BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Vma9p Need Not Be Associated with the Yeast V-ATPase for Fully-Coupled Proton Pumping Activity in Vitro

Stephanie A. Bueler[†] and John L. Rubinstein*[,†,‡,§]

[†]Molecular Structure and Function Program, The Hospital for Sick Children Research Institute, Toronto, Ontario M5G 1X8, Canada
[‡]Department of Biochemistry, The University of Toronto, Toronto, Ontario M5S 1A8, Canada
[§]Department of Medical Biophysics, The University of Toronto, Toronto, Ontario M5G 1L7, Canada

ABSTRACT: Vacuolar-type ATPases (V-ATPases) acidify numerous intracellular compartments in all eukaryotic cells and are responsible for extracellular acidification in some specialized cells. V-ATPases are large macromolecular complexes with at least 15 different subunits, some of which are found in multiple copies. The main roles of all V-ATPase subunits have been established except for the e subunit, encoded by the gene VMA9 in *Saccharomyces cerevisiae*, and the Ac45 subunit, which is not found in the *S. cerevisiae* enzyme. Here we demonstrate that when the *S. cerevisiae* V-ATPase is solubilized with the detergent dodecylmaltoside (DDM), Vma9p is removed. We further demonstrate that after Vma9p has been removed by detergent the purified enzyme is still able to perform fully-coupled ATP-dependent proton pumping. This observation shows that Vma9p is not necessary in vitro for this principal activity of the V-ATPase.

The vacuolar-type ATPase (V-ATPase) from *Saccharomyces cerevisiae* consists of subunits $A_3B_3CDE_3FG_3Hac_xc'_yc''_zde$, where *x*, *y*, and *z* denote unknown stoichiometries. Subunits with uppercase letter names are part of the soluble V_1 region, whereas subunits with lowercase letter names are part of the membrane-bound V_O region. The A_3B_3 subcomplex consists of the catalytic subunits with nucleotide binding sites.[1] ATP hydrolysis in the A_3B_3 oligomer induces rotation of the rotor subcomplex consisting of subunits $DFdc_xc'_yc''_z$. The rotation of the membrane-bound part of the rotor against the membrane-bound a subunit drives the translocation of protons across the lipid bilayer. The three peripheral stalk subcomplexes,[2−4] each consisting of a heterodimer of subunits E and G, prevent the a subunit from rotating along with the central rotor. The soluble N-terminal portion of the a subunit, together with the C subunit, links the three peripheral stalks.[4,5] The H subunit serves to inhibit ATP hydrolysis in the dissociated V_1 region, providing a regulation-by-dissociation mechanism.[6,7] Of all of the subunits of the yeast V-ATPase, only the subunit e has an unclear role.

The e subunit was identified as a hydrophobic component of the V-ATPase from bovine chromaffin granules purified by blue native polyacrylamide gel electrophoresis.[8] The corresponding subunit of the V-ATPase from the plasma membrane of the midgut and Malpighian tubule of the tobacco hornworm *Manducca sexta* was subsequently identified.[9] The bovine and *M. sexta* e subunits were originally thought to correspond to *S. cerevisiae* Vma21p,[8] a protein involved in V-ATPase assembly that is localized to the endoplasmic reticulum membrane.[10] Subsequently, it was shown that Vma9p is the e subunit in *S. cerevisiae*.[11] Vma9p is homologous with Vma21p and could be immunoprecipitated in a V_O assembly complex containing Vma21p.[12]

Previously, single particle electron cryomicroscopy (cryo-EM) maps were determined for the *M. sexta* V-ATPase[4] and the *S. cerevisiae* V-ATPase.[5] The resolution of these maps was not sufficient to allow α-helices to be identified in the membrane region of the complex, as has been done for the *Thermus thermophilus* V/A-ATPase.[13] To give insight into the role of Vma9p in the *S. cerevisiae* enzyme, we designed experiments where we added a protein tag to Vma9p to localize it in the membrane region of the V-ATPase. To our surprise, these experiments and subsequent investigation showed that our preparation of detergent-solubilized V-ATPase lacked the Vma9p subunit, which was presumably removed by the detergent extraction of the complex from the membrane. However, the V-ATPase lacking Vma9p still displayed fully-coupled ATP-hydrolysis-driven proton pumping activity in vitro. These results demonstrate that the role of the Vma9p is not central to the in vitro proton pumping activity of the V-ATPase.

Received: October 21, 2014
Revised: December 1, 2014
Published: December 29, 2014

DOI: 10.1021/bi5013172
Biochemistry 2015, 54, 853−858

METHODS

Yeast Strains and Plasmids. The *S. cerevisiae* strain MM112 (MATα his3-Δ200 leu2 lys2 stv1::LYS2 ura3−52 vph1::LEU2), containing a stv1 and vph1 double deletion, was a gift from Dr. M. Manolson. MM112 was modified by homologous recombination to introduce a 3×FLAG tag (DYKDHDGDYKDHDIDYKDDDDK) at the C terminus of VMA1 followed by the URA3 marker from *C. albicans* to produce yeast strain SABY47. Chromosomal modification by homologous recombination was done as described previously.[5] Yeast strain BY4741 cwh36Δ (MATα his3Δ1 leu2 met15 ura3 cwh36::KanMX), containing a vma9 deletion, was a gift from Dr. P. Kane. A 3×FLAG tag was added to the C terminus of VMA1 to produce strain SABY55 in the same manner described for SABY47. Plasmids with versions of VMA9 and VPH1 were created by restriction free cloning[14] of genes into the plasmid p413−ADH1, which also contains the *S. cerevisiae* HIS3 nutritional marker and was a gift from Dr. J. Fillingham. For constructs including a MBP label, the MalE gene was incorporated by restriction free cloning. For HA-tagged constructs, the HA tag (YPYDVPDYA) and a six-amino-acid linker (ALNVAL) were added with a QuikChange reaction (Agilent Technologies Inc.). Yeast strains SABY49, SABY51, SABY60, SABY61, SABY62, and SABY63 were transformed with plasmids pSAB19, pSAB22, pSAB25, pSAB30, pSAB31, and pSAB32, respectively, using standard lithium acetate transformation. Transformation of yeast with these p413−ADH1-based plasmids was verified by growth on SD-His plates. A summary of yeast strains is given in Table 1.

Table 1. Yeast Strains Used in This Work

strain	plasmid	relevant phenotype
MM112	n/a[a]	ΔVph1pΔStv1p
SABY47	n/a	Vma1p-3×FLAG, ΔVph1pΔStv1p
BY4741 cwh36Δ	n/a	ΔVma9p
SABY55	n/a	Vma1p-3×FLAG, ΔVma9p
SABY49	pSAB19	Vma1p-3×FLAG, Vph1p from plasmid
SABY51	pSAB22	Vma1p-3×FLAG, Vph1p−MBP from plasmid
SABY60	pSAB25	Vma1p-3×FLAG, Vma9p from plasmid
SABY61	pSAB30	Vma1p-3×FLAG, Vma9p−MBP from plasmid
SABY62	pSAB31	Vma1p-3×FLAG, Vma9p−HA from plasmid
SABY63	pSAB32	Vma1p-3×FLAG, Vph1p−HA from plasmid

[a]Not applicable.

Growth of Yeast and Purification of V-ATPase. Yeast with chromosomal modifications only were grown in an 11 L Microferm fermentor (New Brunswick Scientific) with YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose). Yeast strains containing plasmids were grown in 11 L of SD-His (1.74 g/L yeast nitrogen base without amino acids or ammonium sulfate, 5 g/L ammonium sulfate, 2 g/L drop-out medium supplement without histidine, and 20 g/L glucose). V-ATPase was purified via the 3×FLAG tag on Vma1p with M2 sepharose affinity matrix (Sigma) as described previously.[5] The Vma- growth phenotype was detected by growth on YPD agar plates supplemented with 4 mM $ZnCl_2$.

Immunoprecipitation. Yeast membranes prepared as described[5] were resuspended in lysis buffer (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, 80 g/L sucrose, 20 g/L sorbitol, 20 g/L glucose, 5 mM 6-aminocaproic acid, 5 mM benzamidine, 5 mM EDTA, and 10 mg/L PMSF at pH 7.4) and solubilized with 1% (w/v) *n*-dodecyl-β-D-maltopyranoside (DDM) (Affymetrix) for 1 h at 4 °C. Solubilized membranes were clarified by ultracentrifugation, and 40 μL of anti-HA agarose bead slurry (Pierce) was added to the supernatant and incubated for 1 h at 4 °C. Beads were collected by centrifugation and washed three times with 500 μL of TBS (50 mM Tris and 150 mM NaCl at pH 7.5) containing 0.05% (w/v) DDM. Bound protein was eluted from the beads by boiling in SDS sample buffer without a reducing agent.

SDS-PAGE and Western Blotting. Proteins from 12% or 15% acrylamide SDS-PAGE gels were either stained with Coomassie R-250 or transferred to nitrocellulose membranes for Western blotting. For Western blots, membranes were blocked in 3% (w/v) bovine serum albumin in TBS containing 0.1% (w/v) Tween-20 (TBST) for 1 h at room temperature. Membranes were incubated with either anti-FLAG monoclonal antibody (anti-FLAG M2, Sigma-Aldrich) or anti-HA monoclonal antibody (HA.11 clone 16B12, Covance) followed by goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad Laboratories). ECL Western blotting substrate (Pierce) was used to visualize peroxidase-coupled antibodies on photographic film. Membranes to be reprobed with a second primary antibody were stripped by incubating in 60 mM Tris−HCl (pH 6.8), 0.7% (w/v) β-mercaptoethanol, and 2% (w/v) SDS for 30 min at 50 °C. Membranes were washed with water and TBST before blocking again.

Liposome Reconstitution. Twenty milligrams of POPC and DOPA at a ratio of 19:1 (w/w) in chloroform (Avanti) were mixed. The lipids were dried under a stream of nitrogen to form a thin film on the inside of a test tube and placed under vacuum for at least 1 h. The lipid cake was resuspended in buffer (1 mL) containing 20 mM Tricine−NaOH (pH 8.0), 40 mM Na_2SO_4, 20 mM succinic acid, and 5 mM β-mercaptoethanol. The resuspended lipid was subjected to three cycles of being heated to 65 °C and vortexing for 10 s before being frozen and thawed. The heat−freeze−thaw process was repeated five times before extruding the lipids through a 0.2 μm filter (Avanti). Five milligrams of liposomes were mixed with Triton X-100 at a ratio of 1:0.8 (w/w) with 1 mM $MgSO_4$ for 10 min at room temperature. Purified V-ATPase was added to the solution at 20:1 (w/w) lipid:protein and allowed to incubate at room temperature for 1 h. Detergent was removed by adding 80 mg/mL Biobeads (Bio-Rad) and rotating the solution for 2 h at 4 °C. Two subsequent additions of 160 mg/mL Biobeads were made and incubated for 4 h and overnight, respectively. Negative stain electron microscopy with uranyl acetate was performed as described previously.[15]

ATPase Assay. ATP hydrolysis was measured with an enzyme-coupled assay based on one described previously:[16] 50 μL of purified V-ATPase, or proteoliposomes containing reconstituted V-ATPase, was added to 50 mM HEPES (pH 8.0), 3 mM $MgCl_2$, 0.2 mM NADH, 1 mM phosphoenolpyruvate (Sigma-Aldrich), 10 units of pyruvate kinase (Sigma-Aldrich), and 25 units of L-lactic dehydrogenase (Sigma-Aldrich) in a total volume of 0.5 mL. ATP (2 mM) was added to start the reaction and the loss of NADH was monitored at 340 nm on a Ultraspec 2100 pro (GE Healthcare Life Sciences). A 100 μM stock solution of bafilomycin A1 (Bioshop Canada) was made in ethanol and used at 1 μM.

Proton-Pumping Assay. Proton pumping was assayed using 9-amino-6-chloro-2-methoxyacridine (ACMA, Sigma-Aldrich) fluorescence quenching. Proteoliposomes (50 μL)

DOI: 10.1021/bi5013172
Biochemistry 2015, 54, 853−858

were added to 1 mL of ACMA Quench Buffer (20 mM Tricine−KOH, 10 mM $MgCl_2$, and 300 mM KCl at pH 8.0) with 2 μM ACMA from a 200 μM ACMA stock solution prepared in water with 200 μM HCl. ATP was added to 2 mM to begin proton pumping. Fluorescence was excited at 410 nm and emission monitored at 490 nm with a PTI fluorometer (Photon Technology International). One micromolar nigiricin (Tocris Biosciences) was added to uncouple the membranes.

RESULTS

Dodecylmaltoside-Solubilized V-ATPase Does Not Contain Vma9p. In a previous cryo-EM map of the yeast V-ATPase, the c-ring subcomplex in the membrane region could be readily resolved from the other membrane-intrinsic proteins.[5] The yeast strain used for isolating V-ATPase had the STV1 gene removed, and consequently the remaining transmembrane density was presumed to correspond to Vph1p and Vma9p. To localize Vma9p in the map, we prepared a yeast strain with the VMA9 gene removed from the chromosomal DNA and instead carried on a plasmid. The yeast strain also included a 3×FLAG tag at the C terminus of Vma1p (subunit A) to facilitate V-ATPase purification. Yeast strains with defects in the V-ATPase complex are known to be unable to grow on a variety of media, including those supplemented with one of several heavy metal salts.[17,18] Yeast were grown on rich medium containing 4 mM $ZnCl_2$, showing that the strain harboring the VMA9 gene on a plasmid contained a functional V-ATPase[19] and the strain lacking VMA9 did not (Figure 1A). We then prepared a strain where the Vma9p was fused to a C-terminal maltose binding protein (MBP). MBP labels expressed as fusions with proteins of interest have been used previously to locate subunits in low resolution cryo-EM maps.[20] The yeast strain with the Vma9p−MBP fusion protein similarly had a functional V-ATPase complex, as demonstrated by growth on YPD with 4 mM $ZnCl_2$ (Figure 1A). To our surprise, V-ATPase purified from the strain with a Vma9p−MBP fusion did not appear to contain the Vma9p−MBP protein, as judged by SDS-PAGE (Figure 1Ci,ii). One would expect the Vma9p−MBP fusion to have a molecular mass of ∼51 kDa and to appear with a density comparable to other subunits found with a stoichiometry of 1 in the complex (e.g., subunits D, C, or H). We also prepared a yeast strain with a V-ATPase lacking the chromosomal STV1 and VPH1 genes and with a Vph1p−MBP fusion protein produced from a plasmid. This strain also had a functional V-ATPase (Figure 1B). In contrast to V-ATPase from the strain with a Vma9p−MBP fusion protein, the Vph1p−MBP fusion protein was readily distinguishable from unmodified Vph1p on an SDS-PAGE gel (Figure 1Ciii).

To further investigate if Vma9p was associated with the DDM-solubilized V-ATPase preparation, we prepared a yeast strain where the VMA9 gene carried on a plasmid had a C-terminal HA epitope tag. We reasoned that the nine residue HA tag is less likely to lead to dissociation of a subunit from a complex than the much larger MBP label. The strain was viable and harbored a functional V-ATPase, as judged by growth of the yeast on YPD including 4 mM $ZnCl_2$ (Figure 1A). This result agrees with the earlier observation that yeast encoding Vma9p-3×HA grows on medium with $CaCl_2$.[11] For a control, we also prepared a strain with a HA-tagged VPH1 gene on a plasmid. Similar to the Vma9p−HA strain, the Vph1p−HA strain was viable and contained a functional V-ATPase (Figure 1B). V-ATPase was purified from both the Vph1p−HA and the Vma9p−HA strains using the 3×FLAG tag. The resultant

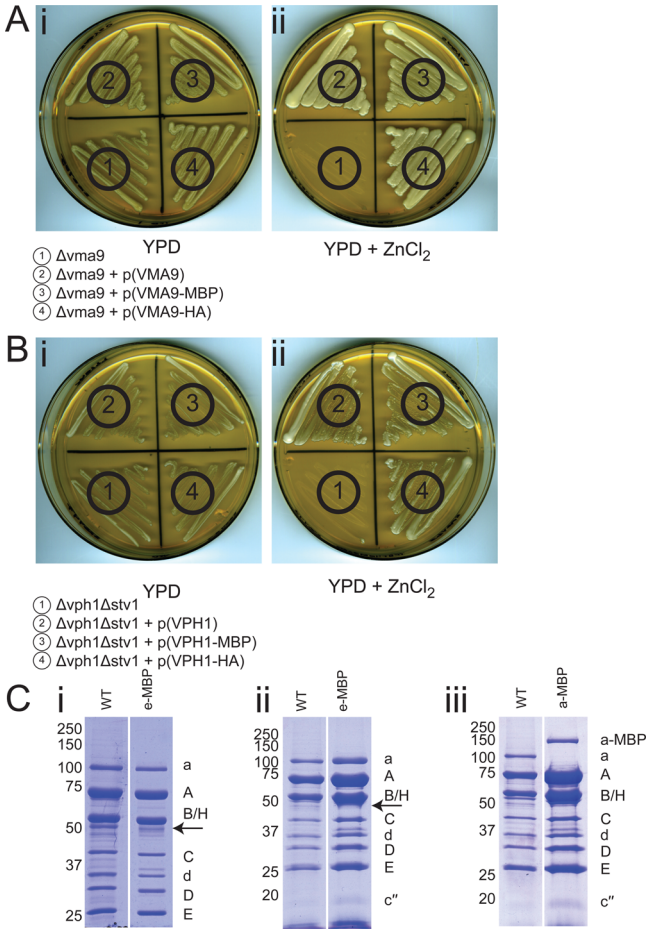

Figure 1. Characterization of yeast strains with tagged versions of Vph1p and Vma9p. (A) Four different yeast strains were spread on (i) nonselective rich medium and (ii) rich medium containing 4 mM $ZnCl_2$, which selects for a functional V-ATPase complex. The strains were (1) SABY55, which lacks Vma9p, (2) SABY60, which encodes Vma9p on a plasmid, (3) SABY61, which encodes a Vma9p−MBP fusion protein on a plasmid, and (4) SABY62, which encodes a Vma9p−HA fusion protein on a plasmid. All four strains grew on the nonselective medium and only the strain lacking Vma9p altogether did not grow on the $ZnCl_2$ medium, suggesting that all Vma9p fusion proteins were functional. (B) Four different yeast strains were spread on (i) nonselective rich medium and (ii) rich medium containing 4 mM $ZnCl_2$. The strains were (1) SABY47, which lacks Vph1p and Stv1p, (2) SABY49, which encodes Vph1p on a plasmid, (3) SABY51, which encodes a Vph1p−MBP fusion protein on a plasmid, and (4) SABY63, which encodes a Vph1p−HA fusion protein on a plasmid. All four strains grew on the nonselective medium and only the strain lacking Vph1p altogether did not grow on the $ZnCl_2$ medium, suggesting that all Vph1p fusion proteins were functional. (C) SDS-PAGE gels stained with Coomassie Brilliant Blue of V-ATPase purified from strain SABY61 do not show the expected ∼52 kDa Vma9p−MBP fusion with either (i) 12% or (ii) 15% polyacrylamide gels. The expected position of the Vmap9p−MBP fusion is indicated with an arrow. In comparison, V-ATPase from strain SABY51 shows an increased mass for the Vph1p−MBP fusion compared to that of wild-type Vph1p (iii).

protein preparation was visualized by SDS-PAGE and probed by Western blotting to detect the HA-tagged subunit. The Coomassie Blue-stained SDS-PAGE gel showed that both V-ATPase complexes could be isolated with similar abundance (Figure 2A). However, although the HA-tagged Vph1p could be detected in the Vph1p−HA strain by Western blotting

DOI: 10.1021/bi5013172
Biochemistry 2015, 54, 853−858

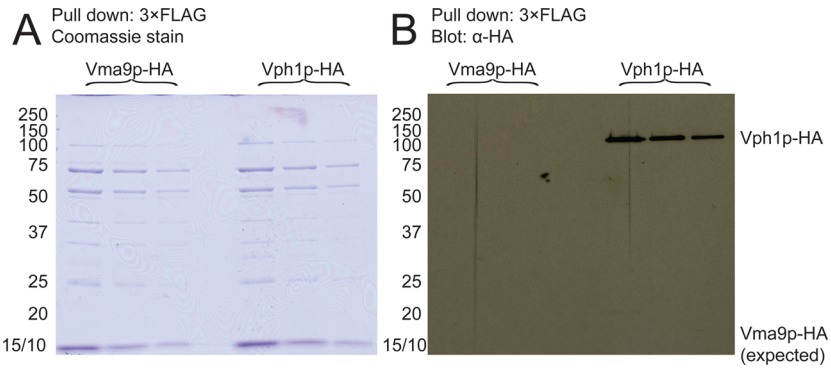

Figure 2. Detection of HA-tagged Vph1p and Vma9p. (A) An SDS-PAGE gel stained with Coomassie Brilliant Blue shows that V-ATPase could be purified with similar yields from strains SABY62 and SABY63, which contain HA-tagged Vma9p and Vph1p, respectively. Three dilutions of the sample are shown for each specimen. (B) Western blotting with an antibody directed against the HA tag is able to detect the Vph1p−HA fusion protein, but not the Vma9p−HA fusion protein, in the two V-ATPase preparations shown in (A).

(Figure 2B), no HA-tagged protein could be detected in the strain with Vma9p−HA. This experiment suggests that the HA-tagged Vma9p is not part of the DDM-solubilized V-ATPase preparation.

One potential source of error in the experiment described above is that the Vph1p−HA protein and Vma9p−HA protein have dramatically different expected masses: ∼135 and ∼9 kDa, respectively. It is therefore possible that, due to different masses or because of other differences in their chemical properties, the Vma9p−HA did not transfer as effectively as the Vph1p−HA to the nitrocellulose membrane used for Western blotting. Therefore, we carried out the converse experiment using anti-HA affinity matrix to immunoprecipitate V-ATPase followed by probing for the presence of the 3×FLAG-tagged Vma1p by Western blotting. The resulting Western blot showed that 3×FLAG-tagged Vma1p could be detected for the Vph1p−HA strain but not for the Vma9p−HA strain (Figure 3A). Due to

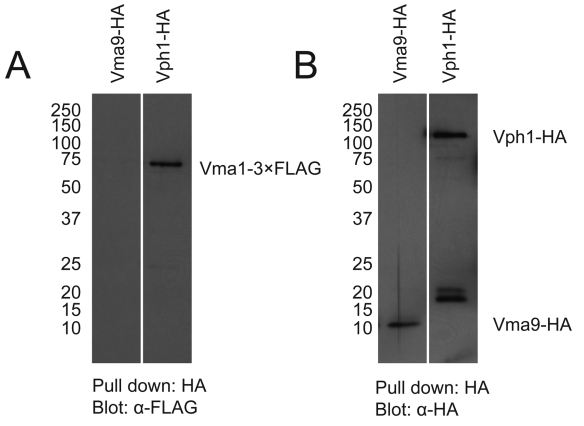

Figure 3. Detection of the 3×FLAG-tagged Vma1p from V-ATPase complexes containing HA-tagged subunits. (A) V-ATPase was immunoprecipitated using the HA tag from the detergent-solubilized membrane extracts of strains SABY62 and SABY63, containing HA-tagged Vma9p and Vph1p, respectively. The presence of V-ATPase was detected by probing the immunoprecipitate with an antibody directed against the 3×FLAG-tagged Vma1p subunit of V-ATPase. V-ATPase could be successfully immunoprecipitated from strain SABY63 but not SABY62. (B) The Western blot from (A) was stripped of antibodies and reprobed with an antibody against the HA epitope. The blot shows that both Vph1p−HA and Vma9p−HA were successfully immunoprecipitated, but only Vph1p−HA was associated with Vma1p-3×FLAG.

the small quantities of material used for this immunoprecipitation experiment, purified V-ATPase could not be visualized by Coomassie staining. This experiment suggested again that Vma9p is not associated with V-ATPase in DDM, with the known association of Vph1p served as a positive control for the assay.

An additional confounding factor for this immunoprecipitation approach is that the HA tag may not be accessible to the anti-HA matrix in detergent-solubilized V-ATPases. Consequently, a Western blot of the HA-tagged immunoprecipitate for each strain was reprobed for the presence of the HA-tagged target. As seen in the blot (Figure 3B), Vma9p−HA could be detected from the Vma9p−HA strain, indicating that it was effectively immunoprecipitated in the experiment shown in Figure 3A. Further, the Vph1p−HA was also detected, along with a lower molecular weight contaminant, explaining the successful coprecipitation of Vma1p-3×FLAG and Vph1p−HA. The Vma9p−HA protein detected was from detergent-solubilized cell membranes, indicating that, like the V-ATPase, Vma9p is in the membrane fraction of cells but is separated from the V-ATPase upon immunoprecipitation or purification in the detergent-solubilized state.

V-ATPase Lacking Vma9p Shows Fully-Coupled ATP Hydrolysis Activity. Although V-ATPase with a HA-tagged Vma9p was shown to be functional by growth of yeast on YPD with 4 mM $ZnCl_2$, it is possible that including the HA-tag destabilizes the interaction of Vma9p with the rest of the complex, leading to its dissociation in detergent. If Vma9p was necessary for V-ATPase activity, its dissociation would become apparent only in functional analysis of the purified V-ATPase in vitro. Therefore, we measured the ATP hydrolysis activity of detergent-solubilized V-ATPase preparations with HA-tagged Vma9p, HA-tagged Vph1p, and no HA-tagged subunits. Detergent-solubilized V-ATPase with Vma1p-3×FLAG and no other modifications has a specific activity of 1.82 ± 0.04 μmol of ATP min^{-1} mg^{-1}.[5] The absence of the Vma9p−HA from the preparation does not affect the rate of ATP hydrolysis. Further, the activity of the different preparations were all indistinguishable (Figure 4A), suggesting that the HA tag attached to Vph1p and Vma9p does not affect enzyme activity. The V-ATPase activity from all of these preparations was inhibited >95% by bafilomycin. Bafilomycin is known to bind to the c subunits (Vma3p, Vma11p, and Vma16p) of V-ATPase,[21] and inhibition of ATP hydrolysis by bafilomycin consequently

DOI: 10.1021/bi5013172
Biochemistry 2015, 54, 853−858

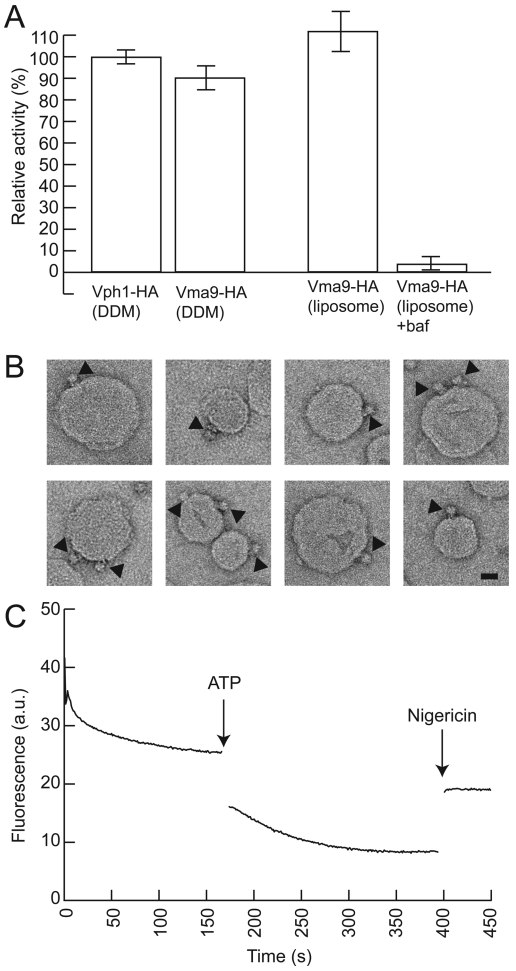

Figure 4. In vitro functional analysis of V-ATPase complexes. (A) Detergent-solubilized V-ATPase with HA-tagged Vma9p and Vph1p had ATPase activities that were indistinguishable from each other. V-ATPase with an HA-tagged Vma9p reconstituted into liposomes had an ATPase indistinguishable from wild-type (100% activity), and its activity could be completely inhibited with 1 μM bafilomycin. (B) Negative stain electron microscopy shows that V-ATPase complexes (black arrows) were successfully reconstituted into liposomes. The scale bar corresponds to 200 Å. (C) V-ATPase with an HA-tagged Vma9p reconstituted into liposomes displayed ATP-dependent quenching of ACMA fluorescence, which could be alleviated with the ionophore nigericin.

demonstrates coupling of the activities of the V_1 and V_O regions of the complex.

V-ATPase Lacking Associated Vma9p Can Pump Protons. To further characterize the V-ATPase lacking Vma9p, we reconstituted V-ATPase from the Vma9p−HA strain asymmetrically into proteoliposomes, with the catalytic V_1 region outside of the liposome. We confirmed the successful reconstitution of V-ATPase by negative stain electron microscopy (Figure 4B). Again, ATP hydrolysis by the liposome-reconstituted enzyme could be inhibited completely with bafilomycin (Figure 4A). Further, V-ATPase reconstituted into liposomes was capabable of ATP-dependent 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence quenching, which could in turn be alleviated by the addition of the protonophore nigericin (Figure 4C). This assay has been used extensively to monitor proton pumping by V-ATPases and F-type ATP synthases.[22,23] The observation that V-ATPase lacking Vma9p is capable of fully-coupled ATP-dependent proton pumping demonstrates that Vma9p need not be physically associated with the V-ATPase complex for this enzyme function. To our knowledge, these experiments are also the first published demonstration of ATP-driven proton pumping by a purified and reconstituted V-ATPase, rather than by vacuolar membranes that have been used to form proteoliposomes.

DISCUSSION

The experiments described above indicate that the DDM-solubilzed *S. cerevisiae* V-ATPase does not contain Vma9p. Subunits of membrane protein complexes can often be dissociated by specific detergents. In studying the F-type ATP synthase from *S. cerevisiae* mitochondria, it was found that subunits e and g were removed when membranes were solubilized with DDM or Brij-35.[24−26] Although it cannot be formally excluded that Vma9p is only removed from the V-ATPase complex when a tag such as MBP or HA is fused to the protein, experiments with the complex containing an HA-tagged e subunit demonstrate that the purified enzyme without Vma9p appears indistinguishable from the wild-type complex in vitro. The preparation of *S. cerevisiae* V-ATPase shows complete sensitivity of its ATPase activity to the inhibitor bafilomycin, for both the detergent-solubilized and the lipid-membrane-reconstituted specimens. Bafilomycin binds to the c-ring of V-ATPase, blocking rotation of the rotor and proton transport.[21] Thus, inhibition of ATPase activity by bafilomycin indicates that the ATP hydrolysis and proton translocation activities of the enzyme are tightly coupled in the preparation. Coupling in the detergent-solubilized enzyme is in contrast to the F-type ATP synthase, where many detergent-solubilized preparations are not sensitive to oligomycin, an inhibitor of proton translocation in that enzyme.[27,28]

Because Vma9p is not necessary for ATP-dependent proton pumping in vitro, the question arises: what is the role for the protein? It is possible that Vma9p may be necessary for proton pumping in vivo. Several V-ATPase subunits have been proposed to have additional roles in the cell not related to proton translocation.[29−33] The remarkable property of Vma9p is that, unlike other V-ATPase subunits, it does not appear to also have an essential role for proton pumping, at least in vitro. As with other V-ATPase subunits, deletion of VMA9 results in the full Vma- growth phenotype in yeast, which do not acidify the vacuole.[11] Vma9p has been shown to be a vacuolar protein, and its presence in the vacuole depends on the presence of the V_O region.[11] Deletion of any V_O region protein or V-ATPase assembly factors prevents Vma9p from exiting the endoplasmic reticulum or interacting with the Vma21p assembly factor, suggesting that Vma9 could also function as an assembly factor.[12] Furthermore, expression of the gene for the bovine equivalent of Vma9p was shown to be tissue-specific, with high level expression occurring in the kidney, adrenal gland, spleen, and lung, low level expression in the heart, and negligible expression in skeletal muscle and the brain cortex.[8] This tissue specificity may be key to understanding the function of Vma9p.

AUTHOR INFORMATION

Corresponding Author

*Email: john.rubinstein@utoronto.ca. Phone: (416) 813-7255. Fax (416) 813-5022.

DOI: 10.1021/bi5013172
Biochemistry 2015, 54, 853−858

Author Contributions

S.A.B. and J.L.R. designed the experiments, S.A.B. performed the experiments, and J.L.R. wrote the manuscript.

Funding

This work was supported by operating grant MOP 81294 to J.L.R. from the Canadian Institutes of Health Research. J.L.R. holds a Canada Research Chair.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Michael Latham for discussions and a critical reading of this manuscript.

■ REFERENCES

(1) Kane, P. M. (2006) The where, when, and how of organelle acidification by the yeast vacuolar H+-ATPase. *Microbiol. Mol. Biol. Rev. 70*, 177−191.

(2) Kitagawa, N., Mazon, H., Heck, A. J. R., and Wilkens, S. (2008) Stoichiometry of the peripheral stalk subunits E and G of yeast V1-ATPase determined by mass spectrometry. *J. Biol. Chem. 283*, 3329−3337.

(3) Diepholz, M., Börsch, M., and Böttcher, B. (2008) Structural organization of the V-ATPase and its implications for regulatory assembly and disassembly. *Biochem. Soc. Trans. 36*, 1027−1031.

(4) Muench, S. P., Huss, M., Song, C. F., Phillips, C., Wieczorek, H., Trinick, J., and Harrison, M. A. (2009) Cryo-electron microscopy of the vacuolar ATPase motor reveals its mechanical and regulatory complexity. *J. Mol. Biol. 386*, 989−999.

(5) Benlekbir, S., Bueler, S. A., and Rubinstein, J. L. (2012) Structure of the vacuolar-type ATPase from Saccharomyces cerevisiae at 11-A resolution. *Nat. Struct. Mol. Biol. 19*, 1356−1362.

(6) Sumner, J. P., Dow, J. A., Earley, F. G., Klein, U., Jager, D., and Wieczorek, H. (1995) Regulation of plasma membrane V-ATPase activity by dissociation of peripheral subunits. *J. Biol. Chem. 270*, 5649−5653.

(7) Kane, P. M. (1995) Disassembly and reassembly of the yeast vacuolar H(+)-ATPase in vivo. *J. Biol. Chem. 270*, 17025−17032.

(8) Ludwig, J., Kerscher, S., Brandt, U., Pfeiffer, K., Getlawi, F., Apps, D. K., and Schagger, H. (1998) Identification and characterization of a novel 9.2-kDa membrane sector-associated protein of vacuolar proton-ATPase from chromaffin granules. *J. Biol. Chem. 273*, 10939−10947.

(9) Merzendorfer, H., Huss, M., Schmid, R., Harvey, W. R., and Wieczorek, H. (1999) A novel insect V-ATPase subunit M9.7 is glycosylated extensively. *J. Biol. Chem. 274*, 17372−17378.

(10) Hill, K. J., and Stevens, T. H. (1994) Vma21p is a yeast membrane protein retained in the endoplasmic reticulum by a di-lysine motif and is required for the assembly of the vacuolar H(+)-ATPase complex. *Mol. Biol. Cell 5*, 1039−1050.

(11) Sambade, M., and Kane, P. M. (2004) The yeast vacuolar proton-translocating ATPase contains a subunit homologous to the Manduca sexta and bovine e subunits that is essential for function. *J. Biol. Chem. 279*, 17361−17365.

(12) Compton, M. A., Graham, L. A., and Stevens, T. H. (2006) Vma9p (subunit e) is an integral membrane V0 subunit of the yeast V-ATPase. *J. Biol. Chem. 281*, 15312−15319.

(13) Lau, W. C. Y., and Rubinstein, J. L. (2012) Sub-nanometer resolution structure of the intact T. thermophilus H(+)-driven ATP synthase reveals the arrangement of its transmembrane helices. *Nature 481*, 214−218.

(14) Van Den Ent, F., and Löwe, J. (2006) RF cloning: a restriction-free method for inserting target genes into plasmids. *J. Biochem. Biophys. Methods 67*, 67−74.

(15) Rubinstein, J. L. (2007) Structural analysis of membrane protein complexes by single particle electron microscopy. *Methods 41*, 409−416.

(16) Lötscher, H. R., deJong, C., and Capaldi, R. A. (1984) Modification of the F0 portion of the H+-translocating adenosine-triphosphatase complex of Escherichia coli by the water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and effect on the proton channeling function. *Biochemistry 23*, 4128−4134.

(17) Nelson, H., and Nelson, N. (1990) Disruption of genes encoding subunits of yeast vacuolar H(+)-ATPase causes conditional lethality. *Proc. Natl. Acad. Sci. U. S. A. 87*, 3503−3507.

(18) Ohya, Y., Umemoto, N., Tanida, I., Ohta, A, Iida, H., and Anraku, Y. (1991) Calcium-sensitive cls mutants of Saccharomyces cerevisiae showing a Pet- phenotype are ascribable to defects of vacuolar membrane H(+)-ATPase activity. *J. Biol. Chem. 266*, 13971−13977.

(19) Manolson, M. F., Wu, B., Proteau, D., Taillon, B. E., Roberts, B. T., Hoyt, M. A., and Jones, E. W. (1994) STV1 gene encodes functional homologue of 95-kDa yeast vacuolar H(+)-ATPase subunit Vph1p. *J. Biol. Chem. 269*, 14064−14074.

(20) Chen, Z., Speck, C., Wendel, P., Tang, C., Stillman, B., and Li, H. (2008) The architecture of the DNA replication origin recognition complex in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A. 105*, 10326−10331.

(21) Bowman, E. J., Graham, L. A., Stevens, T. H., and Bowman, B. J. (2004) The bafilomycin/concanamycin binding site in subunit c of the V-ATPases from Neurospora crassa and Saccharomyces cerevisiae. *J. Biol. Chem. 279*, 33131−33138.

(22) Lee, H. C., and Forte, J. G. (1978) A study of H+ transport in gastric microsomal vesicles using fluorescent probes. *Biochim. Biophys. Acta 508*, 339−356.

(23) Forgac, M., and Berne, M. (1986) Structural characterization of the ATP-hydrolyzing portion of the coated vesicle proton pump. *Biochemistry 25*, 4275−4280.

(24) Velours, J., and Arselin, G. (2000) The Saccharomyces cerevisiae ATP synthase. *J. Bioenerg. Biomembr. 32*, 383−390.

(25) Lau, W. C., Baker, L. A., and Rubinstein, J. L. (2008) Cryo-EM structure of the yeast ATP synthase. *J. Mol. Biol. 382*, 1256−1264.

(26) Rubinstein, J., and Walker, J. (2002) ATP synthase from Saccharomyces cerevisiae: location of the OSCP subunit in the peripheral stalk region. *J. Mol. Biol. 321*, 613−619.

(27) Runswick, M. J., Bason, J. V., Montgomery, M. G., Robinson, G. C., Fearnley, I. M., and Walker, J. E. (2013) The affinity purification and characterization of ATP synthase complexes from mitochondria. *Open Biol. 3*, 120160.

(28) Symersky, J., Osowski, D., Walters, D. E., and Mueller, D. M. (2012) Oligomycin frames a common drug-binding site in the ATP synthase. *Proc. Natl. Acad. Sci. U. S. A. 109*, 13961−13965.

(29) Hurtado-Lorenzo, A., Skinner, M., El Annan, J., Futai, M., Sun-Wada, G. H., Bourgoin, S., Casanova, J., Wildeman, A., Bechoua, S., Ausiello, D. A., Brown, D., and Marshansky, V. (2006) V-ATPase interacts with ARNO and Arf6 in early endosomes and regulates the protein degradative pathway. *Nat. Cell Biol. 8*, 124−136.

(30) Merkulova, M., Hurtado-Lorenzo, A., Hosokawa, H., Zhuang, Z., Brown, D., Ausiello, D. A., and Marshansky, V. (2011) Aldolase directly interacts with ARNO and modulates cell morphology and acidic vesicle distribution. *Am. J. Physiol.: Cell Physiol. 300*, C1442−1455.

(31) Lu, X., Yu, H., Liu, S. H., Brodsky, F. M., and Peterlin, B. M. (1998) Interactions between HIV1 Nef and vacuolar ATPase facilitate the internalization of CD4. *Immunity 8*, 647−656.

(32) Geyer, M., Yu, H., Mandic, R., Linnemann, T., Zheng, Y.-H., Fackler, O. T., and Peterlin, B. M. (2002) Subunit H of the V-ATPase binds to the medium chain of adaptor protein complex 2 and connects Nef to the endocytic machinery. *J. Biol. Chem. 277*, 28521−28529.

(33) Peters, C., Bayer, M. J., Buhler, S., Andersen, J. S., Mann, M., and Mayer, A. (2001) Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. *Nature 409*, 581−588.

DOI: 10.1021/bi5013172
Biochemistry 2015, 54, 853−858