity of the pump cannot be due to a contaminating P-type ATPase (Fig. 6B). H⁺-pumping by the reconstituted epicotyl V-ATPase was completely insensitive to vanadate. Furthermore, the fruit V-ATPase, which was insensitive to oxidation in its native membrane (2), was now as prone to oxidation as the epicotyl V-ATPase (Fig. 7), and the inhibition could be partially reversed by 50 mM DTT.

Fig. 8 shows electron micrographs of tonoplast enriched membrane fractions from lemon fruits and epicotyls, negatively stained with methylamine tungstate. Both membranes showed the typical ball-and-stalk structures of the vacuolar type H⁺-ATPases previously described (9–11). Although the hydrophilic portion of both complexes were roughly comparable in size, the stalk portions of the epicotyl V-ATPases were barely visible, whereas those of the fruit V-ATPases were quite prominent (see lower insets). The thicker stalks of the fruit V-ATPases may reflect the presence of additional subunits. Alternatively, the thinner stalk of most epicotyl V-ATPases may represent artifactual loss of subunits during negative staining.

**DISCUSSION**

The proton pumping activity of native tonoplast-enriched membrane vesicles from lemon juice sacs exhibits an unusual insensitivity to inhibitors of V-ATPases, including nitrate, bafilomycin, NEM, and oxidation (2). Nevertheless, juice sac tonoplasts contain an authentic V-ATPase that, when purified and reconstituted into artificial liposomes, exhibit properties similar to those of other eukaryotic V-ATPases. As in the case of the epicotyl V-ATPase, proton pumping and ATPase activities of the reconstituted juice sac V-ATPase were inhibited by nitrate, NEM, bafilomycin, and oxidation. These results suggest that native membrane lipids play an important role in
tution had specific activities of 2.9 m mol/min (epicotyl) and 3.5 m mol/min (fruit) of nitrate-sensitive ATPase activity equivalents of reconstituted vesicles were loaded. The molecular mass of standard proteins (Std) is indicated on the left of the gel. V-ATPase subunits are identified on the right.

FIG. 4. ATP-dependent proton pumping by purified and reconstituted V-ATPases from lemon juice sacs and epicotyls. Upper panel, seven different fruit (Fr) and epicotyl (Epi) V-ATPase reconstitutions were used to generate 10 distinct proton pumping and leakage profiles. The assay mix was as described under “Experimental Procedures.” The reaction was started with MgSO4. Proton leakage by the proteoliposomes was assessed by stopping the reaction with EDTA after equilibrium was reached, and the residual pH gradient was collapsed with gramicidin. In A–F, the profiles involved proteoliposomes stored frozen or maintained on ice for extended periods of time. In D–F, the profiles were obtained with freshly prepared proteoliposomes. In D–F, the profiles of proteoliposomes with gramicidin. In A–C, the profiles were obtained with freshly prepared proteoliposomes. In A, B, E, and F, the specific activities of the V-ATPases used for reconstitution were in the range of 4.5 ± 0.4 m mol P i/mg min 1 and the protein/lipid ratio was maintained constant at (1.6 ± 0.1) × 10 3 for both the fruit and epicotyl reconstitutions. In C and D, the V-ATPases used for reconstitution had specific activities of 2.9 ± 0.6 m mol P i/mg min 1 and 5.0 ± 0.1 m mol P i/mg min 1 and the protein/lipid ratios were (1.4 ± 0.1) × 10 3 and (2.7 ± 0.1) × 10 3 for the fruit and epicotyl proteoliposomes, respectively. The protein concentrations used for each reaction are given in parentheses (Epicotyl / Fruit). In A, equal protein concentrations were used to generate the pumping profiles. In B and C, the proteoliposome concentrations were adjusted to obtain equal initial rates. In D and E, the proteoliposome concentrations were adjusted so as to build up equal pH gradients at equilibrium. In F, proteoliposomes with high leakage rates are compared. Lower panel, leakage (k L), slip (k S), and the combined leakage and slip (k) rates as determined from the curves A–F according to Ts et al. (7). k was determined by linearizing the initial third of the proton pumping curves, and k S was based on the second half of the leakage curves. In either case, the linear fittings were better than 99%. (If the entire curves were considered, the linear fittings were better than 98%.)

protecting the fruit enzyme from inactivation in vivo. We have previously shown that the juice sac tonoplast is less permeable to protons than the tonoplast of epicotyls (2). Preliminary membrane viscosity measurements indicate that the juice sac tonoplast is more rigid than that of the epicotyl.3 Thus, the specialized lipid composition of the juice sac tonoplast apparently serves two important roles, reducing proton permeability and protecting the V-ATPase against inactivation. Although the purified, reconstituted fruit V-ATPase, unlike the V-ATPase in the native membrane, exhibited an inhibitor profile similar to that of the epicotyl, it differed in two respects: 1) it retained its sensitivity to vanadate, and 2) it pumped protons with twice the efficiency, i.e. half the slip rate, of the epicotyl V-ATPase. The latter observation suggests that intrinsic structural fea-

3 M. L. Müller, A. Brune, L. Taiz, and E. Echeverría, unpublished data.
than that of intact V-ATPases, and possibly carry out proton pumping as well. Further studies are needed to confirm this point.

It is unlikely that the partial vanadate sensitivity associated with the lemon fruit V-ATPase is related to a P-type inhibition mechanism for three reasons: 1) vanadate concentrations required for maximal inhibition of the juice sac V-ATPase were higher than those needed for inhibition of P-type ATPases; 2) the purified, reconstituted fruit V-ATPase was completely inhibited by bafilomycin; and 3) inhibition by vanadate has also been observed in other V-ATPases such as those from osteoclasts (14), yeast, chromaffin granules (15), and plants (16). So far, none of the nucleotide sequences obtained from these materials have shown a P-type motif in their catalytic site (17, 18).

However, as shown by a recent report by David et al. (19), vanadate inhibition of the chicken kidney V-ATPase was dependent on the presence of ADP and was suggested to involve the formation of a vanadate-ADP complex at a nucleotide binding site. Although extremely high concentrations of vanadate were needed to inhibit the chicken kidney V-ATPase (IC50 5 1.58 mM), much higher than those used in this study, a similar mechanism could apply in the case of the lemon fruit and may be favored by the partial dissociation of the catalytic complex. This would be consistent with our previous observation that the proton pumping activity of tonoplast-enriched vesicles from lemon fruits became increasingly sensitive to vanadate after nitrate- and cold-induced release of the catalytic subunit. Although it is generally assumed that the whole catalytic complex is dissociated by treatment with chaotropic agents, the overall unchanged proton pumping activity measured with nitrate-treated lemon fruit vesicles implies that the enzyme remains functional despite the partial loss of subunits (2). Alternatively, it could be that a second nitrate-insensitive proton pump on the membrane becomes activated in response to a reduction in the membrane potential resulting from the inactivation of the V-ATPase. This second, vanadate-sensitive proton pump would thus compensate for the loss of the V-ATPase activity and maintain the total proton pumping activity unchanged.

Based on a measured H+/ATP stoichiometry of 2 (20–22), the maximum ΔpH that a V-ATPase can generate under typical physiological conditions is around 4 pH units (23). This is sufficient for a fully coupled V-ATPase to reach a vacuolar pH of 2.5, provided it is operating at thermodynamic equilibrium and the cytosol is slightly acidified to pH 6.5. This latter condition seems to be fulfilled in the mature lemon fruit as sug-

### Table I

| Treatment        | Activity          |
|------------------|-------------------|
|                  | Tonoplast enriched membranes | Fruit | Reconstituted V-ATPase |
| Control          | 100               | 100   | 100 |
| 100 mM KNO3      | 1 ± 0             | 80 ± 1| 0 ± 0 |
| 1 mM Bafilomycin A1 | 27 ± 12         | 103 ± 4| 0 ± 0 |
| 100 μM NEM       | 1 ± 0             | 82 ± 0| 3 ± 2 |
| 1 mM NaN3        | 71 ± 4            | 81 ± 1| 89 ± 13|

**FIG. 6.** Inhibition of the purified, reconstituted V-ATPases from lemon juice sacs and epicotyls by vanadate and bafilomycin A1. A, the effect of vanadate on the initial rates of proton pumping as measured by ACMA fluorescence quenching. Vesicles were preincubated in the reaction mix for 10 min in the presence of vanadate, prior to starting the reaction with magnesium. B, the effect of Bafilomycin A1 on the initial rates of proton pumping by the reconstituted proteoliposomes under similar conditions. ● ●, epicotyl V-ATPase; ○ ○○ ○, juice sac V-ATPase.

**FIG. 7.** Oxidative inactivation of the purified, reconstituted V-ATPases of epicotyls and juice sacs. The reconstituted proteoliposomes were incubated at 20 °C in the absence of DTT, and the initial rates of proton pumping were assayed at different time intervals. After 4 h, 50 mM DTT were added to the membranes and the activity was monitored for another 2 h. ● ●, epicotyl V-ATPase; ○ ○○ ○, juice sac V-ATPase.
The dark 16-kDa band visible below the 17-kDa proteolipid in the fruit preparation was strongly enriched in six out of 10 fruit proteoliposome preparations and therefore appears to be characteristic of the reconstituted juice sac V-ATPase (the proteins of the other four preparations were run on SDS-polyacrylamide gels which did not resolve the low molecular weight bands). Although the function of this 16-kDa band is unknown, it is tempting to speculate that, together with the 33/34-kDa doublet, it is involved in tighter coupling of the fruit enzyme. A V-ATPase polypeptide, isolated from bovine chromaffin granules, and migrating on SDS-PAGE to an apparent molecular mass of 16 kDa, has recently been shown to be homologous to subunit b of the F$_0$ complex (12). Since the b subunit in F-ATPases connects the catalytic subunit with the a subunit of the channel, it has been implicated in the coupling between the ATP-hydrolytic and proton transport sites (12). If the heavily stained 16-kDa band present in the reconstituted fruit enzyme were related to the b subunit of F-ATPases, its occurrence in multiple copies in the reconstituted fruit V-ATPase could explain the tighter coupling of the enzyme and its ability to build up a higher pH gradient across the membrane.

In electron micrographs of negatively stained tonoplast preparations, the "stalk" portion of the V$_1$ complex of the juice sac V-ATPase appeared to be thicker than that of the epicotyl V-ATPase. This is consistent with the proposal that the catalytic complex of the fruit enzyme is more firmly anchored to the membrane than that of the epicotyl V-ATPase. Multiple copies of a "bridging" subunit might also account for the apparent presence in the fruit tonoplast of partially disassembled V-ATPases capable of both ATP hydrolysis and proton pumping. Finally, the increased stability afforded by a reinforced stalk might explain the failure of nitrate to inhibit proton pumping in native vesicles (2), despite the loss of a major portion of its catalytic subunits.

Acknowledgments—We thank Deborah Bailey and Kira Steinberg for technical assistance in the preparation of the membranes. We also gratefully acknowledge the invitation of Dr. Rafael Ratajczak and Prof. Ulrich Lüttge to carry out the electron microscopy experiments at Darmstadt.

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Fig. 8. Electron micrographs of tonoplast enriched vesicles from lemon juice sacs and epicotyls, negatively stained with methylimine tungstate. Tonoplast-enriched vesicles from epicotyls (A) and juice sacs (B) were prepared as described under "Experimental Procedures" and examined in the electron microscope at a primary magnification of × 50,000 or × 85,000. The bars represent 50 nm in each panel.
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Purification and Reconstitution of the Vacuolar H\(^{+}\)-ATPases from Lemon Fruits and Epicotyls

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J. Biol. Chem. 1997, 272:12762-12770.
doi: 10.1074/jbc.272.19.12762

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