Structure of the Lipid Nanodisc-reconstituted Vacuolar ATPase Proton Channel

DEFINITION OF THE INTERACTION OF ROTOR AND STATOR AND IMPLICATIONS FOR ENZYME REGULATION BY REVERSIBLE DISSOCIATION*

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Eukaryotic vacuolar H⁺-ATPase (V-ATPase) is a multisubunit enzyme complex that acidifies subcellular organelles and the extracellular space. V-ATPase consists of soluble V₁-ATPase and membrane-integral V₀ proton channel sectors. To investigate the mechanism of V-ATPase regulation by reversible disassembly, we recently determined a cryo-EM reconstruction of yeast V₀. The structure indicated that, when V₁ is released from V₀, the N-terminal cytoplasmic domain of subunit a (aNT) changes conformation to bind rotor subunit d. However, insufficient resolution precluded a precise definition of the aNT-d interface. Here we reconstituted V₀ into lipid nanodiscs for single-particle EM. 3D reconstructions calculated at ~15-Å resolution revealed two sites of contact between aNT and d that are mediated by highly conserved charged residues. Alanine mutagenesis of some of these residues disrupted the aNT-d interaction, as shown by isothermal titration calorimetry and gel filtration of recombinant subunits. A recent cryo-EM study of holo V-ATPase revealed three major conformations corresponding to three rotational states of the central rotor of the enzyme. Comparison of the three V-ATPase conformations with the structure of nanodisc-bound V₀ revealed that V₀ is halted in rotational state 3. Combined with our prior work that showed autoinhibited V₁-ATPase to be arrested in state 2, we propose a model in which the conformational mismatch between free V₁ and V₀ functions to prevent unintended reassembly of holo V-ATPase when activity is not needed.

The vacuolar H⁺-ATPase (V-ATPase, V₁V₀-ATPase) is a large multisubunit enzyme complex found in the endomembrane system of all eukaryotic cells, where it acidifies the lumen of subcellular organelles, including lysosomes, endosomes, the Golgi apparatus, and clathrin-coated vesicles (1–4). V-ATPase function is essential for pH and ion homeostasis (2), protein trafficking, endocytosis, mechanistic target of rapamycin (mTOR) (5, 6), and Notch (7) signaling as well as hormone secretion (8) and neurotransmitter release (9). In animals, V-ATPase can also be found in the plasma membrane of polarized cells, where its proton pumping function is involved in bone remodeling, urine acidification, and sperm maturation (1). The essential nature of eukaryotic V-ATPase is highlighted by the fact that complete loss of V-ATPase activity in animals is embryonic lethal (10). On the other hand, partial loss of enzyme function (or hyperactivity) has been associated with numerous widespread human diseases, including, but not limited to, renal tubular acidosis (11), osteoporosis (12), neurodegeneration (13), male infertility (14), deafness (15), diabetes (8), and cancer (16). Furthermore, V-ATPase is targeted by pathogens such as Mycobacterium tuberculosis or Legionella pneumophila (17, 18) to facilitate pathogen entry and survival. Because of its essential nature and key role in so many human diseases, V-ATPase has been identified as a potential drug target (19–21).

V-ATPase can be divided into a soluble catalytic sector, V₁, and a membrane-integral proton channel sector, V₀ (Fig. 1). In the yeast Saccharomyces cerevisiae, V₁ is composed of eight different polypeptides, AB(C)DEFGH, that are arranged in an A₂B₂ catalytic hexamer with a central stalk made of DF and three peripheral stators (EG heterodimers), one of which binds the single-copy H subunit. The ~320-kDa V₀ contains subunits acc’d’de, which are organized in a membrane-integral “proteolipid” ring (c₈c’e” (22, 23)), a membrane-bound subunit a with an integral C-terminal domain (aCT) that is bound at the periphery of the proteolipid ring, and a N-terminal cytoplasmic domain (aNT) that is bound to subunit d (Fig. 1). The stoichiometry, location, and function of subunit e are not known. Eukaryotic V-ATPase belongs to the family of energy-transducing rotary motor ion pumps that also includes F₁F₀-ATP synthase, archaenal A-ATPase, and bacterial A/V-like ATPase (24, 25). In V-ATPase, ATP hydrolysis at three catalytic sites in the A₄B₄ hexamer is coupled to proton translocation via rotation of V₁ subunits DF that are connected to the subunit d-proteolipid ring subcomplex of V₀. Proton translocation is through two aqueous half-channels at the interface of aCT and the proteolipid domain.
olipid ring and involves membrane-embedded essential glutamate and arginine residues in the c subunits and aCT, respectively.

V-ATPase function is regulated in vivo by a unique mechanism referred to as reversible disassembly, a condition under which the enzyme dissociates into membrane-bound V_o and cytoplasmic V_1 sectors (26, 27) (Fig. 1). Reversible dissociation of V-ATPase is well characterized in the model organism S. cerevisiae (28), but more recent data suggest that the mammalian enzyme is regulated by a similar process in some cell types (5, 29–31). Although the assembly status of yeast V-ATPase is mainly governed by nutrient availability (32), the situation in mammalian cells appears to be more complex. Besides glucose levels (30), V-ATPase assembly in animal cells can be induced by a variety of signals, including cell maturation (33) and stimulation by hormones (34) and growth factors (6). Upon enzyme dissociation, the activity of both sectors is silenced; that is, the V_1 no longer hydrolyzes MgATP (35, 36), and the V_o no longer translocates protons (37, 38). Although studies in yeast suggest that V_1 activity silencing depends on the C-terminal domain of subunit H, possibly together with inhibitory MgADP (35, 39, 40), the mechanism by which passive proton transport across V_o is blocked is less well understood.

The structure of eukaryotic V-ATPase has been analyzed by EM, and together with crystal structures of individual subunits and subcomplexes from yeast V-ATPase and related bacterial enzymes, the EM reconstructions have allowed generation of pseudoatomic models of the intact enzyme (22, 41, 42) and its functional V_1 (43) and V_o (38, 44) sectors. Although the resulting structural models together with biochemical data provide valuable information on the mechanism of ATP hydrolysis-driven proton pumping, we only have a limited understanding of the mechanism of reversible enzyme dissociation and reassociation. We recently obtained a cryo-EM reconstruction of yeast V_o (38), and although a comparison with EM models of holo V-ATPase showed that aNT undergoes a large structural change to bind the rotor subunit d (Fig. 1), the resolution of the model was insufficient to precisely define the aNT-d interface.

Here we present a negative-stain 3D EM reconstruction of lipid nanodisc-reconstituted V_o calculated at a resolution of ~15 Å. The model of nanodisc-bound V_o suggests that the interaction between aNT and subunit d is mediated by charge complementation between acidic and basic residues on d and aNT, respectively. Site-directed mutagenesis and isothermal titration calorimetry experiments conducted with recombinant subunits identified acidic and basic patches on d and aNT that mediate the aNT-d interaction. A comparison with the recent EM reconstructions of yeast V-ATPase in three states (22) suggests that, upon enzyme dissociation, free V_o is halted in state 3. We showed previously that autoinhibited, membrane-detached V_T-ATPase is halted in state 2 (45), and we propose that this conformational mismatch to state 3 V_o could function to prevent unintended reassembly of holo V-ATPase under conditions when the proton pumping activity of the enzyme is not needed.

**Results**

*Purification of V_o Membrane Sector and Reconstitution into Lipid Nanodiscs—* We previously developed a procedure for purification of milligram amounts of yeast V-ATPase V_o sector for functional and structural studies (38). Briefly, V_o was solubilized from vacuolar membranes and affinity-captured via a calmodulin binding peptide fused to the C terminus of the vac-
uole-specific isoform of subunit a (Vph1p). For structural studies under more native-like conditions, Vo was reconstituted into lipid nanodiscs as described under “Experimental Procedures” (Fig. 2). Purified detergent-solubilized Vo (Fig. 2a) was mixed with Escherichia coli polar lipids and the recombinant membrane scaffold protein MSP1E3D1 (Fig. 2b), followed by detergent removal with polystyrene beads. In a final purification step, Vo-containing nanodiscs (VoND) were separated from “empty” discs by a second calmodulin affinity binding step followed by size exclusion chromatography on a Superdex S200 column (Fig. 2, c and d). Peak fractions of VoND eluted from the gel filtration column were pooled, and the concentrated preparation was analyzed by SDS-PAGE and silver staining (Fig. 2e).

3D EM Reconstruction of VoND—We initially generated a dataset of ~30,000 particles from EM images of negatively stained VoND. However, attempts to reconstruct a 3D model of the complex using reference-free algorithms were unsuccessful, likely because of the limited size of the complex and the lack of characteristic features required for alignment. Models did not converge on a specific handedness, i.e. orientation of aNT with respect to the membrane sector, and density for aNT was smeared out over the membrane and did not allow us to distinguish its positioning relative to subunit d. We therefore decided to make use of the calmodulin binding peptide on the C terminus of subunit a to introduce an additional asymmetry to aid in alignments and angle determination in the 3D startup procedure. Purified calmodulin (CaM) was incubated with VoND, followed by removal of excess CaM using glycerol gradient centrifugation (Fig. 2, f–h). Negative stain electron microscopy showed that the final VoND-CaM preparation was monodis-
FIGURE 3. Negative stain transmission electron microscopy of VoND-CaM. a, the representative micrograph reveals a monodisperse sample of ∼12-nm particles. b, class averages obtained by reference-free alignment of a dataset of ∼40,000 VoND-CaM projections (center row) with corresponding raw particle images (top row) and reprojections of the final VoND-CaM reconstruction (bottom row). c and d, final 3D reconstructions of VoND-CaM (c) and VoND (d) with corresponding gold standard FSC graphs shown below the models. The red circle on the VoND-CaM reconstruction indicates the density for calmodulin bound to the C terminus of subunit α. Insets in the FSC graphs illustrate the angular distributions of the particle orientations of the two datasets. Scale bars = 20 nm (a) and 10 nm (b).
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perse, with an average diameter of the particles of ~12 nm (Fig. 3a). A dataset of ~40,000 particles, generated from 380 micrographs such as shown in Fig. 3a, was subjected to reference-free alignment procedures as implemented in EMAN2. Fig. 3b shows class averages of side, top, and intermediate view projections (center row), representative raw particle images (top row), and the corresponding reprojections of the final 3D model (bottom row). Class averages, including those shown in Fig. 3b, were used for a 3D startup procedure in EMAN2, and the resulting 3D reconstruction was refined until stable. At this point, the reconstruction was strongly low pass-filtered and used as input for the 3D autorefinement procedure as implemented in the Relion 1.3 software package. The model was then refined until no further improvement was observed. The resolution of the final VoND-CaM 3D reconstruction was estimated to be 14.9 Å (20.3 Å at 0.5 correlation) using the “gold standard” FSC protocol as implemented in Relion 1.3 (Fig. 3c).

The final V_o,ND-CaM model was strongly low pass-filtered to serve as a reference for a new V_o,ND dataset (~47,000 images) using the Relion 3D autorefinement procedure as described for the V_o,ND-CaM dataset. The 3D autorefinement converged to a final model of V_o,ND with an estimated gold standard resolution of ~14.9 Å (16.3 Å at 0.5 correlation, Fig. 3d). As can be seen from Fig. 3, e and f, the final V_o,ND-CaM and V_o,ND models are very similar except for a small density in the V_o,ND-CaM map that is due to the CaM bound at the a subunit C terminus (Fig. 3c, top panel, red circle). However, because the V_o,ND-CaM map showed slightly more detail, we used this model to illustrate the features of nanodisc-bound V_o as summarized in Fig. 4.

Side, top, and bottom views of the V_o,ND-CaM model are illustrated in Fig. 4, a–c, showing the characteristic features as seen in earlier reconstructions of bovine (44) and yeast (38) V_o, including the densities above the membrane (a_NT and subunit d), the cleft between the density for a_CT and the c-ring (Fig. 4a, arrowhead), which opens into a solvent (stain)-accessible pore as seen in the bottom view (Fig. 4c, arrowhead), and the large cavity on the cytoplasmic side of the c-ring (Fig. 4b, arrowhead). Semiautomatic fitting of homology models of the yeast V_o subunits into the EM density is summarized in Fig. 4, d–f. As illustrated in Fig. 4, d and e, a_NT (blue) was positioned with its proximal lobe (which is comprised by the N and C termini of subunit a, domain nomenclature as in Ref. 46) near the connection point to the membrane-bound a_CT (green), placing its distal lobe near the central density corresponding to subunit d (green).
Structure of $\text{Vo}$ in Lipid Nanodiscs Defines $\alpha_{\text{NT}}$-d Interface

Because of its pseudo-3-fold symmetry and the limited resolution of the EM reconstruction, the yeast subunit d homology model could be fit in three orientations corresponding to rotational states 1, 2, and 3, as seen in the recent cryo-EM reconstructions of intact yeast V-ATPase (22). However, only the orientation corresponding to state 3 preserved the contact between the subunit d N-terminal $\alpha$ helix and the cytoplasmic face of the c subunit ring, as seen in the recent cryo-EM model of holo V-ATPase in state 3 (22) (Fig. 4a), and we therefore explored the contacts between $\alpha_{\text{NT}}$ and d predicted by this configuration (Fig. 4h, see below). For filling the density corresponding to the yeast c-ring, we used the crystal structure of the K$_10$ ring from Enterococcus hirae (47) (Fig. 4h, magenta).

Interaction of $\alpha_{\text{NT}}$ and Subunit d—We previously reported a 3D reconstruction from cryo-EM images of detergent-solubilized yeast $\text{Vo}$ that showed $\alpha_{\text{NT}}$ and subunit d in close proximity (38). Although subsequent binding studies using recombinant $\alpha_{\text{NT}}$ (residues 1–372, $\alpha_{\text{NT}(1–372)}$) and d revealed a $K_d$ of the $\alpha_{\text{NT}(1–372)}$-d interaction of $\approx$5 $\mu$M (38), the resolution of the cryo-EM model, was insufficient to define the binding site(s) between $\alpha_{\text{NT}}$ and d in detail. Fig. 4h illustrates the interface between $\alpha_{\text{NT}}$ and subunit d based on the fitting of the homology models into the EM density of the $\text{V}_{10}$ND-CaM model presented here. As can be seen, $\alpha_{\text{NT}}$ and subunit d appear to contact each other via two distinct sites near the proximal and distal lobes of $\alpha_{\text{NT}}$ (Fig. 4h). The contact near the distal lobe (Fig. 4h, bottom right) is mediated by two short $\alpha$ helices, one from $\alpha_{\text{NT}}$ (residues 242–256, yeast subunit a isoform Vph1p) and one from subunit d (residues 144–154). The other site near the proximal lobe of $\alpha_{\text{NT}}$ is mediated by a short helix-turn-helix motif in d (residues 38–58) and a less well defined face in $\alpha_{\text{NT}}$ (Fig. 4h, bottom left). Many of the acidic and basic residues involved in these two contact sites are highly conserved from yeast to human (see isothermal titration calorimetry sections below). Considering the conserved nature of the charged residues, we reasoned that complex formation between $\alpha_{\text{NT}}$ and d could be driven by electrostatic interactions involving the conserved residues. To test this hypothesis, we generated $\alpha_{\text{NT}}$ double (R250A,K251A) and quadruple mutants (K247A,R250A,K251A,E254A) in the short $\alpha$ helix in the distal lobe of $\alpha_{\text{NT}}$ and triple (D144A,E146A,E150A) and quadruple (D37A,D40A,D41A,K43A) mutants in subunit d for in vitro binding experiments using isothermal titration calorimetry (ITC). As a negative control, we generated a triple mutant of d in an area predicted to be outside of the $\alpha_{\text{NT}}$-d contact area (E198A,E199A,E202A).

The wild type and alanine mutants of $\alpha_{\text{NT}(1–372)}$ and subunit d were expressed as N-terminal fusions with maltose binding protein (MBP) connected via a protease cleavage site as described previously (38). The proteins were purified away from MBP via anion exchange and size exclusion chromatography. Purity and proper folding of the resulting $\alpha_{\text{NT}(1–372)}$ and subunit d constructs was confirmed by Coomassie-stained SDS-PAGE and CD spectroscopy, respectively (Fig. 5, a and b). As can be seen from the CD spectra, all $\alpha_{\text{NT}(1–372)}$ and subunit d mutant proteins showed minima at 208 and 222 nm similar to the wild-type subunits and characteristic for highly $\alpha$-helical proteins, indicating that the mutations did not interfere with proper folding of the recombinant subunits.

Isothermal Titration Calorimetry of Mutant $\alpha_{\text{NT}(1–372)}$ and Wild-type d—We first conducted ITC titrations with double and quadruple mutants of the conserved charged residues in
the short α helix of the aNT distal domain that were facing subunit d (Fig. 6a). Titrating subunit d into wild-type aNT(1–372) revealed an exothermic binding reaction (Fig. 6b). Fitting the data with a single-site binding model revealed a 1:1 stoichiometry with a $K_d$ of 6.7 M, similar to what we obtained earlier by titrating aNT(1–372) into subunit d (38). The $H$ and $S$ were 34.2 kJ/mol and 21.6 J/(mol·K)−1, respectively, giving a $G$ of the enthalpy-driven binding reaction of 28.1 kJ/mol.

Titrating subunit d into the double aNT(1–372) mutant (R250A,K251A) produced significantly less heat ($H$ 7.4 kJ/mol), but, at the same time, the $K_d$ was similar compared with the wild-type proteins ($K_d = 4$ μM, Fig. 6c) and appeared to be partly driven by entropy ($ΔS = 77.8$ J/(mol·K)−1, $ΔG = −29.4$ kJ/mol). On the other hand, titrating subunit d into the quadruple mutant of aNT(1–372) (K247A,R250A,K251A,E254A) resulted in a weak endothermic reaction that could not be fit to a single site model without fixing the stoichiometry at 1:1 (Fig. 6d). Under these conditions, the $K_d$ was ~1 mM, indicating that replacement of all four conserved charged residues by alanines disrupted the interaction between aNT(1–372) and d.

The cell contents of the ITC experiments (with titrated wild-type aNT(1–372), double mutant, and quadruple mutant) were subjected to size exclusion chromatography as described under “Experimental Procedures.” Subunit d and aNT(1–372) alone eluted at 65 and 60 ml, respectively (Fig. 6, e and f). Note that although subunit d alone runs as a monodisperse monomer (Fig. 6, e and f), aNT(1–372) exists in a concentration-dependent monomer-dimer equilibrium as reported earlier (38) and as evident from its elution profile and accompanying SDS-PAGE gel.
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(Fig. 6, f and k). The elution profile of the mixture of wild-type \( a_{NT(1–372)} \) and \( d \) revealed two peaks at 58 and 65 ml (Fig. 6g), and analysis by SDS-PAGE showed that the peak around 58 ml (fraction 29) contained close to stoichiometric amounts of \( a_{NT(1–372)} \) and \( d \) (Fig. 6l). The relatively small shift of 2 ml (one fraction) toward larger molecular size is consistent with the moderate \( K_d \) of the \( a_{NT(1–372)} \cdot d \) complex formation of 6.7 \( \mu \text{M} \) and in close agreement with our previous study (38).

In the size exclusion profile of the double mutant titration (subunit \( d \) into \( a_{NT(1–372)} \), R250A,K251A), the interacting peak is shifted slightly to a larger volume at \( \sim 61 \) ml as a shoulder of the subunit \( d \) peak (\( \sim 66 \) ml) (Fig. 6h), indicating a weakening of the \( a_{NT(1–372)} \cdot d \) interaction and consistent with the reduced binding enthalpy and the SDS-PAGE gel of the peak fractions (Fig. 6m). This trend is continued for the titration of the quadruple mutant (subunit \( d \) into \( a_{NT(1–372)} \), K247A,R250A,K251A, E254A), which only shows a shoulder for \( a_{NT} \) unresolved from the subunit \( d \) peak (Fig. 6i) with elution profiles similar to the individual subunits (Fig. 6n). Taken together, the ITC and gel filtration data showed that mutation of the conserved charged residues on the short \( \alpha \) helix in the distal domain of \( a_{NT} \) disrupts the \( a_{NT} \cdot d \) interaction.

Isothermal Titration Calorimetry of Mutant \( d \) and Wild-type \( a_{NT(1–372)} \) As mentioned above, subunit \( d \) appears to contact \( a_{NT} \) at two sites, the short \( \alpha \) helix in the distal domain and at a second site near the proximal domain (Fig. 4h). To verify the fit of subunit \( d \) in the EM model and to test whether the contact site near the proximal lobe of \( a_{NT} \) contributes to the interaction between the two subunits, we generated triple and quadruple mutants of subunit \( d \) by replacing conserved charged residues that are facing \( a_{NT} \) from the two sites on \( d \) (Fig. 7, a and e). ITC titrations of both triple (Fig. 7b) and quadruple (Fig. 7f) alanine mutants of \( d \) with wild-type \( a_{NT(1–372)} \) revealed weak endothermic reactions that could not be fit to a single site binding model without fixing \( n = 1 \). Under these conditions, the \( K_d \) for the two titrations of the triple and quadruple mutants of \( d \) were \( \sim 0.25 \) mM and \( \sim 1.7 \) mM, respectively. Consistent with the ITC titrations, gel filtration profiles (Fig. 7, c and g) and SDS-PAGE of the peak fractions (Fig. 7, d and h) indicated elution of non-interacting subunits. Contrary to the alanine mutations of residues predicted to be in the \( a_{NT} \cdot d \) binding interface (Fig. 7, a–h), mutagenesis of a patch of acidic residues outside the interface (E198A,E199A,E202A) did not interfere with complex formation (Fig. 7, i–l).

Taken together, the ITC and gel filtration experiments showed that both contact sites between \( a_{NT} \) and \( d \) as seen in the EM fit contribute to the binding interaction between the two subunits. However, because disrupting either of the two sites weakened the interaction beyond detection by ITC or gel filtration, this suggests that the individual interactions are weak and that only the combined avidity of the two interactions results in a measurable affinity.

Discussion

We have developed a protocol to reconstitute purified V-ATPase \( V_o \) membrane sector into lipid nanodiscs. When reconstituted into nanodiscs, \( V_o \) is stable, as evident from the lack of subunit \( a \) degradation products (Fig. 2e) sometimes seen in the detergent-solubilized complex (38). Negative stain EM showed that the preparation is monodisperse, and we were able to reconstruct 3D models of the nanodisc-bound \( V_o \) with and without calmodulin bound to the calmodulin binding peptide at the C terminus of subunit \( a \). Fitting of \( a_{NT} \) and subunit \( d \) homology models into the EM density revealed that the two subunits are in contact, as described previously for the negative stain and cryo-EM models of detergent solubilized bovine and yeast \( V_o \) respectively (38, 44). However, the slightly better resolution of the \( V_o \) ND models allowed us to identify two sites of contact between \( a_{NT} \) and \( d \), both involving charged and highly conserved residues. ITC analysis of triple and quadruple alanine mutants of \( a_{NT} \) and \( d \) confirmed the involvement of the charged residues in the interaction, but the analysis also showed that the individual interactions are weak and that only the combined avidity of both binding sites leads to a measurable \( K_d \) of \( \sim 6 \) \( \mu \text{M} \) (Ref. 38 and the data presented here).

As mentioned under “Introduction,” V-ATPase is regulated by a reversible disassembly mechanism that results in membrane-detached \( V_1 \) and membrane-bound free \( V_o \). A relatively moderate affinity between \( d \) and \( a_{NT} \) can be rationalized by the fact that this interaction has to be broken when reassembly of holo V-ATPase is initiated.

A comparison of the \( V_o \) ND 3D models with the recent cryo-EM reconstructions of holo V-ATPase in three states (22) revealed that free \( V_o \) appears to be halted in rotary state 3 based on the orientation of subunit \( d \) relative to \( a_{NT} \). As mentioned under “Results,” because of the limited resolution of the \( V_o \) ND 3D models and because of the pseudo-3-fold symmetry of subunit \( d \), \( d \) could also be fit in the state 2 orientation with a comparable model-map correlation compared with the state 3 fit (the fit of state 1 is of much lower quality, see Fig. 4). The ITC data, however, are only consistent with the state 3 orientation. Furthermore, only the state 3 orientation preserves the contact between the N-terminal \( \alpha \) helix of \( d \) and the cytoplasmic loops of the proteolipid ring, as seen in the models of all three states of the holo enzyme (22). Taken together, the data therefore indicate that free \( V_o \) is halted in a single conformation corresponding to state 3 of holo V-ATPase.

Recently, we determined the 6.2- to 6.5-Å crystal structure of autoinhibited yeast \( V_1 \)-ATPase (40). Interestingly, a comparison of the structure of autoinhibited \( V_1 \) with the structure of \( V_1 \) as part of holo V-ATPase (22) revealed that the membrane-detached \( V_1 \) is halted in state 2 based on the rotational position of the DF rotor relative to the inhibitory H subunit (40). The observation that autoinhibited \( V_1 \) is halted in state 2 together with the findings presented here that free \( V_o \) is halted in state 3 indicates that there is a conformational mismatch between the two complexes as a result of regulated enzyme disassembly. We speculate that this mismatch may serve to prevent unintended reassembly of the enzyme when the disassembled state is required. How this mismatch is relieved when reassembly is required is not known, but it is possible that \( V_1 \) binding to the assembly chaperone regulator of the H\(^{+}\)-ATPase of vacuolar and endosomal membranes (RAVE) (48) changes \( V_1 \) conformation to enable \( V_o \) binding. Another possibility is that the \( V_o \) conformation is altered by interac-
tion with specific phosphoinositides that have been shown to bind to \( \alpha \) and promote enzyme assembly on the vacuolar membrane (49).

Here we have shown that yeast \( \alpha \) can be reconstituted into lipid nanodiscs, resulting in a highly monodisperse preparation that is amenable to structure determination by single-molecule EM. Future studies using cryo-EM will allow high-resolution structural studies of the complex in a more native environment compared with the detergent-solubilized state, allowing, for example, an examination of the interaction with specific lipid molecules that have been shown to be either essential for V-ATPase function (50) or

![Diagram](image-url)

**FIGURE 7.** Isothermal titration calorimetry of the interaction between wild-type \( \alpha(1–372) \) and mutant subunit \( d \). a and e, close-up of the contact between \( d \) and the \( \alpha \) distal (d) and proximal (e) lobe. b–d, IT(c) (b), gel filtration (c), and SDS-PAGE (d) of the \( d \) triple mutant (D44A,E146A,E150A) with \( \alpha(1–372) \). f–h, ITC (f), gel filtration (g), and SDS-PAGE (h) of the \( d \) quadruple mutant (D37A,E40A,D41A,K43A) with \( \alpha(1–372) \). I–l, as a negative control, a subunit \( d \) mutant with acidic residues (Glu-198, Glu-199, and Glu-202) outside of the predicted interface with \( \alpha \) changed to alanines was titrated with wild-type \( \alpha(1–372) \). Fitting the data revealed a \( K_a \) of \( 4 \times 10^{-4} \) M, \( N = 1.2, \Delta H = -12.4 \pm 0.49 \text{ kJ/mol}, \Delta S = 63 \text{ kJ/molK}^{-1} \). Shown are representative ITC titrations of at least two repeats for each mutant. Note that the gel filtration column was repacked after the experiments in Fig. 6, resulting in a slightly different elution volume for the recombinant subunits for the two sets of titrations shown in Figs. 6 and 7.
involved in the mechanism of reversible enzyme disassembly (49). These studies are ongoing in our laboratory.

**Experimental Procedures**

**Reagents**—Undefecyl-β-D-maltoside (UnDM) was from Ana-trace. *E. coli* polar lipid extract was obtained from Avanti. Calmodulin-Sepharose beads were from GE Healthcare or Agilent. CDTA was from Fisher Scientific. All other reagents were of analytical grade.

**Purification of Yeast V₀**—Cell growth of a yeast strain expressing subunit a isoform Vph1p with a C-terminal fusion of calmodulin binding peptide, membrane preparation, and V₀ extraction and purification were performed as described previously (38) with the following modifications. Yeast cells were harvested during the second log phase by centrifugation at 2600 × g, washed in water, and resuspended in lysis buffer (25 mM Tris-HCl (pH 7.4), 500 mM sorbitol, and 2 mM E GTA), and broken in a Bead Beater (Omni International) using zirconium beads (BioSpec). After removing cell debris and mitochondria by low-speed (2500 × g, 10 min) and medium-speed (12,000 × g, 20 min) centrifugation, membranes were collected by ultracentrifugation (370,000 × g, 2 h), washed once in buffer (25 mM Tris-HCl (pH 7.4) and 500 mM sorbitol), and pelleted again (370,000 × g, 1 h). The total protein concentration of the membrane samples was determined by BCA protein assay (Thermo Scientific) of trichloroacetic acid-precipitated membranes. Isolated membranes were diluted to 10 mg/ml and stored at −80 °C until use.

Membranes were solubilized by addition of UnDM from a 20% stock solution in water to a final concentration of 0.6 mg of detergent/mg of membrane protein for 1 h with gentle agitation. Extracted membranes were supplemented with 4 mM CaCl₂ and centrifuged at 180,000 × g for 1 h to remove the insoluble fraction. The supernatant was then applied to a 5-ml calmodulin-Sepharose column pre-equilibrated in calmodulin washing buffer (10 mM Tris-HCl (pH 8), 150 mM NaCl, 2 mM CaCl₂, 10 mM β-mercaptoethanol, and 0.1% UnDM). The column was washed with 5 column volumes each of washing buffer and washing buffer without NaCl and eluted with elution buffer (10 mM Tris-HCl (pH 8), 10 mM CDTA, 10 mM β-mercaptoethanol, and 0.1% UnDM).

**Preparation of Membrane Scaffold Protein**—Membrane scaffold protein MSP1E3D1 (MSP) was expressed in *E. coli* BL21 (DE3) via a pET28a plasmid (Addgene, 20066) as described previously (51) with the following modifications. Briefly, the strain was grown to mid-log phase in terrific broth (25 g/liter Luria-Bertani-Miller broth (EMD Biosciences) supplemented with 0.4% (v/v) glycerol). Expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (BioVectra) for 1 h at 37 °C, followed by 3.5 h at 28 °C. Cells were harvested by centrifugation, resuspended in lysis buffer (25 mM sodium phosphate (pH 8) and 1% Triton X-100) and lysed with a French press (Spectronic Unicam). Lysate was cleared by centrifugation (17,000 × g) and passed over a nickel-nitrilotriacetic acid affinity column (Qiagen). The column was washed in place with each of three buffers: 40 mM Tris-HCl, 300 mM NaCl, and 1% Triton X-100, pH 8); 40 mM Tris-HCl, 300 mM NaCl, 50 mM sodium cholate, and 5 mM imidazole (pH 8); and 40 mM Tris-HCl, 300 mM NaCl, and 10 mM imidazole (pH 8). Protein was eluted with a 10-column volume linear gradient of elution buffer (40 mM Tris-HCl, 300 mM NaCl, and 100 mM imidazole (pH 8)) and dialyzed against 40 mM Tris-HCl, 100 mM NaCl, and 0.5 mM EDTA (pH 7.4). MSP-containing fractions were pooled and concentrated by ultrafiltration using an Amicon cell with an XM50 filter membrane. Purified MSP was stored at −80 °C until use.

**Lipid Nanodisc Reconstitution of V₀**—*E. coli* total lipid extract (Avanti Polar Lipids) was suspended by sonication in disc-forming buffer (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.5 mM EDTA) with the addition of 1 mM DTT (EMD Milipore). Detergent-solubilized V₀ purified MSP, and lipid were combined at a molar ratio of 0.02:1:25 with the addition of protease inhibitors, 1 mM PMSF, 1 mM leupeptin, 1 mM pepstatin, and 1 mM chymostatin (EMD Biosciences) and incubated at room temperature for 1 h with mixing. Prewashed Bio-Beads SM-2 (Bio-Rad) were added at 0.4 g/ml and incubated with mixing for 2 h at room temperature. The self-assembled nanodisc sample was recovered from the Bio-Bead mixture with a syringe. To remove unfilled (empty) nanodiscs (ND) from V₀-containing discs (V₀ND), the reconstituted sample was supplemented with 10 mM CaCl₂ and applied to a 1-ml calmodulin resin column, washed with disc-forming buffer and eluted with the same buffer without CaCl₂ and supplemented with 10 mM CDTA. As a final polishing step, the eluted V₀ND sample was concentrated to 2 ml, applied to a Superdex 200 HR 16/500 column on an AKTA FPLC (GE Healthcare) equilibrated with disc-forming buffer and eluted at 0.5 ml/min.

**Preparation of Calmodulin**—The gene for human calmodulin 1 was synthesized (BioBasic, Markham, ON, Canada) and cloned into a modified pMAL-c2E expression vector with a Prescission protease cleavage site between MBP and the N terminus of calmodulin. Briefly, *E. coli* Rosetta 2 harboring the calmodulin expression plasmid was grown to mid-log phase in rich broth (Luria broth supplemented with 0.2% glucose), and expression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 18 h at 18 °C. Cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM EDTA) and lysed by sonication (Hiel-scher Ultrasonics). The lysate was cleared by centrifugation at 20,000 × g and passed over a pre-equilibrated amylose column (New England Biolabs). Bound protein was washed using the same buffer and eluted with the buffer supplemented with 10 mM maltose. Protein was cleaved using Prescission protease to remove the MBP tag and dialyzed into anion exchange buffer (20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1 mM DTT). The sample was passed over a MonoQ anion exchange column attached to an FPLC and pre-equilibrated in buffer and eluted using a 30-column volume linear gradient of buffer to buffer plus 500 mM NaCl. Calmodulin-containing fractions were pooled, concentrated, and subjected to size exclusion chromatography (Superdex 75 16/500 column). For fluorescence detection of calmodulin, residue Ala-47 was changed to cysteine using QuikChange site-directed mutagenesis with the following primers: A47_fwd, GCC AGA ATC CAA CCG AAT GTG AAC TGC AAG AAG ATA TGA TTA ACG; A47_rev, CGT TAA TCA TAT CTT GCA GTT CAC ATT CGG TTG GAT TCT GCC. For fluorescence detection, calmodulin (A47C) was
reacted with fluorescein maleimide for 1 h in the dark. Excess label was removed by a Sephadex G25 spin column.

Labeling of V$_{ND}$ with Calmodulin—Purified calmodulin was added in a 5:1 molar ratio to V$_{ND}$, and the sample was loaded onto a discontinuous glycerol gradient (15–35% (v/v), 10 mM MOPS (pH 7), and 4 mM CaCl$_2$) for separation of unbound calmodulin. The gradient was subjected to centrifugation at 285,000 ¥ g for 16 h. Afterward, 1-ml fractions were collected by fractionation from the bottom of the gradient and analyzed by SDS-PAGE.

Electron Microscopy—Carbon-coated copper grids were subjected to glow discharge in air for 45 s. Samples of V$_{ND}$ and calmodulin-labeled V$_{ND}$ (V$_{ND}$-CaM) at ~1 mg/ml were diluted 1:100 in 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl and 285,000 ¥ g for 16 h. Afterward, 1-ml fractions were collected by fractionation from the bottom of the gradient and analyzed by SDS-PAGE.

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sequencing (Eurofins) using MalE and M13 primers (New England Biolabs).

Circular Dichroism Spectroscopy—Far UV CD spectra of $\alpha_{NT(1–372)}$ and subunit $d$ constructs were collected on an Aviv 420 spectrometer in 25 mM sodium phosphate (pH 7.4) in a 1-mm path length cuvette at 10 °C. For the subunit $d$ scans, 0.1 mM tris(2-carboxymethyl)phosphine (TCEP) was included in the buffer. Scans of buffer only were subtracted from the spectra.

Isothermal Titration Calorimetry—The interactions between subunit $d$ and $\alpha_{NT(1–372)}$ constructs were determined using a VP-ITC (MicroCal). Proteins were prepared in 20 mM Tris (pH 7), 0.5 mM EDTA, and 1 mM TCEP at 10 °C. Ligand (subunit experiments were carried out by N. J. S. N. J. S. and S. W. wrote the paper. Cardel is acknowledged for advice with Vo purification and Dr. S. W. wrote the paper. Rebecca Oot and Stuti Sharma for helpful discussions.

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