Cellular Environment Is Important in Controlling V-ATPase Dissociation and Its Dependence on Activity*

Jie Qi and Michael Forgac

From the Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts 02111

One mechanism of regulating V-ATPase activity in vivo involves reversible dissociation into its component V₁ and V₀ domains, which in yeast occurs in response to glucose depletion. V-ATPase complexes containing the Vph1p isoform of subunit α (VCC) are targeted to the vacuole, and Stv1p-containing complexes (SCC) are targeted to the Golgi. Overexpression of Stv1p results in mistargeting of SCC to the vacuole. We have investigated the role of the α subunit isoform and cellular environment in controlling dissociation using vacuolar protein sorting (vps) mutants that accumulate proteins in either the prevacuolar compartment (PVC) (vps27Δ) or a post-Golgi compartment (PGC) (vps21Δ). Dissociation of both VCC and SCC depends upon cellular environment, with dissociation most complete in the vacuole and least complete in the PVC. The dependence of dissociation on V-ATPase activity was also investigated using both concanamycin and inactivating mutations. Concanamycin partly blocks dissociation of both VCC and SCC in all three compartments, with inhibition generally greater for SCC than VCC. The R735Q mutant of Vph1p results in loss of both ATPase and proton pump activity, whereas the R735K mutant lacks proton transport but has 10% of wild type ATPase activity. For VCC in the vacuole, dissociation is completely blocked for the R735Q but not the R735K mutant. Significant dissociation of VCC is observed for both mutants in the PVC and PGC, indicating that V-ATPase activity is not absolutely required for dissociation. Similar results were obtained for SCC, although dissociation of SCC is again generally more sensitive to activity than VCC. These results suggest that the cellular environment is important both in controlling in vivo dissociation of the V-ATPase and the dependence of this process on catalytic activity. Moreover, catalytic activity is not absolutely required for V-ATPase dissociation.

The vacular (H⁺)-ATPases (V-ATPases)² are a family of ATP-dependent proton pumps that both acidify intracellular compartments and pump protons across the plasma membrane (1–4). Within intracellular compartments, V-ATPases function in receptor-mediated endocytosis, intracellular membrane traffic, degradation and processing of proteins, and coupled transport of small molecules, such as neurotransmitters. They also assist in the entry of envelope viruses, such as influenza virus, and the killing of cells by toxins, such as diphtheria toxin and anthrax toxin (5). V-ATPases are also present in the plasma membrane of cells, where they function in bone resorption (6), renal acidification (3), sperm maturation (7), pH homeostasis (8), and tumor metastasis (9). V-ATPases are thus potential targets in treating such diseases as osteoporosis and cancer.

The structure and mechanism of V-ATPases resemble that of the F₁F₀-ATP synthase (10). V-ATPases are composed of a peripheral V₁ complex consisting of eight subunits (A–H) that hydrolyzes ATP and an integral V₀ complex consisting of six subunits (a, d, e, c’, c’, and c”) that conducts protons (1–4). The V₀ complex includes a ring of proteolipid subunits (c, c’, and c”) onto which subunit d sits (11). Subunit a is a 100-kDa protein composed of a hydrophobic amino terminus and a hydrophobic carboxyl terminus that is in contact with the proteolipid ring (12). V-ATPases operate by a rotary mechanism (13, 14) in which the c-ring rotates relative to subunit a, with proton transport driven by interactions at the subunit a-proteolipid interface (12).

Subunit a is also important for targeting the V-ATPase to different membranes in the cell. In yeast there are two isoforms of subunit a, Vph1p and Stv1p (15, 16). Vph1p targets V-ATPases to the vacuole, whereas Stv1p causes V-ATPases to be retained in the Golgi (16, 17). The amino terminus of subunit a contains the signal responsible for targeting of V-ATPases (18). In a strain disrupted in both Vph1p and Stv1p, overexpression of Stv1p results in the appearance of a significant number of Stv1p-containing complexes in the vacuole (16–18). V-ATPase complexes containing Vph1p and Stv1p also differ in assembly and activity. Stv1p-containing complexes localized to the vacuole show lower assembly of V₀ and V₁ as well as a 4-fold lower coupling of proton transport to ATP hydrolysis relative to Vph1p-containing complexes (17). In mammals there are four isoforms of subunit a (a₁–a₄) (6, 19, 20). The a₃ isoform is present in the plasma membrane of osteoclasts, and mutations in the a₃ gene cause the disease osteoporosis (21). The a₄ isoform is expressed in the plasma membrane of renal intercalated cells, and mutations cause the disease renal tubule acidosis (22).

An important mechanism of controlling V-ATPase activity in vivo involves reversible dissociation of the V₁ and V₀ domains (1, 2). Reversible dissociation has been shown to regulate V-ATPase activity in both yeast and insect cells (23, 24) and has recently been implicated in control of acidification in renal cells (25) and in dendritic cells (26), where V-ATPases are

*This work was supported by National Institutes of Health Grant GM 34478 (to M. F.). 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.

1. To whom correspondence should be addressed: Dept. of Physiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Tel.: 617-636-6939; Fax: 617-636-0445; E-mail: michael.forgac@tufts.edu.

2. The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine 5’-triphosphatase; PVC, pre-vacuolar compartment; PGC, post-Golgi compartment; MES, 4-morpholineethanesulfonic acid; C₁₂E₉, polyoxyethylene 9-lauryl ether; HA, influenza hemagglutinin; CPY, carboxypeptidase Y.
V-ATPase Dissociation Depends on Cellular Environment

essential for antigen processing. A distinctive feature of dissociation of V-ATPases is that the free V1 domain does not hydrolyze MgATP (27), and free V0 is not passively permeable to protons (28). This property is essential to avoid generation of an uncoupled ATPase activity in the cytosol or an unregulated passive proton conductance in cellular membranes.

In yeast, dissociation of V-ATPase complexes is induced by glucose depletion, occurs rapidly and reversibly, and does not require new protein synthesis (23). Various signaling pathways involved in response to glucose depletion appear not to be involved in this process (29), although dissociation (but not reassembly) requires an intact microtubular network (30). By contrast, reassembly (but not dissociation) requires a novel cytoplasmic complex termed RAVE, which is also important for normal assembly of the V-ATPase (31, 32). Thus dissociation and reassembly appear to be independently controlled processes. Dissociation has also been shown to require catalytic activity (29) and to be blocked by chloroquine, which neutralizes internal acidic compartments (33).

Previous results from our laboratory have shown that Vph1p-containing complexes localized to the vacuole undergo glucose-dependent dissociation, whereas Stv1p-containing complexes localized to the Golgi do not (17). By contrast, Stv1p-containing complexes localized to the vacuole do show dissociation. Trafficking of Vph1p-containing complexes to the vacuole can be blocked using vacuolar protein sorting (Vps27) gene knock-out and for HA-Vph1p or HA-Stv1p expression. Yeast cells were grown in yeast extract/peptone/dextrose medium or synthetic dropout medium (35).

Yeast Gene Replacement—The strains MM112 vps21Δ and MM112 vps27Δ were constructed following the protocol of two-step gene disruption (36). The VPS21 and VPS27 genes were replaced by homologous recombination in MM112 by the marker gene Kan’, and the strains MM112 vps21Δ and MM112 vps27Δ were selected using media containing the antibiotic G418.

Transformation and Selection—The single copy plasmid pRS316 carrying the HA-VPH1 sequence (pRS316-VPH1::HA, also called pSKN12) or the HA-STV1 sequence (pRS316-STV1::HA, also called pSKN11) and the high copy plasmid YEp352 carrying HA-Stv1p (YEp352-STV1::HA, also called pSKN14) were constructed previously (17) and were transformed into yeast strain MM112 by the lithium acetate method (37). The transformants were selected on uracil minus (Ura−) plates. For co-transformation of yeast strains with HA-Stv1p and untagged Vph1p, the VPH1 gene was subcloned into pRS413, a vector containing the HIS3 selectable marker. A yeast strain carrying pSKN14 was transformed with pRS413-Vph1p and then selected on uracil and histidine minus (Ura−His−) plates.

Construction of Mutants—Site-directed mutagenesis was performed to introduce mutations R795K and R795Q on Vph1p-containing complexes localized to the Golgi (16). The transformants were confirmed by DNA sequencing. The EcoRI-BamHI fragments of the HA-Stv1p sequence (pRS316-VPH1::HA) were digested with EcoRI-BamHI and were ligated into yeast plasmid pRS316 carrying the HA-STV1 sequence (pRS316-STV1::HA), also called pSKN11. The mutagenic oligonucleotides are designed as 5′-GATCATATCTACACTGGCGTGGCTACA-3′ and 5′-GATCATTATCTAAAACACTTGGCGCTAT-3′ for R795Q and R795K, respectively. Mutations were confirmed by DNA sequencing. The EcoRI-BamHI region of HA-Vph1p was substituted by the corresponding sequence from either the R795Q or R795K mutant of Vph1p constructed previously (38).

In Vivo Dissociation of Yeast V-ATPases in Response to Glucose Deprivation—In vivo dissociation of yeast V-ATPases was induced by glucose deprivation as described before (23) with some modification. The yeast strains MM112, MM112 vps21Δ, and MM112 vps27Δ expressing either normal levels of Vph1p (using pSKN12) or high levels of Stv1p (using pSKN14) were grown overnight, diluted in fresh media to an absorbance of 600 nm of 0.1−0.2, and grown to an absorbance of 0.6−0.8. Cells were harvested and converted to spheroplasts by addition of 0.3 mg/ml Zymolyase in lysis buffer (YPD, 0.1 m Tris/MES, pH 7.5, 0.7 m sorbitol, 2 mM dithiothreitol) and incubated for 30 min at 30 °C. Cells were then split into 2 aliquots and incubated in YEP (without glucose) and YEP medium (with 2% glucose) at 30 °C for 15 min. Spheroplasts were pelleted and lysed in PBS, pH 7.2 containing 1% C12E8, protease inhibitors, and 1 mM dithiobis(succinimidyl propionate). For concanamycin A inhibi-
bition experiments, the inhibitor was added to the medium to a final concentration of 1 μM in the final 10 min of spheroplasting and then kept at this concentration until spheroplasts were lysed.

V-ATPase complexes were immunoprecipitated from the lysate using the antibody 8B1 against subunit A and protein A-Sepharose followed by electrophoresis on 7.5% acrylamide gels and transfer to nitrocellulose. Western blotting was then performed using the horseradish peroxidase-conjugated monoclonal antibody 3F10 against HA to blot the a subunit of the V₀ domain or the antibody 8B1 against the A subunit to blot the V₁ domain followed by a horseradish peroxidase-conjugated secondary antibody. Dissociation of the V-ATPase complex is reflected as a reduction in the amount of subunit a (in V₀ complexes) co-immunoprecipitated by the antibody directed against subunit A (in the V₁ complexes). Densitometry analysis was performed using the software ImageJ (rsb.info.nih.gov/ij/).

To determine the linearity of the signal obtained in the Western blotting experiments, a titration was performed analyzing the signal obtained with varying amounts of the immunoprecipitates using both the anti-HA and anti-A subunit antibodies. Over the intensity range employed in these experiments (relative intensities of ~1,000 to 16,000 units), the signal obtained was linear with the amount of HA-Vph1p loaded but showed some deviation from linearity for subunit A. This may be because detection of subunit A involved both a primary antibody (8B1) and a horseradish peroxidase-conjugated secondary antibody, whereas detection of HA-Vph1p was done directly using a horseradish peroxidase-conjugated anti-HA antibody (3F10), eliminating the necessity of a secondary antibody. Quantitation of assembly was done by measuring the amount of subunit a precipitated, which was linear. The slight nonlinearity of signal with the amount of subunit A was not a problem because samples were matched for the amount of subunit A within a given experiment and nearly matched between experiments.

**Immunosolization of Compartments Carrying HA-Stv1p**—The strains MM112, MM112 vps27Δ, and MM112 vps21Δ were co-transformed with pPRS413-VPH1 and YEp352-STV1-HA, cultured, harvested, and converted to spheroplasts as described under the procedure for measurement of in vivo dissociation. Spheroplasts were lysed by osmotic shock in the absence of detergent in solubilization buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1% glycerol), followed by sedimentation twice for 1 min at 3,000 × g to remove unbroken cells and nuclei. Supernatants were preclarified by addition of protein G-agarose, incubation for 1 h, and sedimentation at 3,000 × g for 1 min. Supernatants were then incubated with the anti-HA antibody (3F10) plus protein G-agarose overnight at 4 °C. The compartments carrying HA-Stv1p were then precipitated by sedimentation, and the proteins were eluted from the beads with SDS sample buffer and separated by SDSPAGE. The presence of both HA-Stv1p and Vph1p in the immunoprecipitates was detected by Western blotting using the monoclonal antibodies against HA (3F10) and Vph1p (10D7), respectively.

**Other Procedures**—Vacular membrane vesicles were isolated using the protocol described previously (39). Quinacrine staining of yeast was performed as described previously (40).

Protein concentrations were determined by the method of Lowry et al. (41). ATPase activity was measured using a coupled spectrophotometric assay in the presence or absence of 1 μM concanamycin A as described previously (33). ATP-dependent proton transport was measured in transport buffer (25 mM MES/Tris, pH 7.2, 5 mM MgCl₂) using the fluorescence probe 9-amino-6-chloro-2-methoxyacridine in the presence or absence of 1 μM concanamycin, as described previously (42).

**RESULTS**

Stv1p-containing Complexes Localized to the Golgi Show Reduced Assembly with V₁ Relative to Vph1p-containing Complexes Localized to the Vacuole—Previous results from our laboratory have demonstrated that V₀ complexes containing the Stv1p isoform of subunit a, when localized to the vacuole through overexpression, showed significantly lower levels of assembly with the V₁ domain than V₀ complexes containing Vph1p, thus giving rise to the ~10-fold difference in ATPase activity for these two complexes (17). Because the vacuolar environment is different in a number of respects from that of the Golgi, it was of interest to determine whether this reduced assembly with V₁ was also observed for Stv1p-containing complexes present in their normal cellular environment, namely the Golgi. HA-tagged Stv1p was expressed at low levels in the yeast strain MM112 using the single copy plasmid pRS316. As a control, HA-tagged Vph1p was also expressed in MM112 using the same plasmid. Complexes containing either HA-Vph1p or HA-Stv1p were immunoprecipitated using the antibody 3F10 directed against the HA tag. After SDS-PAGE, the precipitants were Western blotted using the anti-HA antibody and an antibody directed against subunit A of the V₁ domain. Because subunit A is co-immunoprecipitated with subunit a only when the V₁ and V₀ domains are assembled, the amount of subunit A precipitated using the anti-HA antibody is a reflection of the degree of assembly of the V₁ and V₀ domains. As can be seen in Fig. 1 (left two lanes), Vph1p-containing complexes localized to the vacuole show much better assembly with V₁ than do Stv1p-containing complexes localized to the Golgi. Thus, the difference in assembly behavior of Vph1p- and Stv1p-containing complexes is not an artifact of mislocalization of Stv1p to the vacuole. For comparison, the degree of assembly of Stv1p localized to the vacuole through overexpression is also shown (Fig. 1, left two lanes).
V-ATPase Dissociation Depends on Cellular Environment

3rd lane). For this experiment, the amount of antibody used to precipitate HA is limiting, such that no significant difference between the HA signal in the 2nd and 3rd lanes of Fig. 1 is observed. To determine whether Stv1p-containing complexes localized to the Golgi might be unstable during solubilization and immunoprecipitation, the experiments were repeated in the presence of the specific V-ATPase inhibitor concanamycin, which has been shown to inhibit both Vph1p and Stv1p-containing V-ATPases (17). As can be seen in Fig. 1 (4th and 5th lanes), the difference in assembly of Vph1p-containing complexes in the vacuole and Stv1p-containing complexes localized to the Golgi was observed even in the presence of concanamycin, although Stv1p-containing complexes in the vacuole show much higher assembly with V1 in the presence of concanamycin (Fig. 1, 6th lane). This latter result rules out the possibility that the HA epitope in the HA-Stv1p-containing complexes is not accessible in intact V1V0.

Cellular Environment Influences Glucose-dependent Dissociation of V-ATPase Complexes Containing Either Isoform of Subunit a—To examine the dependence of in vivo dissociation of the V-ATPase on cellular environment, experiments were performed using yeast strains disrupted in two genes of the carboxypeptidase Y (CPY) pathway. V-ATPase complexes containing Vph1p employ the CPY pathway for delivery to the vacuole (34, 43, 44). Yeast strains disrupted in the VPS27 gene accumulate proteins in a PVC as a result of a defect in budding of vesicles from this compartment (43). In strains disrupted in VPS21, proteins accumulate in Golgi-derived vesicles because of the inability of these vesicles to fuse with the PVC (34). The ability of HA-tagged Vph1p- and Stv1p-containing complexes to undergo dissociation in response to glucose depletion was examined in wild type cells as well as cells disrupted in either VPS27 or VPS21. HA-Stv1p was overexpressed using a high copy plasmid, under which conditions a significant amount of HA-Stv1p is mistargeted to the vacuole in a strain that is wild type for the CPY pathway (17).

To test whether Vph1p and overexpressed Stv1p are localized to the same compartments in the vps21Δ and vps27Δ strains, HA-tagged Stv1p and untagged Vph1p were co-expressed in the same yeast strain. Following cell lysis by osmotic shock in the absence of detergent, the compartments containing HA-Stv1p were immunoprecipitated with the monoclonal antibody 3F10 against the HA tag plus protein G-agarose. The immunoprecipitates were then analyzed by Western blotting as described above. As shown in Fig. 2, lanes 1–3, Vph1p is co-immunoprecipitated with HA-Stv1p in wild type cells as well as the vps21Δ and vps27Δ strains. By contrast, no significant co-immunoprecipitation was observed if immunoprecipitation was performed on cell lysates from cells expressing either Vph1p or HA-Stv1p, which were mixed before immunoprecipitation (Fig. 2, lane 4). These results suggest that Vph1p and overexpressed HA-Stv1p are present in the same compartments in both the wild type cells and the vps21Δ and vps27Δ mutant strains.

To determine the amount of glucose-dependent dissociation occurring in each strain, spheroplasts were incubated in the presence or absence of glucose for 15 min at 30 °C followed by detergent solubilization and immunoprecipitation of V1 and V1V0 complexes using an antibody specific for subunit A. Following SDS-PAGE, Western blotting was performed using both the antibody against subunit A and an antibody against the HA epitope tag present in subunit a. The amount of HA-Vph1p or HA-Stv1p co-immunoprecipitated with subunit A thus reflects the degree of association between the V1 and V0 domains. Representative results are shown for both HA-Vph1p (Fig. 3A) and HA-Stv1p (Fig. 3C). Band intensities were determined by densitometry, and the results from at least three independent determinations are shown in the bar graphs of Fig. 3, B and D. The values shown represent the ratio of the degree of assembly observed in the absence of glucose divided by the degree of assembly observed in the presence of glucose. As can be seen, Vph1p-containing complexes showed the greatest degree of glucose-dependent dissociation when localized to the vacuole and the least amount of dissociation when localized to the PVC. These results are in agreement with earlier findings using nontagged Vph1p (17). Stv1p-containing complexes show nearly the same dependence of dissociation on cellular environment, with dissociation being most complete in the vacuole and least complete in the PVC. Thus Stv1p-containing complexes appear to show the same behavior with respect to glucose-dependent dissociation as Vph1p-containing complexes, not only when localized to the vacuole (17) but also when present in other cellular compartments.

Cellular Environment Affects the Dependence of in Vivo Dissociation on Catalytic Activity—Previous results have demonstrated that glucose-dependent dissociation of Vph1p-containing V-ATPase complexes localized to the vacuole depends upon catalytic activity (29). To determine whether this dependence of dissociation on activity extends to Stv1p-containing complexes and to both Vph1p- and Stv1p-containing complexes localized to other compartments, the effect of the specific V-ATPase inhibitor concanamycin on dissociation was examined. As can be seen in Fig. 3, A and B, concanamycin was able to partially block dissociation of Vph1p-containing complexes localized to the vacuole, the PVC, and the post-Golgi compartments, with the greatest effect observed for V-ATPases present in the vacuole. Similar results were obtained for Stv1p-containing complexes (Fig. 3, C and D), although more com-
FIGURE 3. Dependence of glucose-dependent dissociation of Vph1p- or Stv1p-containing complexes on intracellular environment and the presence
or absence of concanamycin. Yeast strains MM112, MM112 vps21Δ, and MM112 vps27Δ expressing either HA-Vph1p or HA-Stv1p were grown to an
absorbance of 0.6–0.8, converted to spheroplasts, and incubated in the presence or absence of 2% glucose for 15 min. Spheroplasts were lysed in the presence
of C12E9, and both V1 and V1V0 complexes were immunoprecipitated using the antibody 8B1 against subunit A. Following SDS-PAGE, Western blotting was
performed using both the antibody against subunit A and the anti-HA antibody (3F10) directed against the HA-tagged subunit a. The degree of assembly of V1
and V0 is reflected in the amount of subunit a co-precipitated with the antibody against subunit A. Where indicated V-ATPase activity was inhibited by addition
of 1 μM concanamycin A during the final 10 min of spheroplasting and kept at this concentration through lysis. The results of a representative experiment are
shown on the left (A for Vph1p and C for Stv1p), and the average of three independent determinations are shown on the right (B for Vph1p and D for Stv1p).
Band intensities were determined by densitometry, and the results are expressed as the ratio of assembly observed in the absence of glucose divided by the
assembly observed in the presence of glucose, where assembly is quantitated as the ratio of the intensity of subunit a divided by the intensity of subunit A. Error
bars correspond to average deviations. w.t., wild type.
V-ATPase Dissociation Depends on Cellular Environment

Dissociation Behavior of Inactive Mutants of the V-ATPase When Localized to Different Intracellular Compartments—As noted above, in most cases concanamycin did not show complete inhibition of in vivo dissociation of the V-ATPase, particularly for Vph1p-containing complexes. Because of the possibility that concanamycin may not have equal accessibility to V-ATPases in all compartments, it was of interest to determine the effect of inactivating mutations on glucose-dependent dissociation. We had previously shown that Arg-735 in TM7 of Vph1p is essential for proton transport by the V-ATPase (38). Any substitution of the arginine at this position (including the conservative R735K mutation) leads to complete loss of proton transport activity (38). Moreover, all mutants except R735K affect the dependence of dissociation on catalytic activity.

Reversible dissociation of V-ATPase complexes represents an important but as yet incompletely understood process for regulating acidification in vivo. Because V-ATPases reside in multiple cellular compartments, it might be predicted that not all of the V-ATPases in the cell would show the same degree of dissociation in response to a given stimulus. This prediction is supported by the observation that the degree of dissociation of the V-ATPase in yeast in response to glucose depletion does depend upon the cellular environment. Thus, V-ATPase complexes localized to the vacuole show the greatest degree of dissociation, and those localized to the PVC or post-Golgi compartments show intermediate dissociation behavior, whereas those localized to the Golgi (at least for complexes containing Stv1p) do not dissociate at all. The failure of Golgi-localized V-ATPases to dissociate in response to glucose depletion is not due to an intrinsic property of Stv1p, because Stv1p localized to compartments other than the Golgi dissociates efficiently. It might be argued that Stv1p-containing complexes are already associated to such a low level that no further dissociation is possible. However, because Stv1p shows similar low levels of assembly in both the Golgi and the vacuole, even in the presence of glucose (Fig. 1), but shows dissociation when localized to the vacuole but not the Golgi, this possibility is ruled out. The ability of the cell to retain Golgi-localized V-ATPases in an assembled state, even in the absence of glucose, may indicate that the Golgi V-ATPase, even though less active than that present in the vacuole, nevertheless serves an indispensable function.

In addition to providing a more alkaline pH to the Golgi compartment, does the lower level of assembly of Stv1p-containing V-ATPases localized to the Golgi serve any additional function? One possibility is that the free V0 domains present as a result of this lower assembly participate in membrane fusion.
reactions occurring in this compartment. The $V_0$ domain has been postulated to participate directly in membrane fusion based upon studies of homotypic vacuole fusion in yeast (45, 46). In this model, $V_0$ domains in adjacent membranes pair in a trans complex that follows trans-SNARE pairing but that promotes fusion of the tethered membranes. Recent studies of vesicle fusion in *Drosophila* and *Caenorhabditis elegans* (47, 48) suggest that this role may not be restricted to yeast. The presence of additional free $V_0$ domains in Golgi membranes may reflect the high degree of membrane fusion occurring in this compartment. Additional studies will be required to determine whether free $V_0$ domains participate in fusion of vesicles involved in movement of proteins between Golgi stacks.

It was previously observed that *in vivo* dissociation of V-ATPase complexes depends upon catalytic activity. This was demonstrated for Vph1p-containing complexes localized to the vacuole (29). In this paper we demonstrate that this dependence of dissociation on activity is, in most cases, not absolute. Thus, although treatment with concanamycin in all cases at least partially blocks dissociation of both Vph1p- and Stv1p-containing complexes, in most cases this inhibition of dissociation is incomplete. For Vph1p-containing complexes, in particular, 20–40% of dissociation is still observed, even in the presence of concanamycin. This is also true for inactivating mutants of Vph1p, where only the R735Q mutant localized to the vacuole shows complete inhibition of dissociation. For
Stv1p, both concanamycin and inactivating mutations generally lead to more complete inhibition of dissociation than for Vph1p. Even for Stv1p-containing complexes, however, partial dissociation is observed when these complexes are localized to the post-Golgi compartment. These results clearly indicate that catalytic activity is not absolutely required for in vivo dissociation of the V-ATPase complex.

The molecular basis for the dependence of dissociation on cellular environment remains uncertain. It has previously been observed that neutralization of acidic compartments with chloroquine leads to at least partial inhibition of dissociation (33). This suggests that one environmental factor affecting dissociation is the luminal pH, such that when the luminal pH becomes too alkaline, dissociation is inhibited. Inconsistent with this conclusion is the observation that Stv1p, when localized to the vacuole, shows very low levels of proton transport, yet still dissociates efficiently in response to glucose depletion. Moreover, completely inactive mutants of Vph1p still show dissociation when localized to compartments other than the vacuole. The absence of quinacrine staining of these compartments in strains expressing mutant forms of Vph1p suggests that they are not being acidified by some other mechanism. Thus, although luminal pH may partly control dissociation in certain cases, this cannot be the only environmental factor affecting this process. It is likely that specific proteins or lipids localized to different cellular compartments help control in vivo dissociation.

It is important to note that the studies described employ yeast mutant strains that are disrupted in normal intracellular trafficking, and it is therefore possible that some of the differences observed in dissociation behavior are a secondary consequence of disruption of trafficking, such as the failure of an important regulatory molecule to reach its correct cellular destination. Although this possibility cannot be ruled out, it is important to note that the vps21Δ and vps27Δ mutants used in these studies have fewer pleiotropic effects than other yeast-trafficking mutants. For example, class C vps mutants, which are defective in proteins involved in multiple membrane fusion events (44, 49), are characterized by the absence of identifiable vacuoles. By contrast, vps21Δ and vps27Δ mutants can form and maintain identifiable vacuoles. Moreover, although CPY cannot be targeted to the vacuole in these mutants, processing of CPY in the endoplasmic reticulum and Golgi is normal (33, 43). In addition, targeting of alkaline phosphatase to the vacuole via a pathway distinct from the CPY pathway as well as vacuolar processing of alkaline phosphatase is normal in the vps21Δ and vps27Δ mutants (34, 50). These results suggest that it is less likely that the observed differences in dissociation are a secondary consequence of defective trafficking.

An additional question emerging from these results is why the dependence of dissociation on activity is in turn a function of the cellular environment. One possibility is that for dissociation to occur, the V-ATPase must adopt a particular conformational state, and by inhibiting activity, the enzyme is prevented from achieving the necessary conformational state and is hence blocked in its dissociation. It is possible, however, that as a result of environmental factors within the cell (interaction with unique proteins or lipids localized to particular organelles), the V-ATPase adopts different stable conformations in the inhibited state. Thus the inhibited conformation in the vacuole may be incompatible with dissociation, whereas the inhibited state in the PVC may be identical to the state necessary for dissociation to occur. This may also explain why Vph1p and Stv1p show a different dependence of dissociation on activity. Additional probes of the conformational state of the complex are required to address this possibility.

In summary, our results suggest that both dissociation of the V-ATPase complex and the dependence of dissociation on activity is a function of the cellular environment and the subunit isoform present, and that dissociation of the V-ATPase is not absolutely dependent upon catalytic activity.

Acknowledgments—We thank Dr. Daniel Jay for the use of the fluorescence microscope; Dr. Takao Inoue for helpful discussions; and Drs. Daniel Cipriano, Ayana Hinton, and Yanru Wang and also Sarah Bond, Kevin Jefferies, and Kathleen Forgac for careful reading of the manuscript and helpful discussions. E. coli strains were provided by National Institutes of Health Grant DK34928.

REFERENCES

1. Nishi, T., and Forgac, M. (2002) Nat. Rev. Mol. Cell Biol. 3, 94–103
2. Kane, P. M. (2006) Microbiol. Mol. Biol. Rev. 70, 177–191
3. Wagner, C. A., Finberg, K. E., Breton, S., Marshansky, V., Brown, D., and Geibel, J. P. (2004) Physiol. Rev. 84, 1263–1314
4. Nelson, N. J. (2003) J. Bioenerg. Biomembr. 35, 281–289
5. Abrami, L., Lindsay, M., Parton, R. G., Leppla, S. H., and van der Goot, F. G. (2004) J. Cell Biol. 166, 645–651
6. Toyomura, T., Murata, Y., Yamamoto, A., Oka, T., Sun-Wada, G. H., Wada, Y., and Futai, M. (2003) J. Biol. Chem. 278, 22023–22030
7. Pietrement, C., Sun-Wada, G. H., Silva, N. D., McKee, M., Marshansky, V., Brown, D., Futai, M., and Breton, S. (2006) Biochem. Biophys. Acta. 1717, 185–194
8. Nanda, A., Brumell, J. H., Nordstrom, T., Kjeldsen, L., Sengelov, H., Borregaard, N., Rotstein, O. D., and Grinstein, S. (1996) J. Biol. Chem. 271, 15963–15970
9. Sennoune, S. R., Bakunts, K., Martinez, G. M., Chua-Tuan, J. L., Kebir, Y., Attaya, M. N., and Martinez-Zaguilan, R. (2004) Am. J. Physiol. 286, C1443–C1452
10. Yoshida, M., Muneyuki, E., and Hisabori, T. (2001) Nat. Rev. Mol. Cell Biol. 2, 669–677
11. Iwata, M., Imamura, H., Stambouli, E., Ikeda, C., Tamakoshi, M., Nagata, K., Makyio, H., Hankamer, B., Barber, J., Yoshida, M., Yokoyama, K., and Iwata, S. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 59–64
12. Kawasaki-Nishi, S., Nishi, T., and Forgac, M. (2003) J. Biol. Chem. 278, 41908–41913
13. Imamura, H., Nakano, M., Noji, H., Muneyuki, E., Okhuma, S., Yoshida, M., and Yokoyama, K. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2312–2315
14. Hirata, T., Iwamoto-Kihara, A., Sun-Wada, G. H., Okajima, T., Wada, Y., and Futai, M. (2003) J. Biol. Chem. 278, 23714–23719
15. Manolson, M. F., Proteau, D., Preston, R. A., Stenbit, A., Roberts, B. T., Hoyt, M. A., Preuss, D., Mulholland, J., Botstein, D., and Jones, E. W. (1992) J. Biol. Chem. 267, 14294–14303
16. Manolson, M. F., Wu, B., Proteau, D., Taillon, B. E., Roberts, B. T., Hoyt, M. A., and Jones, E. W. (1994) J. Biol. Chem. 269, 14064–14074
17. Kawasaki-Nishi, S., Nishi, T., and Forgac, M. (2001) J. Biol. Chem. 276, 17941–17948
18. Kawasaki-Nishi, S., Bowers, K., Nishi, T., Forgac, M., and Stevens, T. H. (2001) J. Biol. Chem. 276, 47411–47420
19. Nishi, T., and Forgac, M. (2000) J. Biol. Chem. 275, 6824–6830
20. Oka, T., Murata, Y., Namba, M., Yoshimizu, T., Toyomura, T., Yamamoto, A., Sun-Wada, G. H., Hamasaki, N., Wada, Y., and Futai, M. (2001) J. Biol. Chem. 276, 40050–40054
21. Frattini, A., Orchard, P. J., Sobacchi, C., Gilliani, S., Abinun, M., Mattsson,
J. P., Keeling, D. J., Andersson, A. K., Wallbrandt, P., Zecca, L., Notarangelo, L. D., Vezzoni, P., and Villa, A. (2000) Nat. Genet. 25, 343–346
22. Smith, A. N., Skaug, J., Choate, K. A., Nayir, A., Bakkaloglu, A., Ozen, S., Hulton, S. A., Sanjad, S. A., Al-Sabban, E. A., Lifton, R. P., Scherer, S. W., and Karet, F. E. (2000) Nat. Genet. 26, 71–75
23. Kane, P. M. (1995) J. Biol. Chem. 270, 17025–17032
24. Sumner, J. P., Dow, J. A., Earley, F. G., Klein, U., Jager, D., and Wieczorek, H. (1995) J. Biol. Chem. 270, 5649–5653
25. Sautin, Y. Y., Lu, M., Gaugler, A., Zhang, L., and Gluck, S. L. (2005) Mol. Cell. Biol. 25, 575–589
26. Trombetta, E. S., Ebersold, M., Garrett, W., Pypaert, M., and Mellman, I. (2003) Science 299, 1400–1403
27. Parra, K. J., Keenan, K. L., and Kane, P. M. (2000) J. Biol. Chem. 275, 21761–21767
28. Zhang, J., Myers, M., and Forgac, M. (1992) J. Biol. Chem. 267, 9773–9778
29. Parra, K. J., and Kane, P. M. (1998) Mol. Cell. Biol. 18, 7064–7074
30. Xu, T., and Forgac, M. (2001) J. Biol. Chem. 276, 24855–24861
31. Seol, J. H., Shevchenko, A., Shevchenko, A., and Deshaies, R. J. (2001) Nat. Cell Biol. 3, 384–391
32. Smardon, A. M., Tarsio, M., and Kane, P. M. (2002) J. Biol. Chem. 277, 13831–13839
33. Shao, E., and Forgac, M. (2004) J. Biol. Chem. 279, 48663–48670
34. Gerrard, S. R., Bryant, N. J., and Stevens, T. H. (2000) Mol. Biol. Cell 11, 613–626
35. Sherman, F. (2002) Methods Enzymol. 350, 3–41
36. Johnston, M., Riles, L., and Hegemann, J. H. (2002) Methods Enzymol. 350, 290–315
37. Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425
38. Kawasaki-Nishi, S., Nishi, T., and Forgac, M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12397–12402
39. Inoue, T., and Forgac, M. (2005) J. Biol. Chem. 280, 27896–27903
40. Conibear, E., and Stevens, T. H. (2002) Methods Enzymol. 351, 408–432
41. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
42. Fang, Y., and Forgac, M. (1992) J. Biol. Chem. 267, 5817–5822
43. Piper, R. C., Cooper, A. A., Yang, H., and Stevens, T. H. (1995) J. Cell Biol. 131, 603–617
44. Bowers, K., and Stevens, T. H. (2005) Biochim. Biophys. Acta 1744, 438–454
45. Peters, C., Bayer, M. J., Buhler, S., Andersen, J. S., Mann, M., and Mayer, A. (2001) Nature 409, 581–588
46. Bayer, M. J., Reese, C., Buhler, S., Peters, C., and Mayer, A. (2003) J. Cell Biol. 162, 211–222
47. Hiesinger, P. R., Fayyazuddin, A., Mehta, S. Q., Rosenmund, T., Schulze, K. L., Zhai, R. G., Verstreken, P., Cao, Y., Zhou, Y., Kunz, J., and Bellen, H. J. (2005) Cell 121, 607–620
48. Liegeois, S., Benedetto, A., Garnier, J. M., Schwab, Y., and Labouesse, M. (2006) J. Cell Biol. 173, 949–961
49. Peterson, M. R., and Emr, S. D. (2001) Traffic 2, 476–486
50. Piper, R. C., Bryant, N. J., and Stevens, T. H. (1997) J. Cell Biol. 138, 531–545