Affinity Purification and Structural Features of the Yeast Vacuolar ATPase V₀ Membrane Sector*

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Background: Vacuolar ATPase is regulated by reversible disassembly into V₁-ATPase and the V₀ proton channel.
Results: Upon enzyme disassembly, subunit α cytoplasmic domain changes conformation to bind subunit d.
Conclusion: The conformational change of subunit α may play a role in blocking passive proton flow through free V₀.
Significance: The interaction of αNT and d in free V₀ ensures the efficient reassembly of the holo enzyme.

The membrane sector (V₀) of the proton pumping vacuolar ATPase (V-ATPase, V₁V₀-ATPase) from Saccharomyces cerevisiae was purified to homogeneity, and its structure was characterized by EM of single molecules and two-dimensional crystals. Projection images of negatively stained V₀ two-dimensional crystals showed a ring-like structure with a large asymmetric mass at the periphery of the ring. A cryo-EM reconstruction of V₀ from single-particle images showed subunits α and d in close contact on the cytoplasmic side of the proton channel. A comparison of three-dimensional reconstructions of free V₀ and V₀ as part of holo V₁V₀ revealed that the cytoplasmic N-terminal domain of subunit α (αNT) must undergo a large conformational change upon enzyme disassembly or (re)assembly from V₀ V₁, and subunit C. Isothermal titration calorimetry using recombinant subunit d and αNT revealed that the two proteins bind each other with a Kd of ~5 μM. Treatment of the purified V₀ sector with 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] resulted in selective release of subunit d, allowing purification of a V₀Δd complex. Passive proton translocation assays revealed that both V₀ and V₀Δd are impermeable to protons. We speculate that the structural change in subunit α upon release of V₁ from V₀ during reversible enzyme dissociation plays a role in blocking passive proton translocation across free V₀ and that the interaction between αNT and d seen in free V₀ functions to stabilize the V₀ sector for efficient reassembly of V₁V₀.

Vacuolar H⁺-ATPases (V-ATPases, V₁V₀-ATPases)² are large, multisubunit protein complexes that are found in the endomembrane system of all eukaryotic organisms, where they function to acidify the lumen of intracellular organelles (1–3). In specialized cells of higher eukaryotes, V-ATPases are also found in the plasma membrane, where the enzyme is pumping protons into the extracellular milieu (1, 4). Aberrant V-ATPase function has been shown to be associated with numerous widespread human conditions, including renal tubular acidosis (5), sensorineural deafness (6), osteoporosis (7), diabetes (8), microbial (9) and viral infection (10), infertility (11), and cancer (12). V-ATPase has therefore been deemed a valuable drug target (13–15). However, lack of detailed structural information has limited the application of structure-based drug discovery so far.

V-ATPase can be divided into an ATP-hydrolyzing catalytic headpiece, V₁, and a membrane-embedded proton-translocating sector, V₀. The subunit composition of the enzyme from the model organism Saccharomyces cerevisiae is A₈B₃(C)DₓE₃FₓGₓH for V₁ (16) and αcₑc’ede for V₀ (17, 18). Crystal structures of the bacterial V₁-ATPase from Enterococcus hirae show the three A and B subunits arranged in an alternating fashion around a central cavity within which are located the N- and C-terminal ends of subunit D (19). The proton channel is formed at the interface of the ring of the c, c’, and c” subunits (“proteolipid” ring) and the C-terminal domain of subunit α (αC₇) (17, 20). V-ATPase is a member of the family of rotary molecular motor enzymes that, next to V-ATPase, includes F-ATP synthase, found in bacteria, mitochondria, and chloroplasts; archaeal A-ATP synthase; and bacterial A/V-like ATPase (21–23). In eukaryotic V-ATPase, ATP hydrolysis taking place at three catalytic sites located at the interface of the A and B subunits on the membrane extrinsic V₁ is coupled to proton translocation across the V₀, via a central rotor formed by the DF heterodimer of the V₁ and the subunit d-proteolipid ring subcomplex of the V₀. Three peripheral stalks, formed by subunit EG heterodimers together with the single-copy H and C subunits, form the stator that links the catalytic sector to the membrane-embedded proton channel via dNT, and that functions to withstand the torque generated during rotary catalysis (Fig. 1A). However, unlike the related F-, A- and bacterial V-type motors, eukaryotic vacuolar ATPase is regulated by a reversible disassembly and reassembly mechanism employed by the organism to modulate the activity of the complex in

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The 3D cryo-EM reconstruction has been deposited at the EMDB with accession number EMD-2975.

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2 The abbreviations used are: V-ATPase, vacuolar ATPase; DDM, dodecyl maltoside; UnDM, undecyl-β-o-maltoside; LPPG, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol); DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; TAP, tandem affinity purification; BME, β-mercaptoethanol; SAXS, small-angle X-ray scattering; MBP, maltose binding protein; ITC, isothermal titration calorimetry; P(3,5)P₂, phosphatidylinositol 3,5-bisphosphate.

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response to, e.g., nutrient availability or developmental state (24, 25). The mechanism of reversible disassembly has been studied extensively in the yeast system, and it is known that V-ATPase dissociation results in a cytoplasmic V₁ and a membrane-bound V₀ sector that have no magnesium ATPase and passive proton translocation activity, respectively. The rotor is shown in green, the stator in red/orange, the catalytic core in cyan/blue, and subunit H in purple.

Molecular Architecture of the Yeast V-ATPase Proton Channel

Experimental Procedures

Reagents—Dodecyl maltoside (DDM), undecyl-β-D-maltoside (UnDM), and CHAPS were from Anatrace. 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (LPPG), phosphatidylcholine, phosphatidic acid, and 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) were obtained from Avanti. Calmodulin-Sepharose beads were from GE Healthcare or Agilent, and 9-amino-6-chloro-2-methoxyacridine dye was from Invitrogen. The anti-TAP tag polyclonal antibody directed against the amino acid sequence upstream of the tobacco etch virus protease cleavage site was from GenScript. All other reagents were of analytical grade.

Yeast Strains and Growth—Yeast strain YSC1178-7502926 with a TAP tag fused at the C terminus of subunit a (a\textsubscript{TAP}) was from Open Biosystems. To disrupt the subunit B (Vma2p) gene in YSC1178-7502926, a fragment of genomic DNA containing the NAT1 fragment was amplified by PCR from genomic DNA of strain BY4741 vma2\textsubscript{H9004}::nat1 using oligonucleotides vma2-840\textsubscript{H11032}GAATCGGCTAGAGATTACAAC-3\textsubscript{H11032} and vma2-c4 5\textsubscript{H11032}CAT-GTTCTTCGAGACCGGGTTG G-3\textsubscript{H11032}. The resulting 1.2-kb product was used to transform YSC1178-7502926. Transformed colonies were selected on yeast extract, peptone, dextrose buffered to pH 5.0 but not on yeast extract, peptone, dextrose buffered to pH 7.5 \textsubscript{H11001} \textsubscript{60 mM CaCl\textsubscript{2}}. Western blot analysis of whole-cell lysates using anti-TAP and anti-subunit B antibodies were performed to confirm the presence and absence of a\textsubscript{TAP} and subunit B, respectively. The resulting strain was grown in yeast extract.
peptone, dextrose supplemented with 50 mM KH₂PO₄ and 50 mM succinic acid (pH 5). For large-scale biomass production, cells from 8–10 liters of flask culture (A₅₆₀ ~ 7) were collected by centrifugation, transferred to a 10-liter fermenter, and grown to the second diauxic log phase. Cells were harvested by centrifugation, washed once with distilled water, and stored at −80 °C until use. Final cell weight was 8–10 g/liter of culture.

**Isolation of Membranes**—All steps were performed at 4 °C unless noted otherwise. Cells were resuspended in lysis buffer (20 mM Tris-HCl, 150 mM NaCl (pH 7.4) (TBS) supplemented with 8% sucrose, 2% sorbitol, and 2% glucose), and an inhibitor mixture was added to a final concentration of 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 0.5 μg/ml chymostatin, and 1 mM PMSF. 1 mM EDTA was added before disrupting cells in a homemade bead beater using 0.5 mm Zirconia beads (BioSpec), keeping the temperature below 14 °C inside the chamber. Cell debris was removed by low-speed centrifugation (1200 × g, 10 min), and crude membranes were collected by ultracentrifugation at 130,000 × g for 1 h and washed once in lysis buffer. The final membrane pellet was resuspended in the presence of the inhibitor mixture mentioned above. Protein concentration was measured, and membranes were frozen at −80 °C until use.

**Vₒ Purification**—Isolated membranes were diluted to a final concentration of 10 mg/ml in lysis buffer, and inhibitor mixture was added. Extraction was carried out by adding DDM from a 20% stock solution in water to a final concentration of 2 mg of detergent/1 mg of protein, followed by gentle stirring for 1 h. Extracted membranes were cleared by ultracentrifugation at 106,000 × g for 1 h, and the pellet was discarded. The supernatant was collected carefully, avoiding the upper lipid layer, and CaCl₂ was added to a final concentration of 4 mM. The mixture was incubated with 4 ml of Calmodulin beads for 1 h at 4 °C under gentle agitation. The beads were collected in a chromatography column and washed with 20 column volumes of 10 mM Tris-HCl (pH 8), 10 mM β-mercaptoethanol (BME), 2 mM CaCl₂, 0.1% DDM, 150 mM NaCl, and 20 column volumes of the same buffer without NaCl. The column was eluted with 10 mM Tris-HCl (pH 8), 10 mM BME, 0.5 mM EGTA, and 0.1% DDM. Fractions were analyzed by 13% SDS-PAGE, and fractions containing Vₒ were pooled and concentrated in a 100-kDa Vivaspin concentrator (Sartorius Stedim Biotech).

**Glycerol Gradient Centrifugation and Removal of Subunit d**—1 mg of purified Vₒ was applied to the top of a discontinuous glycerol gradient (15–35% (v/v), 10 mM Tris-HCl (pH 8), 10 mM BME, 0.5 mM EGTA, and 0.01% phosphatidylcholine:phosphatidic acid (19:1)) and centrifuged at 200,000 × g for 16 h at 4 °C. For removal of subunit d, 0.05% LPPG was included in the gradient. Otherwise, 0.5% CHAPS was used. Fractions were collected from the bottom and analyzed by SDS-PAGE.

**Reconstitution in Liposomes**—200 μg of Vₒ (in CHAPS) or subunit d-depleted Vₒ (Vₒ,Δd) (in LPPG) was mixed with 15 mg of phosphatidylcholine:phosphatidic acid (19:1) and adjusted with CHAPS to 6%. In some experiments, 9% ergosterol was included in the reconstitution mix. Samples were applied to a Sephadex G50 column (50 cm × 1.6 cm) and eluted with high-potassium buffer (20 mM HEPES (pH 7), 2 mM BME, 0.2 mM EGTA, 10% glycerol, 100 mM K₂SO₄, and 0.5 mg/ml fatty acid-free BSA) at a flow rate of 0.5 ml/min. The eluate was collected in 1-ml fractions, and turbid fractions were analyzed by 13% SDS-PAGE and silver staining.

**Passive Proton Translocation Assay**—Proton translocation assays were performed as described by Qi and Forgac (34). Briefly, assays were conducted in a 3-ml cuvette. 30 μl of each fraction was preincubated in high-sodium buffer (20 mM HEPES (pH 7), 2 mM BME, 0.2 mM EGTA, 10% glycerol, 150 mM NaCl, and 0.5 mg/ml fatty acid-free BSA) for 5 min at 30 °C in the presence of 2 μM 9-amino-6-chloro-2-methoxyacridine. After 300-s incubation, the process was started by addition of 1 μM valinomycin, followed by 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone. Different fractions were probed after 1, 4, and 10 days. Six independent preparations of Vₒ and Vₒ,Δd were analyzed.

**Flotation Assays**—To verify lipid vesicle reconstitution of Vₒ and Vₒ,Δd, fractions from the G50 column (fraction 29 for Vₒ and fraction 28 for Vₒ,Δd) were adjusted with sucrose to 53% and placed at the bottom of an 11-ml tube. A sucrose step gradient (40, 20, and 0% (w/v) in 20 mM HEPES (pH 7), 2 mM BME, 0.2 mM EGTA, 10% glycerol, and 150 mM NaCl) was layered on top of the Vₒ sample and centrifuged at 200,000 × g for 16 h at 4 °C. Fractions were collected from the top of the tube and analyzed by 13% SDS-PAGE and silver staining.

**Mass Spectrometry of Vₒ Subunits**—The Vₒ sector was precipitated with 1% trichloroacetic acid, and the centrifuged pellet was washed with water. The pellet was extracted with a 1:1 mixture of water and trifluoroethanol, and the soluble fraction was analyzed by electrospray ionization mass spectrometry using a Q-TOF Micro mass spectrometer (Waters, Inc.) in positive ion mode. Charge envelopes between 800–2500 m/z were deconvoluted using MaxEnt2 as implemented in MassLynx4.1. Calibration of the instrument was carried out with phosphoric acid and sodium/cesium iodide. Analysis of gel bands by peptide sequencing was done at the Upstate Medical University mass spectrometry core facility using a Thermo LTQ Orbitrap mass spectrometer.

**Small-angle X-ray Scattering Analysis**—Small-angle x-ray scattering (SAXS) data were collected at the Cornell High-energy Synchrotron Source (MacCHESS) F2 beam line operating at a wavelength of 1.2524 Å at 4 °C. For SAXS data collection, Vₒ was purified using UnDM instead of DDM. Vₒ was diluted into 10 mM Tris-HCl (pH 8), 10 mM BME, 0.5 mM EGTA, and 0.05% UnDM to 1, 2, 4, 6, 8, and 10 mg/ml. 30-μl samples were exposed twice for 180 s without an obvious decay in signal. Signal averaging, buffer subtraction and Guinier analysis were done in Bioxtras RAW (35). Molecular weight was estimated using lysozyme as the standard. Thirty data points were used for Guinier analysis for each tested concentration (qRg was ~1.1 in all cases).

**Two-dimensional Crystallization and EM Analysis**—Purified Vₒ was diluted to 3 mg/ml in 10 mM Tris-HCl (pH 6.5), 10 mM BME, 0.5 mM EGTA, 10% glycerol, and 1 mM DTT, and sonicated DOPC was added to reach a lipid-to-protein ratio of 0.3 (w/w). After 24 h, the detergent was removed by stepwise addition (every 3 days) of equal amounts of polysyrène beads (BioBeads SM2, Bio-Rad) for a total of 10 days so that the final ratio of beads to liquid was ~1:1. Samples were kept at 4 °C, and 1 mM sodium azide was added to inhibit bacterial growth. Vₒ
two-dimensional crystals were spotted on glow discharge-treated, carbon-coated copper grids and stained with 1% uranyl acetate. Micrographs were recorded on a 4096 × 4096 charge-coupled device (TVIPS F415MP) at 20,000–40,000 electron optical magnification and an underfocus of 1.5 μm. The quality of the crystalline areas was assessed from calculated power spectra, and areas showing isotropic reflections to ~20-Å resolution were excised and analyzed with the 2dx package of programs (36) and/or by correlation averaging as implemented in IMAGIC 5 (37, 38).

Cryo EM and Single-particle Image Analysis—The solubilized Vₐₐ sector was vitrified at 1–2 mg/ml on glow-discharged, holey, carbon-coated copper grids (C-flat, 2/2 μm). Grids were mounted in a Gatan 626 cryoholder and imaged in a JEOL JEM-2100 transmission electron microscope operating at 120 kV. Micrographs were recorded on a 4096 × 4096 charge-coupled device (TVIPS F415MP) at an electron optical magnification of ×40,000 and an underfocus of between 1.5–2.5 μm. The calibrated pixel size on the specimen level was 2.62 Å. A total of 12,035 particles was extracted as 144 × 144 pixel images using the “boxer” program of the EMAN1.9 software package (39). The dataset was contrast transfer function-corrected using “ctfit” as implemented in EMAN1.9. All subsequent image analysis was done with the IMAGIC 5 package of programs (37). Images were bandpass-filtered to remove low (<6.4 × 10⁻³ Å⁻¹) and high (>0.15 Å⁻¹) spatial frequencies, and a soft-edge circular mask was applied before subjecting the images to reference-free alignment (40). Averages from the reference-free alignment were then used in subsequent multireference alignment, and the multireference alignment was iterated to obtain averages of the most abundant projections. Three-dimensional reconstruction was initiated by one round of projection-matching using the low pass-filtered three-dimensional reconstruction of the bovine V-ATPase membrane sector (29) as a reference model. Because the bovine Vₐₐ sector subunit Ac45 is not found in yeast Vₐₐ, the density corresponding to Ac45 was removed with the volume eraser tool as implemented in EMAN1.9. All subsequent image analysis was done with the IMAGIC 5 package of programs (37). The final EM density was fitted manually with cryoEM visualization software Chimera (41). Cycles of projection-matching alignment and three-dimensional reconstruction were iterated with increasing numbers of references until no further improvement was observed. The resolution of the final model was estimated using the 0.5 Fourier shell correlation criterion (42). The final EM density was fitted manually with crystal structures of bacterial homologs for the yeast Vₐₐ subunits, including the aₐNT₈ homolog from *Meiothermus ruber* (I₈NT, PDB code 3RRK), the subunit d homolog from *Thermus thermophilus* (subunit C, PDB code 1RS2), and the c subunit ring homolog from *E. hirae* (K₁₀c, PDB code 2BL2). Although the primary sequence conservation between the yeast and bacterial subunits is limited (11%, 16%, and 26% for aₐNT₈ and I₈NT, d and C, and c and K, respectively), their secondary and tertiary structure is highly conserved, as evidenced by the fact that the yeast subunit structures can be modeled on the basis of the bacterial A/V-ATPase subunit crystal structures using the Phyre2 server (43) with 100% confidence.

Expression and Purification of aₐNT(1–372) and Subunit d—Plasmid pRS316 containing the open reading frame for subunit d (Vma6p) was a gift from the laboratory of Dr. Karlett Parra (University of New Mexico). The coding sequence for subunit d was PCR-amplified using primers GCTCAGTG ACCGATGG AAGGCCGTGATTTCAATATT (forward) and CGAGTCC TGCAGTCAATCAATAACGG GAAATATAATT (reverse), and the resulting PCR product was ligated into pGEM T-easy. Subsequent cloning of the subunit d coding sequence into a modified plasmid pMAL-c2e (New England Biolabs, enterokinase cleavage site replaced by the human rhinovirus 3C site) for bacterial protein expression was done by BioBasic, Inc. (Markham, Ontario). The resulting construct consisted of subunit d with an N-terminal fusion of maltose binding protein (MBP) separated by a protease cleavage site (human rhinovirus 3C protease) for removal of MBP. pMAL-c2E harboring MBP subunit d was expressed in *Escherichia coli* strain Rosetta2. Cells were grown in rich broth (Lennox broth plus 0.2% thio-β-d-galactopyranoside for 4 h at 37 °C. Purification was done following the recommended protocol for MBP-tagged proteins (New England Biolabs). The PreScission Protease-cleaved (GE Healthcare) fusion was dialyzed against 25 mM Tris-HCl, 1 mM EDTA, 1 mM tris(2-carboxyethyl)phosphine (pH 7), followed by anion exchange chromatography on a 1-mL mono Q-Sepharose column attached to an AKTA FPLC (GE Life Sciences). Under these buffer conditions, subunit d bound to the column and was eluted using a 0–500 mM sodium chloride gradient in the same buffer. Protein-containing fractions were pooled, concentrated to 1–2 ml, and subjected to size-exclusion chromatography over Superdex S75 (16 × 500 mm). An expression construct for the N-terminal domain of subunit a (Vph1p) consisting of residues 1–372 (aₐNT(1–372)) was generated as described previously (44). The cleavable MBP tag resulted in an N-terminal extension for both subunit d and aₐNT(1–372) constructs with the amino acid sequence GPKVP. Constructs were confirmed by DNA sequencing. A detailed biochemical and biophysical characterization of recombinant subunit d and aₐNT(1–372) will be presented elsewhere.

Isothermal Titration Calorimetry (ITC)—ITC measurements of the interaction of aₐNT(1–372) with subunit d using a Microcal VP-ITC isothermal titration calorimeter were done as described previously (44, 45), with the following modifications. Prior to the titration, both proteins were dialyzed (in the same container) against 2 liters of 25 mM Tris-HCl (pH 7), 0.5 mM EDTA, and 1 mM tris(2-carboxyethyl)phosphine. aₐNT(1–372) was concentrated to 375 μM and titrated into 25 μM subunit d at 10 °C using a total of 30 injections with 10.7% saturation/injection. A heat of dilution titration of 375 μM aₐNT(1–372) into dialysis buffer was subtracted from the aₐNT(1–372) into subunit d titration. A second titration was carried out with 320 μM aₐNT(1–372) in the syringe using, again, 25 μM subunit d in the ITC cell. Both titrations produced very similar results. Protein concentrations were determined from A₂₈₀ using calculated extinction coefficients. ITC data were fitted to a one-site model using the VP-ITC programs in Originlab.

Other Methods—Membrane protein concentrations were measured by BCA method (Thermo Scientific) and improved by TCA precipitation as in Lowry-TCA (46) using fatty acid-free BSA as the standard.
Results

Purification of the $V_o$ Proton Channel Sector—To isolate the yeast $V_o$ sector for structural and functional studies, we attempted several strategies, including C- and N-terminal histidine tags fused to subunits $d$ and $c''$ as well as a TAP tag fused to the C terminus of subunit $a$ or $c$. In the end, the best results were obtained using only the second affinity step of the TAP procedure, where $V_o$ (containing TAP-tagged subunit $a$) is detergent-solubilized from vacuolar membranes and captured by a calmodulin column by way of the calmodulin binding peptide in the tag. To eliminate possible co-purification of (partially) assembled $V_1$-$V_o$ holo enzyme, yeast was grown in a 10-liter fermenter. The yield of $V_o$ sector purified as described under “Experimental Procedures” was $\sim 2$–$3$ mg/150 g of cells. SDS-PAGE of fractions 1–4 eluted from the calmodulin affinity column (10 µg loaded) shows bands for subunits $a$, $d$, $c''$, $c$, $c''$, and $e$.

Fig. 2A shows SDS-PAGE of the final preparation obtained after concentrating fractions 2–4 of the calmodulin column (lane 1, 10 µg of protein loaded), and lane 2 shows SDS-PAGE of holo $V$-ATPase (47) for comparison. Mass spectrometry analysis of the subunit $a$ band from SDS-PAGE gels such as shown in Fig. 2A and 2B, only produced subunit $a$-derived peptides, with no peptides from protein A being detected, indicating that the protein A moiety of the TAP tag was lost because of proteolytic degradation following cell lysis and detergent extraction of membranes. Loss of protein A was confirmed by immunoblot analysis (using an antibody directed against the C-terminal end of the calmodulin binding peptide) that showed that the apparent molecular mass of the subunit $a$ band decreased in size from an initial $\sim 130$ kDa at the washed membrane stage to the final $\sim 116$ kDa after the elution from the calmodulin affinity column (data not shown). Electrospray ionization/TOF mass spectrometry analysis of denatured $V_o$ revealed the presence of proteins with masses of $39,903$ Da (subunit $d$, expected $39,791$ Da), $16,255$ Da (subunit $c$, expected $16,219$ Da), and $8,250$ Da (subunit $e$, expected $8,249$ Da). The mass differences suggest N-terminal acetylation for subunit $c$ and an $\sim 100$-Da modification.
for subunit d. Possibly because of their large size or lower abundance, no peaks for subunits a (100,143 Da without protein A), c' (16,902 Da), and c'' (22,464 Da) were observed in the deconvoluted charge series (data not shown).

**Fig. 2** shows negative-stain transmission electron microscopy analysis of detergent-solubilized Vo. The image shows homogeneously sized particles with a diameter of ~10–15 nm, indicating that the preparation contains intact Vo sectors that are stable in the detergent used for purification (DDM). Furthermore, Guinier plots of small angle x-ray scattering profiles obtained from solutions of Vo sector purified in UnDM (chosen here for its smaller micelle size) showed that the preparation is monodisperse at concentrations of up to 10 mg/ml. The molecular mass of Vo as estimated by SAXS was 544 ± 33 kDa, with a calculated radius of gyration of ~51 ± 3.5 Å. The expected mass of Vo (assuming a subunit ratio of ac'c'de, see next paragraph and “Discussion”) is ~320 kDa, resulting in a difference between the measured and expected mass of ~244 kDa. Considering the average size of UnDM micelles of 35 kDa (micelle size reported by Anatrace) suggests that each Vo sector binds six to seven detergent micelles. Taken together, the data show that highly purified, stable, and monodispersed yeast Vo sector can be obtained via affinity chromatography using a calmodulin peptide fused to the C terminus of subunit a.

**Two-dimensional Crystallization of the Yeast Vo Sector**—Fig. 3 summarizes the transmission electron microscopy analysis of yeast Vo domain two-dimensional crystals. The two-dimensional crystals were obtained by mixing purified Vo at 3 mg/ml with 1 mg/ml DOPC with subsequent removal of detergent (DDM) with polystyrene beads. A, overview of a large, single-layer sheet of reconstituted Vo, containing several crystalline domains (negatively stained with 1% uranyl acetate). B, larger view of a crystalline area, with the power spectrum shown in C, D, IQ plot after unbending using the 2dx package of programs (36). E, projection map with unit cell outlined by the rectangle. The cell dimensions are 101 × 236 Å with 2 molecules/cell. The crystals belong to plane group P1 with alternating up and down orientation of the molecules. At the current resolution of ~24 Å, the projections obtained in negative stain show a ring-like structure (arrow) with a two-domain asymmetric mass at the periphery (arrowheads). We interpret the ring to represent the proteolipid ring and the asymmetric mass ac'T. F, cross-section of the E. hirae K10 ring as seen perpendicular to the plane of the membrane, filtered to a resolution of 16 Å.

**FIGURE 3.** Electron crystallography of yeast Vo sector two-dimensional crystals. The two-dimensional crystals were obtained by mixing purified Vo at 3 mg/ml with 1 mg/ml DOPC with subsequent removal of detergent (DDM) with polystyrene beads. A, overview of a large, single-layer sheet of reconstituted Vo, containing several crystalline domains (negatively stained with 1% uranyl acetate). B, larger view of a crystalline area, with the power spectrum shown in C, D, IQ plot after unbending using the 2dx package of programs (36). E, projection map with unit cell outlined by the rectangle. The cell dimensions are 101 × 236 Å with 2 molecules/cell. The crystals belong to plane group P1 with alternating up and down orientation of the molecules. At the current resolution of ~24 Å, the projections obtained in negative stain show a ring-like structure (arrow) with a two-domain asymmetric mass at the periphery (arrowheads). We interpret the ring to represent the proteolipid ring and the asymmetric mass ac'T. F, cross-section of the E. hirae K10 ring as seen perpendicular to the plane of the membrane, filtered to a resolution of 16 Å.
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Cryo-electron Microscopy and Three-dimensional Reconstruction—Fig. 4 summarizes single-molecule cryo-transmission electron microscopy analysis of the detergent-solubilized V$_o$ sector. V$_o$ was vitrified at a concentration of 2 mg/ml and visualized at $-170$ °C. Individual side view projections are shown at the top right, and averages obtained by reference-free alignment of characteristic side and top views are shown at the bottom right. Bar = 20 nm. B, side view (left) and top view (right) of the three-dimensional reconstruction of yeast V$_o$ calculated from a dataset of 12,000 single molecules at a resolution of 18 Å. The gray rectangle in the side view indicates the position of the lipid bilayer. C, fitting of crystal structures into the EM reconstruction. The structural models used were the a$_{NT}$ homolog from M. ruber (PDB code 3RRK, blue), the subunit d homolog from T. thermophilus (PDB code 1RSZ, green), and the K$_{10}$ proteolipid ring from E. hirae (PDB code 2BL2, magenta). D, side view (left panel) and cross-section (center panel) of holo yeast V-ATPase cryo-EM reconstruction (emd-5476 (32)) for comparison. In free V$_o$ (right panel, arrow), the “distal” end of a$_{NT}$ is in contact with subunit d, whereas, in holo V$_o$, the distal domain of a$_{NT}$ is in a more peripheral position in contact with C$_{pose}$ and EG2 (center panel, arrow). For details, see text.

FIGURE 4. Three-dimensional reconstruction of the yeast V$_o$ sector from cryo-EM. A, the purified yeast V$_o$ domain was vitrified at a concentration of 2 mg/ml and visualized at $-170$ °C. Individual side view projections are shown at the top right, and averages obtained by reference-free alignment of characteristic side and top views are shown at the bottom right. Bar = 20 nm. B, side view (left) and top view (right) of the three-dimensional reconstruction of yeast V$_o$ calculated from a dataset of 12,000 single molecules at a resolution of 18 Å. The gray rectangle in the side view indicates the position of the lipid bilayer. C, fitting of crystal structures into the EM reconstruction. The structural models used were the a$_{NT}$ homolog from M. ruber (PDB code 3RRK, blue), the subunit d homolog from T. thermophilus (PDB code 1RSZ, green), and the K$_{10}$ proteolipid ring from E. hirae (PDB code 2BL2, magenta). D, side view (left panel) and cross-section (center panel) of holo yeast V-ATPase cryo-EM reconstruction (emd-5476 (32)) for comparison. In free V$_o$ (right panel, arrow), the “distal” end of a$_{NT}$ is in contact with subunit d, whereas, in holo V$_o$, the distal domain of a$_{NT}$ is in a more peripheral position in contact with C$_{pose}$ and EG2 (center panel, arrow). For details, see text.
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4D, left and center panels) reveals that \( \alpha_{NT} \) in the holo enzyme adopts a different conformation where the distal domain of \( \alpha_{NT} \) is bound to \( \text{C}_\text{foot} \) (subunit domain nomenclature as in Refs. 50 and 52, respectively) and the N termini of one of the three EG heterodimer peripheral stalks (EG2; Fig. 4D, center panel, arrow). In line with this observation, we have previously characterized the binding interactions between \( \alpha_{NT} \), \( \text{C}_\text{foot} \), and the EG heterodimer and we found that these interactions are of moderate affinity (44). Taken together, the comparison of \( \text{V}_o \) and holo \( \text{V}_1 \text{V}_o \) suggests that \( \alpha_{NT} \) undergoes a large conformational rearrangement upon enzyme disassembly, going from a conformation in free \( \text{V}_o \), that binds the \( d \) subunit to a more peripheral conformation that binds \( \text{C}_\text{foot} \) and EG2 in holo \( \text{V}_1 \text{V}_o \).

In Vivo Interaction of Recombinant Subunit \( d \) and \( \alpha_{NT(1–372)} \)−To test whether the interaction between \( \alpha_{NT} \) and subunit \( d \) as seen in the EM reconstruction is specific and can be quantified in vitro, we performed ITC experiments with recombinant subunits. For these experiments, subunit \( d \) and \( \alpha_{NT(1–372)} \) were expressed in \( E. \text{coli} \) as N-terminal fusions with MBP and affinity-purified on amylose resin. MBP was cleaved, and the resulting subunits were further purified using ion exchange and size-exclusion chromatography. Fig. 5A shows SDS-PAGE of \( \alpha_{NT(1–372)} \) (lane 1) and subunit \( d \) (lane 2). Both proteins are stable and highly soluble at pH 7, and, although recombinant subunit \( d \) elutes with an apparent molecular mass of \( \sim 42 \text{ kDa} \) from a S200 gel filtration column (expected, 40,267 Da), suggesting a globular monomeric protein, \( \alpha_{NT(1–372)} \) exists in a concentration-dependent monomer-dimer equilibrium, as already described for the shorter \( \alpha_{NT(104–372)} \) construct (Ref. 44 and data not shown). Fig. 5B shows a representative ITC experiment in which 375 \( \mu \text{M} \) \( \alpha_{NT(1–372)} \) was titrated into 25 \( \mu \text{M} \) subunit \( d \). As can be seen from the titration, complex formation between \( \alpha_{NT(1–372)} \) and subunit \( d \) was exergonic, and fitting the data to a one-site model revealed an \( N \) value of 0.98 (consistent with a 1:1 stoichiometry of complex formation), a \( K_\text{D} \) of 2.1 \( \times 10^5 \pm 3.5 \times 10^4 \text{ M}^{-1} \) (\( K_\text{D} \) is \( \sim 4.8 \text{ M} \)), a \( \Delta H \) of \(-4.2 \pm 0.22 \text{ kcal/mol} \), a \( \Delta S \) of 9.7 \text{cal/(K·mol)} \), and a \( \Delta G \) of \(-6.9 \text{ kcal/mol} \). After the titration, the ITC cell content was resolved by gel filtration (S200, 16 \( \times 500 \text{ mm} \)), and fractions were analyzed by SDS-PAGE (Fig. 5C). As can be seen from the gel, \( \alpha_{NT(1–372)} \) and subunit \( d \) co-elute around fraction 31 (62 ml), corresponding to an apparent molecular mass of \( \sim 72 \text{ kDa} \) (84 kDa expected for the \( \alpha_{NT(1–372)} \)-\( d \) complex; subunit \( d \) alone elutes at an apparent molecular mass of \( \sim 42 \text{ kDa} \), see above). Together, the ITC and gel filtration data suggest that subunit \( d \) and \( \alpha_{NT(1–372)} \) bind each other in a specific manner, albeit with moderate affinity.

Preparation and Functional Analysis of the \( \alpha_{C',c'}c'e' \) Subcomplex (\( \text{V}_{c',d} \))−In living cells, V-ATPase activity is regulated by reversible dissociation into \( \text{V}_1 \)-ATPase and membrane integral \( \text{V}_o \) sectors (Fig. 1B). Upon enzyme dissociation, the activity of both \( \text{V}_1 \) and \( \text{V}_o \) is silenced so that \( \text{V}_1 \) loses the ability to hydrolyze magnesium ATP and \( \text{V}_o \) becomes impermeable to protons. Considering the interaction described above between \( \alpha_{NT} \) and subunit \( d \) seen in free \( \text{V}_o \), but not \( \text{V}_1 \), we speculated that this interaction may contribute to the inhibition of passive proton translocation through isolated \( \text{V}_o \), by blocking rotation of the \( c' \)-ring past \( \alpha_{CT} \). To assess the role subunit \( d \) might be playing in blocking proton translocation through isolated \( \text{V}_o \), we developed a procedure to selectively remove subunit \( d \) from \( \text{V}_o \) to generate the \( \alpha_{C',c'}c'e' \) subcomplex (\( \text{V}_{c',d} \)). Fig. 6A and B, shows SDS-PAGE of glycerol density centrifugation of \( \text{V}_o \) sector, in presence of the ionic detergent LPPG and CHAPS, respectively. As can be seen from Fig. 6A, in the presence of LPPG, subunit \( d \) remains at the top of the gradient separated from \( \alpha_{C',c'}c'e' \), whereas, in CHAPS, subunit \( d \) migrates as part of intact \( \text{V}_o \). To determine whether removal of subunit \( d \) allows passive proton
translocation through the resulting \( V_o \Delta d \), \( V_o \) and \( V_o \Delta d \) were reconstituted into liposomes in the presence of potassium chloride-containing buffer. Liposomes were collected by centrifugation and subjected to SDS-PAGE and silver staining (Fig. 6C). \( V_o \) and \( V_o \Delta d \)-containing liposomes were subjected to a fluorescence-based assay to test for passive proton conductance. Fig. 6D shows a representative assay for passive proton conductance. As can be seen from the Fig. 6D, both \( V_o \) and \( V_o \Delta d \)-containing liposomes showed the same slow quenching upon addition of the potassium ionophore valinomycin and a sharp drop in the fluorescence signal following addition of the protonophore carbonyl cyanide \( p \)-trifluoromethoxyphenylhydrazone, which verified the presence of a proton gradient. This result indicates that removal of subunit \( d \) does not alleviate the inhibition of proton translocation across the \( V_o \), suggesting that the interaction of \( a_{CT} \) and subunit \( d \) in free \( V_o \) is not (solely) responsible for activity silencing in the isolated V-ATPase membrane sector.

**Discussion**

Transmembrane proton transport across the vacuolar ATPase \( V_o \) sector involves rotation of the proteolipid ring past \( a_{CT} \). A major difference between eukaryotic vacuolar ATPase and the related F- and A-type motors is the mode of regulation of V-ATPase, which involves dissociation into free ATPase and proton channel sectors triggered by nutrient availability or developmental cues (Fig. 1). However, unlike F- and bacterial A/V-like ATPase ion channels, which, when detached from the ATPase, catalyze passive transmembrane proton transport (53, 54), the eukaryotic \( V_o \) sector becomes impermeable to protons.
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upon dissociation of the $V_1$, preserving any existing proton gradient across the organelle membrane (55). In case of the F-ATPase ion channel, $F_o$, it has been shown that rotation of the proteolipid ring relative to the $a$ subunit is essential for passive proton conductance (56), and, because the mechanism of ion transport is highly conserved between F- and V-type motors, that means that rotation of the proteolipid ring in free $V_o$ appears to be blocked by an unknown mechanism. Previously, we generated an EM reconstruction of free $V_o$ sector from bovine brain V-ATPase that suggested an interaction between $a_{NT}$ and subunit $d$, although the linker connecting $a_{NT}$ and $a_{CT}$ was not resolved in the negative stain model (29). Later, we (57) and others (34) speculated that the interaction between $a_{NT}$ and $d$ may serve to silence passive proton transport by linking the rotor and stator of the motor. To address the mechanism of activity silencing as well as other aspects of $V_o$ structure and function, we developed a protocol to isolate milligram amounts of yeast $V_o$ proton channel sector using affinity chromatography. Biochemical experiments show that the complex is stable in low and intermediate critical micelle concentration (cmc) detergents such as UnDM, DDM, and CHAPS, respectively, as evident from glycerol gradient centrifugation. Negative-stain detergents such as UnDM, DDM, and CHAPS, respectively, as in low and intermediate critical micelle concentration (cmc) bilayers, we were able to generate two-dimensional crystals of the complex using our earlier negative-stain, three-dimensional EM reconstruction of the bovine $V_o$ (29) as a starting model. Although the resolution of the yeast $V_o$ reconstruction presented here is limited to about 18 Å (likely because of the relatively small size of the $V_o$ (320 kDa), the presence of a featureless detergent belt, and the lack of overall symmetry), the EM density allows placing of the crystal structures of equivalent subunits from related bacterial enzymes, namely the $E. hirae$ $K_{10}$ ring (48) and $a_{NT}$ and subunit $d$ homologs from $M. ruber$ (50) and $T. thermophilies$ (51), respectively. The resulting pseudo-atomic model shows $a_{NT}$ and subunit $d$ in close proximity, suggesting that the two polypeptides bind each other in free $V_o$, consistent with what had already been described for the bovine complex (29, 57). However, the cryo-EM model of yeast $V_o$ presented here provides more detail by showing the tether connecting $a_{CT}$ and $a_{CT}$ that was not resolved in the bovine model (29). Interestingly, a comparison of EM reconstructions of free $V_o$ and $V_o$ as part of holo V-ATPase (Fig. 4D) revealed that $a_{NT}$ must undergo a large conformational change during regulated enzyme disassembly, from a conformation in holo V-ATPase, where the distal domain of $a_{NT}$ binds $C_{foot}$ and EG2 (18, 31, 44), to a conformation in free $V_o$, where $a_{NT}$ binds subunit $d$. As mentioned above, we initially reasoned that the $a_{NT}$-$d$ interaction may play a role in blocking passive proton conductance. However, as summarized in Fig. 6, removal of subunit $d$ by the ionic detergent LPPG to produce $V_o$$\Delta d$, followed by proton conductance assays, showed no difference in the behavior of the $V_o$ and $V_o$$\Delta d$ complexes. This result suggests that the interaction of $d$ with $a_{CT}$ is not (solely) responsible for blocking proton flow across free $V_o$, consistent with earlier experiments by Qi and Forcag (34) that showed that proteolytic removal of $a_{NT}$ on vacuum vesicles did not render the membrane permeable to protons. Taken together, this means that there must be other (or additional) mechanisms that prevent proton leakage through free $V_o$. As illustrated in Fig. 7, one possibility is that the conformational change in $a_{NT}$ upon enzyme dissociation is transmitted to $a_{CT}$, thereby disrupting the path of protons along the interface between $a_{CT}$ and the proteolipid ring. Another mechanism for blocking passive proton transport could lie within the structure of the proteolipid ring itself. V-ATPase proteolipids have four transmembrane $\alpha$ helices but only one essential proton carrying carboxylate (59), resulting in a larger distance between proton binding sites compared with F-ATP synthase. The large gap between proton binding sites (Fig. 7) could represent too high of a barrier to overcome without the driving force from ATP hydrolysis, resulting in kinetic inhibition of proton flow from the vacuole into the cytoplasm.
However, if the $a_{NT}$-$d$ interaction in free $V_o$ is not involved in blocking passive proton translocation, what then, if any, might its physiological role be? It has been shown that removing the tether linking $a_{NT}$ and $a_{CT}$ prevents assembly of holo $V$-ATPase (resulting in free cytoplasmic $V_i$ and vacuolar membrane-bound $V_o$ that lacks subunit $d$), a defect that can be partially rescued upon overexpression of subunit $d$ (60). This finding suggests that the interaction of $d$ with the proteolipid ring is relatively weak and that the additional interaction with $a_{NT}$ is needed to increase avidity for $d$ during $V_o$ biogenesis and for retaining $d$ upon regulated enzyme disassembly. In line with this model is the relatively weak affinity ($K_{d} \sim 5 \text{ \mu m}$) between $a_{NT}$ and $d$, as measured by ITC using recombinant subunits because this interaction must be readily reversible for enzyme reassembly. Interestingly, recent studies have shown that the vacuole-specific phosphoinositide P(3,5)P$_2$ plays a role in regulating V-ATPase (re)assembly and that P(3,5)P$_2$ is able to directly bind $a_{NT}$ (61). One possibility is that the P(3,5)P$_2$ headgroups compete with subunit $d$ for $a_{NT}$ binding, thereby helping to change the conformation of $a_{NT}$ from the free $V_o$ state to a more peripheral conformation in preparation for enzyme reassembly.

Currently, there is no high-resolution structure available for an intact membrane domain of any of the rotary motor enzymes, and this lack of structural information has limited our understanding of the mechanism of ion translocation and activity silencing in case of the eukaryotic V-ATPase. The protocol described here allows isolation of highly purified and stable $V_o$, paving the way for obtaining a high-resolution structure of a rotary motor ATPase proton channel sector using crystallographic or single-molecule techniques. Studies toward that aim are ongoing in our laboratory.

**Author Contributions**—S. C. C. and S. W. designed the study and wrote the manuscript. S. C. C. performed the $V_o$ purification and structural characterization with technical assistance from S. W. S. W. performed the ITC experiments, including recombinant protein purification. E. M. generated the subunit B deletion strain.

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**References**

1. Forgac, M. (2007) Vacular ATPases: rotary proton pumps in physiology and pathophysiology. *Nat. Rev. Mol. Cell Biol.* 8, 917–929
2. Kane, P. M. (2007) The long physiological reach of the yeast vacuolar H$^+$-ATPase. *J. Bioenerg. Biomembr.* 39, 415–421
3. Sun-Wada, G. H., Wada, Y., and Futai, M. (2004) Diverse and essential roles of mammalian vacuolar-type proton pump ATPase: toward the
Molecular Architecture of the Yeast V-ATPase Proton Channel

physiological understanding of inside acidic compartments. Biochim. Biophys. Acta 1658, 106–114

4. Blake-Palmer, K. G., and Karet, F. E. (2009) Cellular physiology of the renal H+-ATPase. Curr. Opin. Nephrol. Hypertens. 18, 433–438

5. Smith, A. N., Skaug, I., Choate, K. A., Nayir, A., Bakkalakoglu, A., Ozen, S., Hulton, S. A., Sanjed, S. A., Al-Sabban, E., Lifton, R. P., Scherer, S. W., and Karet, F. E. (2000) Mutations in ATP6N1B, encoding a new kidney vacuolar proton pump 116-kd subunit, cause recessive distal renal tubular acidosis with preserved nearness. Nat. Genet. 26, 71–75

6. Karet, F. E., Finberg, K. E., Nelson, R. D., Nayir, A., Mocan, H., Sanjed, S. A., Rodriguez-Soriano, J., Santos, F., Cremers, C. W., Di Pietro, A., Hoffbrand, B. I., Winiarski, J., Bakkalakoglu, A., Ozen, S., Dusunsel, R., Goodyer, P., Hulton, S. A., Wu, D. K., Skvorak, A. B., Morton, C. C., Cunningham, M. I., Jha, V., and Lifton, R. P. (1999) Mutations in the gene encoding B1 subunit of H+-ATPase cause renal tubular acidosis with sensorineal deafness. Nat. Genet. 21, 84–90

7. Thudium, C. S., Jensen, V. K., Karsdal, M. A., and Henriksen, K. (2012) Disruption of the V-ATPase functionality as a way to uncouple bone formation and resorption: a novel target for treatment of osteoporosis. Curr. Protein Pept. Sci. 13, 141–151

8. Sun-Wada, G. H., Toyomura, T., Murata, Y., Yamamoto, A., Futai, M., and Wada, Y. (2006) The α3 isoform of V-ATPase regulates insulin secretion from pancreatic β-cells. J. Cell Sci. 119, 4531–4540

9. Dong, W., Bach, H., Sun, J., Hmama, Z., and Av-Gay, Y. (2011) Mycobacterium tuberculosis protein tyrosine phosphatase (PtpA) excludes host vacuolar H+-ATPase to inhibit phagosomal acidification. Proc. Natl. Acad. Sci. U.S.A. 108, 19371–19376

10. 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

11. Brown, D., Smith, P. J., and Breton, S. (1997) Role of V-ATPase-rich cells in acidification of the male reproductive tract. J. Exp. Biol. 200, 257–262

12. Sennoune, S. R., Bakunts, K., Martinez, G. M., Chua-Tuan, J. L., Kebrí, Y., Attaya, M. N., and Martinez-Zaguilán, R. (2004) Vacuolar H+-ATPase in human breast cancer cells with distinct metastatic potential: distribution and functional activity. Am. J. Physiol. Cell Physiol. 286, C1443–1452

13. Johnson, R. M., Allen, C., Melman, S. D., Walker, A., Young, S. M., Sklar, L. A., and Parra, K. J. (2010) Identification of inhibitors of vacuolar proton-translocating ATPase pumps in yeast by high-throughput screening flow cytometry. Anal. Biochem. 398, 203–211

14. Kartner, N., and Manolson, M. F. (2014) Novel techniques in the development of osteoporosis drug therapy: the osteoclast ruffled-border vacuolar H+-ATPase as an emerging target. Expert. Opin. Drug Discov. 9, 505–522

15. Fais, S., De Milito, A., You, H., and Qin, W. (2007) Targeting vacuolar H+-ATPases as a new strategy against cancer. Cancer Res. 67, 10627–10630

16. Kitagawa, N., Mazon, H., Heck, A. J., and Wilkens, S. (2008) Stoichiometry of the peripheral stalk subunits γ and γ of yeast V1-ATPase determined by mass spectrometry. J. Biol. Chem. 283, 3329–3337

17. Powell, B., Graham, L. A., and Stevens, T. H. (2000) Molecular characterization of the yeast vacuolar H+-ATPase proton pore. J. Biol. Chem. 275, 23654–23660

18. Zhao, J., Benlekbir, S., and Rubinstein, J. L. (2015) Electron cryomicroscopy observation of rotational states in a eukaryotic V-ATPase. Nature 521, 241–245

19. Arias, S., Sajio, S., Suzuki, K., Mizutani, K., Kakinuma, Y., Ishizuka-Katsuura, Y., Ohsawa, N., Terada, T., Shirouzu, M., Yokoyama, S., Iwata, S., Yamato, I., and Murata, T. (2013) Rotation mechanism of Enterococcus hirae V1-ATPase based on asymmetric crystal structures. Nature 493, 703–707

20. Kawasaki-Nishi, S., Nishi, T., and Forgac, M. (2001) Arg-735 of the 100-kDa subunit of the yeast V-ATPase is essential for proton translocation. Proc. Natl. Acad. Sci. U.S.A. 98, 12397–12402

21. Muench, S. P., Trinick, J., and Harrison, M. A. (2011) Structural divergence of the rotary ATPases. Q. Rev. Biophys. 44, 311–356

22. Wilkens, S. (2005) Rotary molecular motors. Adv. Protein Chem. 71, 345–382

23. Stewart, A. G., Sobti, M., Harvey, R. P., and Stock, D. (2013) Rotary ATPases: models, machine elements and technical specifications. Bioarchi-
47. Zhang, Z., Inoue, T., Forgac, M., and Wilkens, S. (2006) Localization of subunit C (Vma5p) in the yeast vacuolar ATPase by immuno electron microscopy. FEBS Lett. 580, 2006–2010
48. Murata, T., Yamato, I., Kakinuma, Y., Leslie, A. G., and Walker, J. E. (2005) Structure of the rotor of the V-Type Na\(^+\)-ATPase from Enterococcus hirae. Science 308, 654–659
49. 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
50. Srinivasan, S., Vyas, N. K., Baker, M. L., and Quiocho, F. A. (2011) Crystal structure of the cytoplasmic N-terminal domain of subunit I, a homolog of subunit a, of V-ATPase. J. Mol. Biol. 412, 14–21
51. 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) Crystal structure of a central stalk subunit C and reversible association/dissociation of vacuole-type ATPase. Proc. Natl. Acad. Sci. U.S.A. 101, 59–64
52. Drory, O., Frolow, F., and Nelson, N. (2004) Crystal structure of yeast V-ATPase subunit C reveals its stator function. EMBO Rep. 5, 1148–1152
53. Schneider, E., and Altenedorf, K. (1985) All three subunits are required for the reconstitution of an active proton channel (F0) of Escherichia coli ATP synthase (F1F0). EMBO J. 4, 515–518
54. Mosher, M. E., Peters, L. K., and Fillingame, R. H. (1983) Use of a unc transducing bacteriophages in genetic and biochemical characterization of H\(^+\)-ATPase mutants of Escherichia coli. J. Bacteriol. 156, 1078–1092
55. Zhang, J., Myers, M., and Forgac, M. (1992) Characterization of the V0 domain of the coated vesicle H\(^+\)-ATPase. J. Biol. Chem. 267, 9773–9778
56. Suzuki, T., Ueno, H., Mitome, N., Suzuki, J., and Yoshida, M. (2002) F(0) of ATP synthase is a rotary proton channel: obligatory coupling of proton translocation with rotation of c-subunit ring. J. Biol. Chem. 277, 13281–13285
57. Wilkens, S., Zhang, Z., and Zheng, Y. (2005) A structural model of the vacuolar ATPase from transmission electron microscopy. Micron 36, 109–126
58. Allegretti, M., Klusch, N., Mills, D. J., Vonck, J., Kühnbrandt, W., and Davies, K. M. (2015) Horizontal membrane-intrinsic α-helices in the stator a-subunit of an F-type ATP synthase. Nature 521, 237–240
59. Hirata, R., Graham, L. A., Takatsuki, A., Stevens, T. H., and Anraku, Y. (1997) VMA11 and VMA16 encode second and third proteolipid subunits of the Saccharomyces cerevisiae vacuolar membrane H\(^+\)-ATPase. J. Biol. Chem. 272, 4795–4803
60. Ediger, B., Melman, S. D., Pappas, D. L., Jr., Finch, M., Applen, J., and Parra, K. J. (2009) The tether connecting cytosolic (N terminus) and membrane (C terminus) domains of yeast V-ATPase subunit a (Vph1) is required for assembly of V0 subunit d. J. Biol. Chem. 284, 19522–19532
61. Li, S. C., Diakov, T. T., Xu, T., Tarsio, M., Zhu, W., Couoh-Cardel, S., Weissman, L. S., and Kane, P. M. (2014) The signaling lipid PI(3,5)P(2) stabilizes V1-V0 sector interactions and activates the V-ATPase. Mol. Biol. Cell 25, 1251–1262
62. Wang, Y., Cipriano, D. J., and Forgac, M. (2007) Arrangement of subunits in the proteolipid ring of the V-ATPase. J. Biol. Chem. 282, 34058–34065
63. Wang, Y., Inoue, T., and Forgac, M. (2004) TM2 but not TM4 of subunit c’’ interacts with TM7 of subunit a of the yeast V-ATPase as defined by disulfide-mediated cross-linking. J. Biol. Chem. 279, 44628–44638