Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 8547–8551, August 1994
Plant Biology

Vacuolar H^+-pumping ATPase variable transport coupling ratio controlled by pH

(vacuolar adenosinetriphosphatase/tonoplast energization/patch clamp/stoichiometry/vacuolar pH)

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Communicated by André T. Jagendorf, April 25, 1994 (received for review January 24, 1994)

ABSTRACT **The eukaryote endomembrane system contains a class of H^+-pumping ATPases (H^+-ATPases) of the vacuolar type (V-ATPases) that are responsible for the acidification of organelles. Their action is critical to numerous physiological processes, but the regulatory mechanisms that may control activity are not yet fully understood. The ratio of H^+ transported per ATP hydrolyzed (n) has been determined thermodynamically for the red beet V-ATPase by using patch clamp. The value of n was found to range from 1.75 to 3.28 and was strictly dependent on cytoplasmic and lumenal pH. This suggests a mechanism by which V-ATPases are regulated by and might therefore control cytoplasmic and lumenal pH. Furthermore, the substantial capacity of plant vacuoles for H^+ accumulation to pH 3 or lower can only be explained by the finding that n can adopt a value of <2.**

Acidification of organelles is critical to membrane trafficking, solute compartmentalization, and cytoplasmic pH homeostasis (1–3). Proton-pumping from the cytoplasm into the organelle lumen is accomplished by H^+-pumping ATPases (H^+-ATPases) of the vacuolar-type (V-ATPases), which are widely distributed among the non-energy-coupling endomembranes of eukaryotes (4). The operational transport coupling ratio [n—i.e., H^+-transported per ATP hydrolyzed (5)] is the prime thermodynamic determinant of the capacity of these enzymes for pH gradient formation across the endomembrane and has significant consequences for mechanistic aspects of ion pumping in this and related classes of H^+-translocating ATPase (6). Previous estimates of n center around a fixed value of 2, principally derived from kinetic methods whereby H^+ translocation is compared with ATP hydrolysis (7–11). Such kinetic estimates may be distorted by unquantified H^+ recirculation and uncoupling of ATP hydrolysis and are commonly restricted to a single pH at which scalar production or consumption of H^+ is minimized. Here, we demonstrate the utility of a thermodynamic determination of n based on the measurement of the reversal potential, E_{rev} (12), of the H^+-ATPase in a single intact organelle (in this instance, the higher plant vacuole) using a patch-clamp configuration analogous to the "whole-cell" mode (13).

For the generalized pump reaction

$$n[H^+]_c + ATP \rightleftharpoons n[H^+]_v + ADP + P_i, \quad [1]$$

where brackets signify chemical activity and subscripts c and v denote the cytoplasmic and vacuolar (lumenal) compartments, respectively, E_{rev} (the membrane potential at equilibrium) of the pump current is given as

$$E_{rev} = (RT/nF)\cdot\ln([P_i][ADP][H^+]_v^n/K_{ATP}[ATP][H^+]_c^n), \quad [2]$$

where K_{ATP} is the apparent equilibrium constant for ATP hydrolysis, and R, T, and F have their usual meanings. Membrane potential ($\Delta\Psi$) is here defined with reference to the lumenal compartment [i.e., as $\Psi_c - \Psi_v$ (14)]. Therefore, the value of E_{rev} can be determined for a given transmembrane pH gradient and mass action ratio. Hence, n can be estimated. In practice, E_{rev} can be measured to an accuracy within 10 mV, which means that when all other parameters in Eq. 2 are known, n is defined to within 0.1. The results show that n is a flexible rather than a fixed parameter and can attain a range of noninteger values that are strictly dependent on cytosolic and lumenal pH.

MATERIALS AND METHODS

Vacuole Preparation and Patch-Clamp Protocol. Red beet plants (*Beta vulgaris*) were grown, and storage tissue vacuoles were isolated as described (15). Fresh vacuoles were prepared for each experiment and varied in diameter from 15 to 25 μm. Patch-clamp methodology has been described (13); the specific protocol followed was as described (12, 15) with the following two exceptions. (*i*) Pipets were dipped into a small volume of Sigmacote (Sigma). Immersion was to approximately 0.5 cm past the shoulder of the pipet, and an air flow through the tip was maintained while the pipet remained in solution. Each pipet was allowed to air-dry and used within an hour of treatment (this replaced conventional coating and fire-polishing). (*ii*) Pulse duration of the bipolar staircase voltage clamp was increased from 4 to 9 s. Seal resistances were from 4 to 15 GΩ. Whole-vacuole current–voltage (*I–V*) recordings were initiated when membrane current was stable at a 0-mV holding potential (and vacuolar pigmentation was lost) and were performed during continuous perfusion of the bathing medium at 20 ± 1°C. The basal bathing and pipet media (respectively equivalent to cytoplasmic and lumenal solutions) contained 100 mM choline-chloride, 5 mM $MgCl_2$, 0.1 mM EGTA, and 20 mM Tris. D-Sorbitol was added to both bathing and pipet solutions to raise their osmolarities 100–150 mosM above that of an expressed cell sap sample. pH was adjusted with 3,3-dimethylglutarate. The *I–V* characteristic of the vacuolar membrane was determined in the presence of ATP, ADP, and P_i (unless otherwise stated) and then determined again in the additional presence of an excess concentration (600 nM) of the specific and irreversible V-ATPase inhibitor bafilomycin A_1 (16, 17). Adenosine nucleotides were added to the bathing medium as Tris salts; P_i, as the free acid. Bafilomycin A_1 was added in a dimethyl

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Abbreviations: H^+-ATPase, H^+-pumping ATPase; V-ATPase, vacuolar-type H^+-ATPase; n, transport coupling ratio (ratio of H^+ transported per ATP hydrolyzed); c, cytoplasmic compartment; v, vacuolar compartment; E_{rev}, reversal potential; K_{ATP}, equilibrium constant for ATP hydrolysis; K_{ref}, reference equilibrium constant; *I–V*, current–voltage; DMSO, dimethyl sulfoxide.

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sulfoxide (DMSO) solution; DMSO was added to all other solutions to the same final concentration (which did not exceed 0.1%, vol/vol). DMSO had no discernable effect on the membrane *I–V* relationship when presented to the cytoplasmic membrane face as a test substance at the highest concentration used (in this instance, pipet and control bathing solution did not contain DMSO).

Analysis of *I–V* Relationships. Liquid-junction potentials were corrected as described (18). Empirical nonlinear least-squares fits using a third-order polynomial were made for each *I–V* relationship (12). The *I–V* characteristic of the vacuolar membrane V-ATPase was obtained by subtraction of the line fitted to the *I–V* relationship of the energized membrane determined in the presence of bafilomycin A_1 from that determined in the absence solely of that inhibitor. The resultant difference *I–V* relationship was thus specifically a bafilomycin-sensitive current; its E_{rev} was the interpolated zero-current intercept.

Estimation of K_{ATP}. Values of K_{ATP} in Eq. 2 were calculated by using the method and association constants as described (19). The ligands considered were ATP^{4-}, ADP^{3-}, and HPO_4^{2-}. The value of the equilibrium constant for the reference reaction (K_{ref}) from (19) was adjusted from the value estimated for 25°C to 20°C with the van't Hoff isochore and had a resultant value of 9.8×10^5 M. Apparent values for K_{ATP} were then determined for each set of ionic conditions by substitution into the relationship

$$K_{ATP} = K_{ref} \times ([HATP^{3-}]/[\Sigma ATP])/([H_2PO_4^-]/[\Sigma P_i]) \times ([HADP^{2-}]/[\Sigma ADP]), \quad [3]$$

where Σ specifies the total ATP, ADP, or P_i present regardless of ionic form.

ATPase Hydrolysis Assay. The potency of bafilomycin inhibition of ATP hydrolysis by the vacuolar membrane V-ATPase over the pH range employed in the patch-clamp experiments was determined by using partially purified uncoupled vacuolar vesicles. Material was taken from red beet storage tissue [grown as specified (15)] and prepared as described (20). The ATPase hydrolysis assay and measurement of P_i production were based on protocols as described (21). The hydrolysis assay mixture contained 100 mM choline-chloride, 50 mM KCl, 3 mM $MgSO_4$, 30 mM Tris, 3 mM Tris ATP salt, and 0.005 mM gramicidin (pH 5–9). Bafilomycin (in DMSO) was added to a final concentration of 100 nM; control solutions contained an equal concentration of DMSO. The reaction was started by the addition of 10–20 μg of membrane protein to a final assay volume of 300 μl and ran for 20 min at 37°C. Results were taken from three replicates at each pH.

RESULTS

Isolation of the V-ATPase Current in Hydrolytic and Synthetic Modes. In the selective presence of 5 mM ATP and with pH_c 8.0 and pH_v 5.5, the bafilomycin-sensitive current was outward from the cytoplasm through the entire range of clamped potentials from −90 to +90 mV (Fig. 1). The mean (± SEM) current when the membrane was clamped at 0 mV amounted to +24 (± 10) $mA \cdot m^{-2}$ in five vacuoles from separate preparations. This bafilomycin-sensitive current was freely reversible. Thus, in the selective presence of 5 mM ADP and 10 mM P_i, the bafilomycin-sensitive current at 0 mV was inward to the cytoplasm and had a mean value of −28 (± 10) $mA \cdot m^{-2}$ in four trials at pH_c 7.6 and pH_v 4.8. Activity of a second phosphoanhydrolase at the vacuolar membrane (an H^+/K^+-pumping inorganic pyrophosphatase), which (through its P_i-dependent reversal) could distort measurements made in the presence of cytosolic P_i (compare with ref. 29), was prohibited by the omission of K^+ from both the pipet and bathing solutions (12, 15). Bafilomycin A_1 had no discernable effect on the membrane *I–V* relationship in the absence of ATP, ADP, and P_i. Addition of 5 mM ADP alone had no effect on the membrane *I–V* characteristic, and 10 mM P_i is known to evoke a negligible current (12). Thus, the bafilomycin-sensitive current evoked by the presence of both of these ligands can be identified reasonably as that mediated by the V-ATPase. Although both the forward (hydrolytic) and reverse (synthetic) currents through the V-ATPase were voltage-sensitive (Fig. 1), in neither set of experiments did the bafilomycin-sensitive current cross the voltage axis. This failure to find a defined E_{rev} for the V-ATPase is anticipated for these conditions, since in both forward and reverse modes, the ligands required to catalyze current reversal were not present.

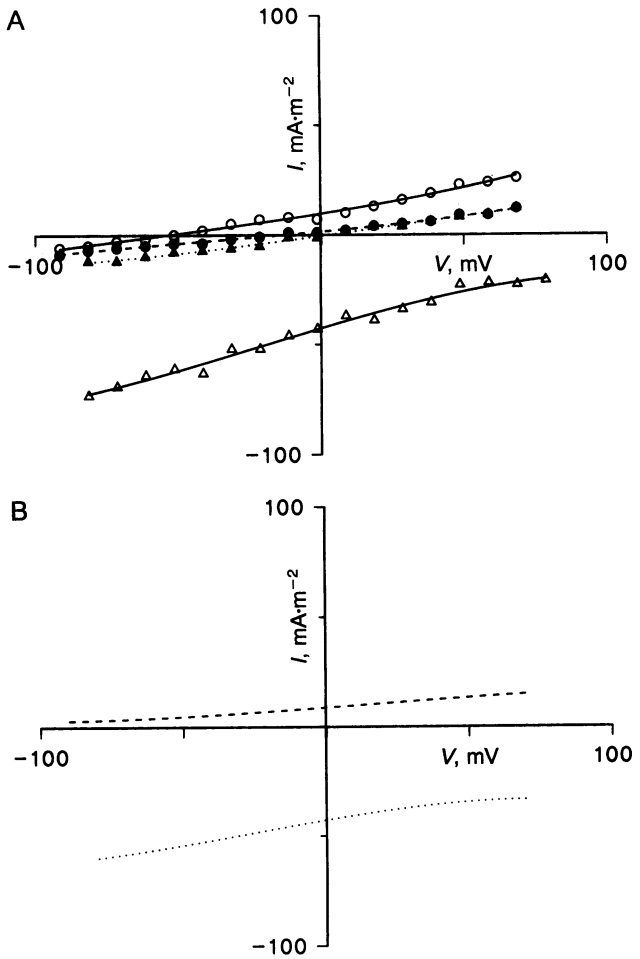

FIG. 1. *I–V* relationships of hydrolytic (- - -) and synthetic (· · ·) modes of V-ATPase. (*A*) Whole membrane *I–V* relationships for two separate vacuoles in the selective presence of 5 mM ATP (○) or 5 mM ADP and 10 mM P_i (△), with subsequent *I–V* relationships measured in the additional presence of 600 nM bafilomycin A_1 (●, ▲). (*B*) *I–V* difference relationships of the respective bafilomycin A_1-sensitive components from *A*. The bathing (cytoplasmic) and pipette (lumenal) solutions were pH 8.0 and 5.5, respectively, for the hydrolytic mode (- - -) and were pH 7.6 and 4.8, respectively, for the synthetic mode (· · ·). The diameter of both vacuoles was approximately 15 μm (surface area, 7.07×10^{-10} m^2).

The Measured E_{rev} of the V-ATPase. With all ligands present, the activities used in the kinetic reversal experiments also generated *I–V* difference relationships for the V-ATPase with E_{rev} within measurable range (± 90 mV). Fig. 2 shows that the bafilomycin-sensitive difference current displayed a clear E_{rev} in the presence of 5 mM ATP, 5 mM ADP, and 10 mM P_i, with a 2.8-unit pH difference across the membrane. A summary of E_{rev} values, observed over a range

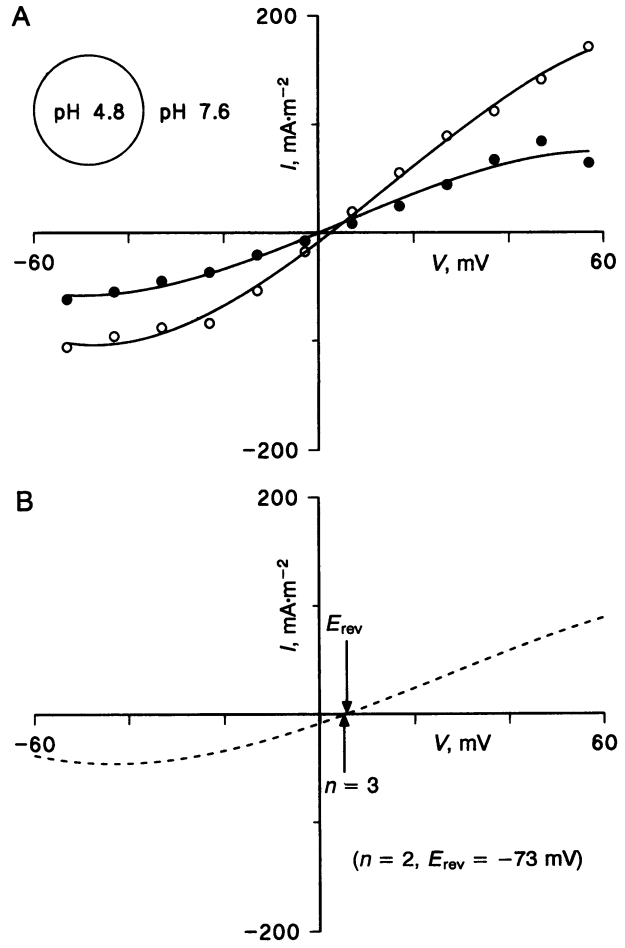

FIG. 2. Determination of E_{rev} for the V-ATPase from bafilomycin A_1-sensitive *I–V* relationships. (*A*) Whole-membrane *I–V* relationships obtained from a single vacuole in the presence of 5 mM ATP, 5 mM ADP, and 10 mM P_i, at lumenal pH 4.8 and cytoplasmic pH 7.6 in the absence (○) and subsequent presence (●) of 600 nM bafilomycin A_1. (*B*) Bafilomycin A_1-sensitive *I–V* relationship. The E_{rev} of the enzyme-mediated current is shown as E_{rev} (- - -) along with the projected E_{rev} values for the H^+-ATPase with integral values of $n = 2$ or 3 calculated from Eq. 2. The vacuole diameter was approximately 15 μm; the surface area was 7.07×10^{-10} m^2.

of values of pH_c and pH_v, and the resultant values of *n* are shown in Table 1. The hydrolytic assays of ATPase activity in partially purified uncoupled vacuolar vesicles demonstrated that inhibition by bafilomycin was to a residual level, which was pH-independent over the pH range 5–9 (Fig. 3).

Table 1. pH dependence of E_{rev} and *n* of V-ATPase

Data set	pH_c/pH_v	E_{rev}, mV: Predicted $n = 2$	Predicted $n = 3$	Observed	*n* (H^+/ATP)
A	7.6/4.80	−73	+5	+3 ± 2 (3)	2.95 (2.92, 2.99)
B	7.6/3.92	−22	+57	+39 ± 7 (3)	2.70 (2.59, 2.81)
C	7.0/4.80	−95	−21	−8 ± 1 (4)	3.28 (3.26, 3.31)
D	8.0/4.32	−32	+50	−24 ± 9 (5)	2.07 (1.99, 2.15)
E	8.0/3.26	+30	+112	−5 ± 3 (4)	1.75 (1.73, 1.77)

Observed values of E_{rev} are derived from bafilomycin A_1-sensitive *I–V* relationships of the type shown in Figs. 2 and 4 and are given as means ± SEM (number of independent trials in parentheses). Estimates of *n* have been calculated from the mean values of E_{rev} according to Eq. 2, with the values in parentheses derived from the error limits on E_{rev}. All trials shown were performed in the presence of 5 mM ATP, 5 mM ADP, and 10 mM P_i.

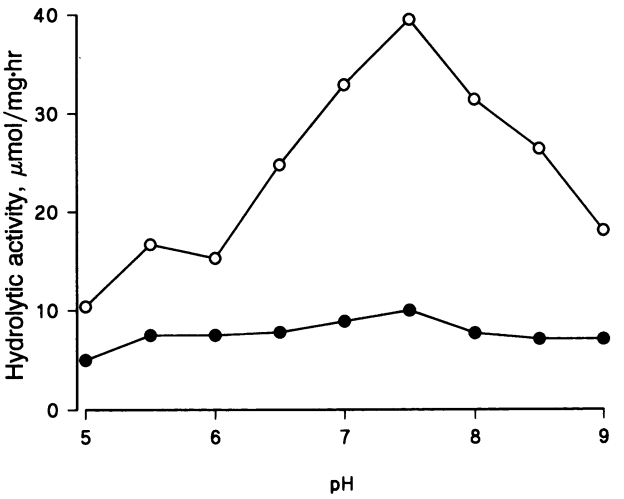

FIG. 3. Effect of 100 nM bafilomycin A_1 on the pH hydrolytic activity profile of the V-ATPase in uncoupled partially purified vacuolar vesicles. Hydrolytic activity evoked by 3 mM Tris ATP was assayed as described in text. ○, Control; ●, with 100 nM bafilomycin A_1. Each data point is the mean of three replicates.

Such activity might be attributed to nonspecific phosphatases, but (as the vesicles were not preincubated with bafilomycin) it is likely that an initial P_i release by the V-ATPase has been detected. The use of an excess concentration of bafilomycin and the sampling of current at steady state serves to minimize the impact of any residual V-ATPase activity in the patch-clamp experiments. Moreover, incomplete inhibition would impact solely on the kinetic rather than the thermodynamic properties of the V-ATPase (i.e., the E_{rev} of any residual V-ATPase would, of necessity, be also that of the bafilomycin-sensitive V-ATPase).

In control conditions, in which the respective values of pH_c 7.60 and pH_v 4.80 are close to those thought to occur in the majority of plant cells *in vivo* (22), *n* is close to 3 (Table 1, data set A). However, lowering pH_v by 0.88 unit resulted in a small but significant decrease in *n* to a noninteger value of 2.70 (Table 1, data set B). Modulation of cytoplasmic pH had a more profound effect on *n*: for pH_v 4.80, decreasing pH_c from 7.6 to 7.0 resulted in an increase of *n* from 2.95 to 3.28 (Fig. 4 and Table 1, data set C compared with set A), while increasing pH_c to 8.0 with a 0.5-unit drop in pH_v decreased *n* dramatically to 2.07 (Table 1, data set D). Comparison of set D with set B shows, furthermore, that markedly different values of *n* occurred in the presence of a single transmembrane pH gradient (here, 3.68 unit). Such results emphasize that *n* is not determined uniquely by the pH difference but by the absolute pH on each side of the membrane.

All of the trials shown in Table 1 were performed in the presence of 5 mM ATP, 5 mM ADP, and 10 mM P_i; the interpulse holding potential of the voltage clamp was 0 mV. Reduction of ATP to 1 mM for the conditions shown in data set D elicited a mean positive shift in E_{rev} of 36 mV (four trials), but E_{rev} compensation by the mass-action ratio component of Eq. 2 resulted in no significant change in the estimate of *n*. Trials in which the holding potential was set at −50 mV but all other conditions were as data set A yielded a small (10 mV) mean negative shift in E_{rev} (eight trials).

DISCUSSION

The current mediated by the vacuolar membrane ATPase may be isolated by using bafilomycin A_1 as a specific inhibitor of the enzyme. Moreover, the enzyme is operative in the K^+-free conditions required to prevent the simultaneous

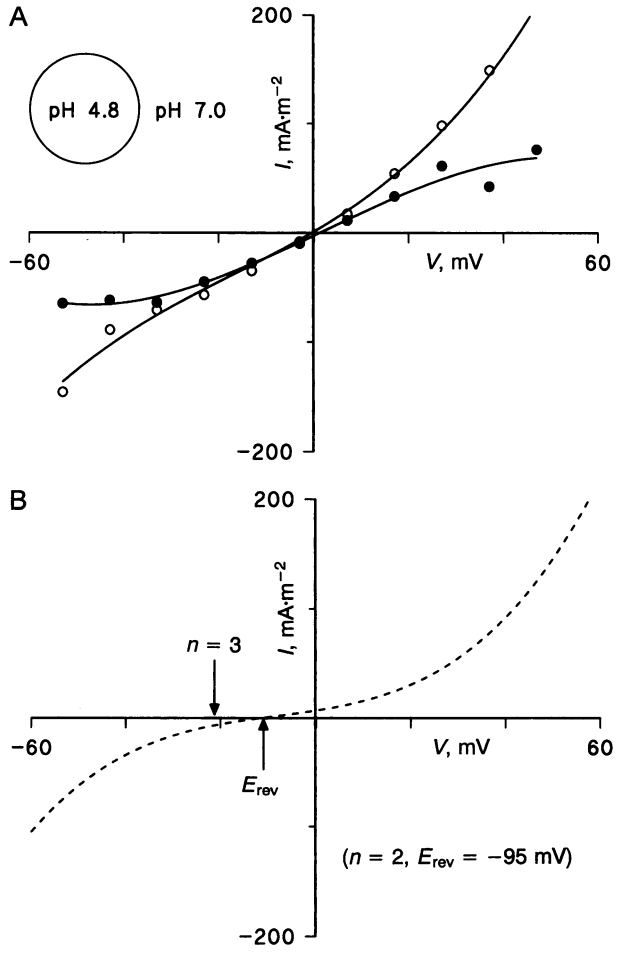

FIG. 4. Determination of E_{rev} for the V-ATPase from the bafilomycin A_1-sensitive *I–V* relationship at pH_c 7.0. (*A*) Whole-membrane *I–V* relationships obtained in the absence (○) and subsequent presence (●) of 600 nM bafilomycin A_1. Ligand concentrations were as given in the legend to Fig. 2; lumenal pH was 4.8, and cytoplasmic pH was 7.0. (*B*) The bafilomycin A_1-sensitive *I–V* relationship derived from the data in *A*. The vacuole diameter was approximately 15 μm; the surface area was 7.07×10^{-10} m².

P_i-elicited action of the inorganic pyrophosphatase. The finding that the operational coupling ratio, *n*, of the V-ATPase is not a fixed parameter has significant consequences for our understanding of tonoplast energization. The value of *n* close to 3 at pH_c 7.60 and pH_v 4.80 (Table 1, data set A) conforms with that determined from analysis of V-ATPase-generated short-circuit currents in turtle bladder at pH_c 7.4 (23). However, were a value of $n = 3$ sustained in all circumstances by the plant V-ATPase, the minimum vacuolar pH that could be generated *in vivo* can be estimated to be 4.9 units [taking the cytoplasmic concentrations of ATP, ADP, and P_i as 2.3, 0.31, and 5 mM, respectively (19, 24, 25); pH_c as 7.6; and $\Delta\Psi$ as −20 mV (26)]. It should be noted that the inorganic pyrophosphatase (which resides on the same membrane) has an overall ionic coupling ratio of 3 [1.3 H^+:1.7 K^+ (12)], which puts a strict thermodynamic limit on its capacity to acidify the lumen (19). Examples in which vacuolar pH is lower than 4.9 are reasonably commonplace, especially in fruits and in the leaves of plants that accumulate oxalic acid (27). Thus, for $n = 2$ at pH_c 8.0 (from Table 1, data set D), the *in vivo* thermodynamic limit on pH_v decreases to 3.6 units. Nevertheless, even this capacity of an ATPase pumping $2H^+$-ATP to generate a low pH_v does not match values in some fruits (e.g., unripe grapes, blackberries, citrus fruits), where pH_v is in the range 2.7–3.0 (estimated from expressed cell sap). To explore the possibility that the V-ATPase is competent in the generation of a lumenal pH of about 3, the pipet (lumenal) pH was lowered to 3.26 while the bathing (cytosolic) pH remained at 8.0. The results (Table 1, data set E) support the finding that lumenal pH is also a determinant of *n*: in this case *n* is lowered to a noninteger value of 1.75. The corresponding *in vivo* thermodynamic limit on pH_v then becomes 2.88. However, there is as yet no experimental evidence to suggest that such low vacuolar pH values are associated with high cytoplasmic pH.

Our results demonstrate that V-ATPase of plant cells is likely to possess as many as four potential H^+ transport/binding sites. However, the transport activity of the enzyme is subject to partial uncoupling which, although compromising the efficiency of the transport reaction, enables the generation of the large transmembrane pH differences, which characterize the ionic relations of plant vacuoles. It is apparent that this uncoupling is mediated primarily by an increase in pH_c but that decreasing the pH_v also contributes to this effect. It is feasible that a physiological ATP/ADP ratio may in turn exert an effect on *n*, but the result presented here, in which a 5-fold change in the ATP/ADP ratio failed to have a significant effect on that parameter, does not support such a possibility.

The mechanistic implications of the variable transport coupling ratio remain to be explored. At least four different genes encode the oat 16-kDa subunit of the V-ATPase (28), raising the possibility that enzyme isoforms with different integer coupling ratios coresiding in the same membrane could yield mean measured E_{rev} and hence noninteger *n* values. Alternatively, both the noninteger and variable characteristics of *n* could arise from the activity of a single isoform. Support for this latter explanation derives from success in describing the V-ATPase *I–V* relationships with kinetic models permitting translocation of one or more H^+ through parallel pathways in the reaction cycle (J.M.D., D.S., and D. Gradmann, unpublished data). It seems reasonable to assume that cytoplasmic pH will determine the extent of occupancy of the H^+ transport sites, with the enzyme competent in catalysis even when all sites are not filled. Likewise, lumenal pH could determine the effective value of *n* through variable dissociation. It seems clear, however, that extensive kinetic controls on these slip reactions (defined here as ATP hydrolysis without the translocation of the full potential complement of H^+) will be required in many circumstances to prevent futile cycling or uncontrolled H^+ leakage.

We thank Mr. C. Abbott for plant cultivation, Dr. A. Pope for a gift of bafilomycin A_1, and the Agricultural and Food Research Council (U.K.) for financial support.

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