REVIEW

Breaking up and making up: The secret life of the vacuolar H^+-ATPase

Rebecca A. Oot, Sergio Couoh-Cardel, Stuti Sharma, Nicholas J. Stam, and Stephan Wilkens*

Department of Biochemistry and Molecular Biology, SUNY Upstate Medical University, Syracuse, New York 13210

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Abstract: The vacuolar ATPase (V-ATPase; V_1V_o-ATPase) is a large multisubunit proton pump 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. V-ATPase function is essential for pH and ion homeostasis, protein trafficking, endocytosis, mechanistic target of rapamycin (mTOR), and Notch signaling, as well as hormone secretion and neurotransmitter release. V-ATPase can also be found in the plasma membrane of polarized animal cells where its proton pumping function is involved in bone remodeling, urine acidification, and sperm maturation. Aberrant (hypo or hyper) activity has been associated with numerous human diseases and the V-ATPase has therefore been recognized as a potential drug target. Recent progress with moderate to high-resolution structure determination by cryo electron microscopy and X-ray crystallography together with sophisticated single-molecule and biochemical experiments have provided a detailed picture of the structure and unique mode of regulation of the V-ATPase. This review summarizes the recent advances, focusing on the structural and biophysical aspects of the field.

Keywords: vacuolar ATPase; V-ATPase; V1Vo-ATPase; rotary motor enzyme; rotary catalysis; protein structure; reversible disassembly; X-ray crystallography; cryo electron microscopy; protein–protein interactions

Introduction

The vacuolar H^+-ATPase (V-ATPase, V_1V_o-ATPase) is a ubiquitous multisubunit enzyme complex that acidifies subcellular compartments in all eukaryotic cells and the extracellular space in some specialized tissues of higher organisms.[1–4] V-ATPase's proton pumping activity plays a vital role in numerous essential cellular processes such as pH and ion homeostasis, protein trafficking, autophagy, endocytosis, mTOR, and Notch signaling, as well as bone remodeling, urine acidification, hormone secretion, and neurotransmitter release.[5–9] While complete loss of V-ATPase function is embryonic lethal,[10] aberrant (hypo- or hyper-) activity is associated with a wide spectrum of human diseases including renal tubular acidosis,[11] sensorineural deafness,[12] osteoporosis,[13] diabetes,[8] microbial[14,15] and viral infection,[16] male infertility,[17] cancer,[18] and AIDS.[19] Because of its involvement in multiple widespread diseases, V-ATPase is a potential drug target.[20–23]

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*Correspondence to: Stephan Wilkens; Department of Biochemistry & Molecular Biology, SUNY Upstate Medical University, Syracuse, NY 13210. E-mail: wilkenss@upstate.edu

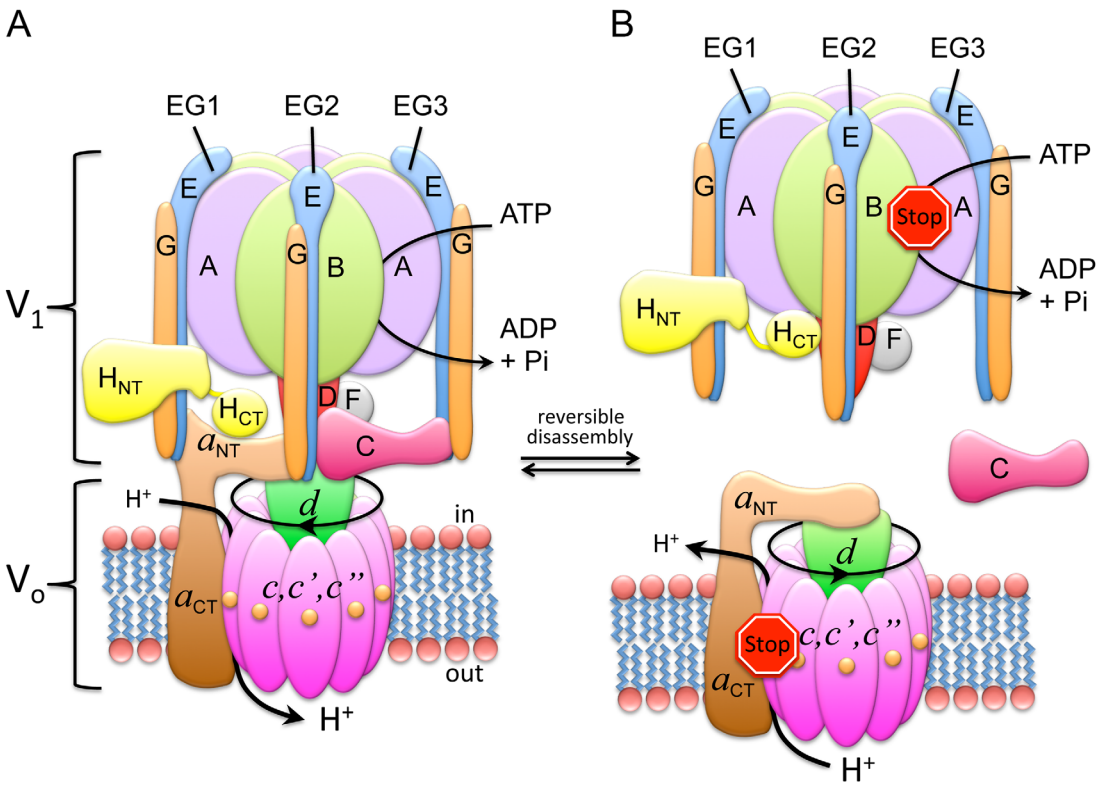

Figure 1. V-ATPase subunit arrangement and regulation by reversible dissociation. (A) Subunit architecture of yeast V-ATPase. (B) Regulation by reversible dissociation. In yeast, under conditions of starvation, V_1-ATPase disengages from the membrane bound V_o and the activity of both sectors is silenced. Activity silencing is accompanied by a large conformational change in V_1's H subunit (H_{CT}) and the N-terminal domain of the V_o *a* subunit (a_{NT}). Illustration adapted from Ref. 73.

However, identifying and optimizing drugs that are designed to modulate V-ATPase activity rather than causing complete inhibition will require a detailed understanding of the enzyme's structure and its catalytic and regulatory mechanisms.

V-ATPase is made up of ~14 unique subunits that are present in different stoichiometries, resulting in ~30 polypeptides with a total relative molar mass of close to one million. The complex can be divided into two functional sectors, a water soluble ATP hydrolyzing catalytic V_1 made of subunits A-H, and a membrane embedded V_o proton channel composed of subunits *a,d,e,f*, and two to three "proteolipid" isoforms referred to as subunits *c*, (*c*′), and *c*″ [Fig. 1(A)]. While V-ATPases from higher organisms can contain additional components such as Ac45 (ATP6AP1) or (pro)renin receptor (ATP6AP2), the core V-ATPase complex is highly conserved from simple eukaryotes (such as baker's yeast) to higher animals, including human. Most of the subunits in higher organisms are expressed as multiple isoforms, some of which are organelle or tissue specific.[24] However, in yeast only subunit *a* is expressed as two isoforms, with isoform Vph1p found in the vacuole and Stv1p in the Golgi.[25] The well-defined subunit composition, together with the ease of genetic manipulation, has made yeast a powerful model system for structural and mechanistic studies of intact V-ATPase and its V_1 and V_o sectors isolated from the native source. The composition of the V-ATPase from the yeast *Saccharomyces cerevisiae* is $A_3B_3(C)DE_3FG_3H$ for the V_1 and *a*,c_8,*c*′,*c*″,*d*,*e*,*f* for the V_o [Fig. 1(A)]. Subunit *f* was only very recently found associated with yeast V_o,[26] but since deletion of the corresponding gene did not produce the well characterized deletion phenotype,[27] its designation as a V-ATPase subunit requires further study.

V-ATPase is a molecular motor enzyme, employing a rotary mechanism of energy coupling that is shared with the related F-, A-, and A/V-type ATPases/ATP synthases.[28–30] In the V-ATPase, stepwise rotation of a central rotor domain made of V_1 subunits DF and V_o subunits *d* and *c* couples the energy of ATP hydrolysis released from three catalytic sites on V_1 to the uphill transport of protons across the lipid bilayer. However, unlike the related rotary motor ATPases, V-ATPase activity *in vivo* is regulated via a unique mechanism referred to as "reversible dissociation" (or disassembly), a process that results in a transient pool of free cytosolic V_1 and membrane integral V_o sectors that are functionally silenced [Fig. 1(B)]. V-ATPase regulation by reversible dissociation was initially discovered in yeast[31] and insect[32] but more recent studies show that the process is conserved in higher animals including human.[6,33–35]

This short review summarizes recent insights into the V-ATPase's unique mode of regulation by

reversible disassembly, focusing on the structural aspects of the process. Considering recent progress with structure determination of the autoinhibited V_1 and V_o sectors from yeast, a model of the molecular mechanism of V-ATPase disassembly is proposed. The review is concluded by a summary of some of the unresolved questions in the field.

V-ATPase Structure

Throughout this review, V-ATPase will refer to the eukaryotic enzyme unless stated otherwise. Where appropriate, the eukaryotic V-ATPase from the yeast *S. cerevisiae* and its functional sectors will be designated by the prefix "Sc" as in ScV_1V_o, ScV_1, and ScV_o. In analogy, the bacterial enzyme (from e.g. *Enterococcus hirae*) will be written as EhV_1V_o, EhV_1, and EhV_o. By convention, subunits of the catalytic and membrane sectors will be in capital letters (ABCDEFGH) and lower case italics (*acc'c"def*), respectively.

The structures of the holo V-ATPase and its two separated sectors have been studied extensively using negative stain and cryo electron microscopy (cryoEM) together with single-particle image averaging and three-dimensional (3-D) volume reconstruction. Early on, tagging of the enzyme with subunit specific probes such as monoclonal antibodies helped to elucidate the overall subunit arrangement and stoichiometry. At the same time, crystal structures of individual subunits or subunit complexes of yeast V-ATPase (subunits H, C, and EG), and subcomplexes from related bacterial enzymes such as the sodium pumping V-like ATPase from *E. hirae* (EhV_1V_o) and the A-ATPase from *Thermus thermophilus* (TtA_1A_o) were becoming available. Fitting of the crystal structures into the low to medium-resolution EM maps allowed building of partial and eventually complete pseudo atomic models of the V-ATPase and its V_1 and V_o sectors isolated from various sources including bovine brain,[36–38] insect midgut,[39] and yeast vacuoles.[40–43] The early models not only confirmed the V-ATPase's structural similarity to the related F-ATP synthases, which was already evident from primary sequence analysis of the catalytic and proteolipid subunits,[44] but the EM models also revealed the increased complexity of the V-ATPase compared to its ATP synthase counterparts. Recently, advances in electron detector technology have allowed determination of cryoEM maps of insect and yeast V-ATPase at subnanometer resolution.[45,46] These maps revealed secondary structure elements in both the soluble V_1 and, for the first time, in the membrane embedded V_o. Together with the available crystal structures, the density maps allowed building of pseudo atomic models of the complex in different catalytic states.

Overall architecture of holo V-ATPase

The A and B subunits of V_1 come together in an alternating fashion to form a "catalytic hexamer" (A_3B_3) with catalytic nucleotide binding sites located at three of the six AB interfaces[47] [Fig. 2(A)]. Located within the hexamer and protruding ~30 Å towards the membrane is part of the central rotor that is comprised by subunits D and F, which provide the functional link between V_1 and V_o[48] [Fig. 2(B)]. Structural support between ATPase and proton channel is provided by the "peripheral stator", which consists of three heterodimers formed by subunits E and G ("peripheral stalks" EG1-3) [Fig. 2(C,D)], the single copy subunits H and C [Fig. 2(E,F)], and subunit *a* [Fig. 2(G); only the N-terminal domain of subunit *a* (a_{NT}) is shown]. The three EG heterodimers link the top of the catalytic hexamer to V_1 subunits H and C and the membrane integral a subunit of the V_o [Fig. 1(A)]. The yeast EG heterodimer in complex with the head domain of subunit C has been crystallized in two conformations[49] [Fig. 2(C,D)]. The structures show EG to be folded in an unusual right-handed coiled-coil (formed by the subunits' N-termini) and a C-terminal globular domain that provides the binding energy to the A_3B_3 hexamer. The H and C subunits are two domain polypeptides with N- and C-terminal domains for subunit H (H_{NT} and H_{CT})[50] [Fig. 2(E)] and foot and head domains for subunit C (C_{foot} and C_{head})[51] [Fig. 2(F)]. Subunit *a* of the V_o can be divided into a cytosolic N-terminal (a_{NT}) and a membrane-integral C-terminal domain (a_{CT}). The a_{NT} X-ray crystal structure of the related A-ATPase from *M. ruber* showed the protein to be folded in two domains designated as proximal and distal lobes ($a_{NT(prox)}$ and $a_{NT(dist)}$)[52] [Fig. 2(G)]. a_{NT} provides the scaffold to connect the peripheral stator components H, C, and EG1-3 to the membrane.

The ATPase is functionally coupled to the proton channel via binding of the central DF rotor to subunit *d*. A homolog of subunit *d* from *T. thermophilus* A-ATPase (TtC) has been crystallized [Fig. 2(H)] and is an all α helical protein that links the central DF rotor to the cytoplasmic loops of the *c* subunits ("proteolipids"). The *c* subunits are arranged in a ring (referred to as proteolipid ring or *c*-ring) as shown for the crystal structure of the related EhK_{10}[53] [Fig. 2(I)]. V-ATPase proteolipids contain four transmembrane α helices (TMH) except isoform *c"*, which has an additional α helix at its N-terminus that is not essential for function.[54] From sequence analysis it has been proposed that V-ATPase four TMH proteolipids have originated by gene duplication from ancestral two TMH *c* subunits found in F- and A-type motors.[55] As of today, there is no high-resolution crystal structure of eukaryotic holo V-ATPase or of any of its bacterial or archaeal

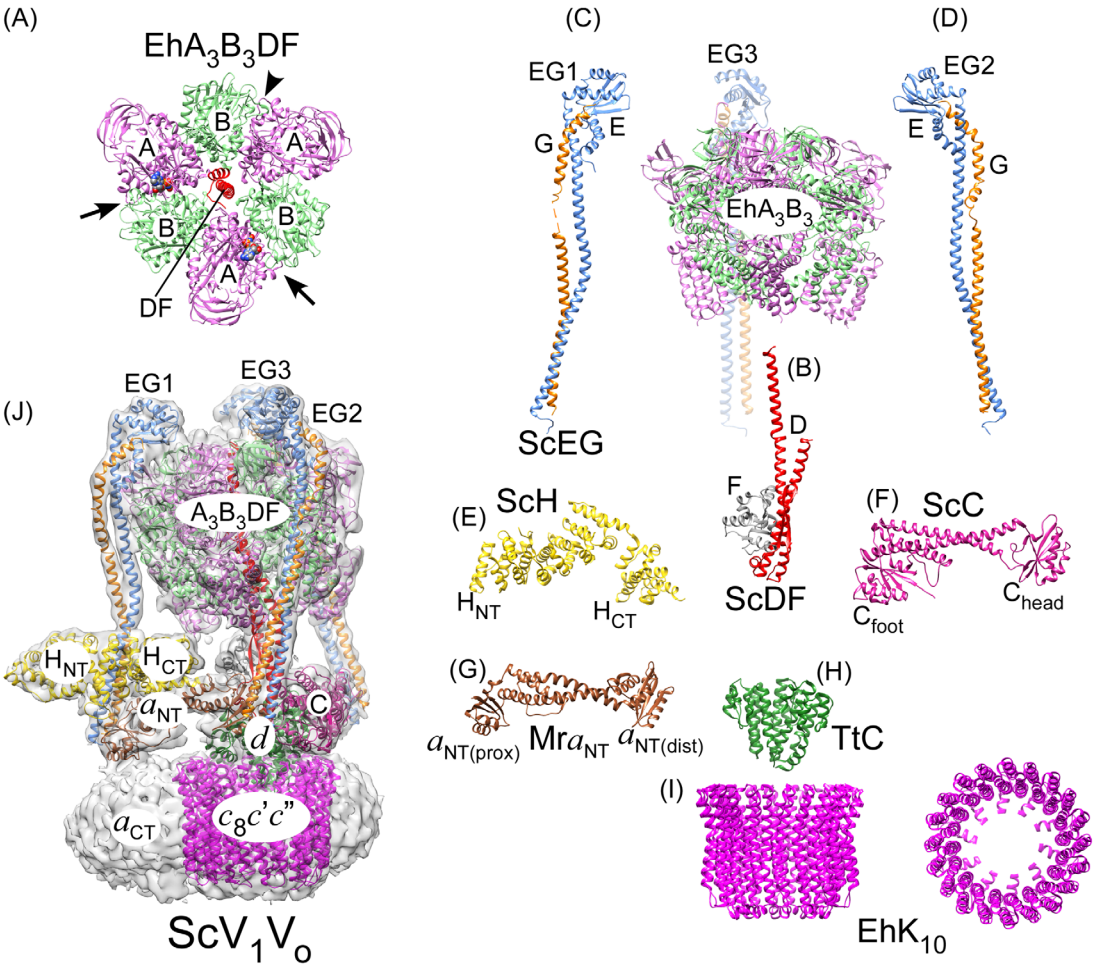

Figure 2. Overall subunit architecture of holo V-ATPase. (A) Crystal structure of EhA_3B_3DF (3vr6; a side-view of the structure is also shown on the right).[47] The arrows indicate closed catalytic sites with AMPPNP bound and the arrowhead points to the open site. (B) Crystal structure of ScDF (4rnd).[48] (C,D) Crystal structures of $ScEGC_{head}$ in two conformations (4dl0, 4efa; C_{head} not shown).[49] (E) Crystal structure of ScH (1ho8).[50] (F) Crystal structure of ScC (1u7l).[51] (G) Crystal structure of the a_{NT} homolog from *M. ruber* (Mra; 3rrk).[52] (H) Crystal structure of the *d* homolog from *T. thermophilus* (TtC; 1r5z).[103] (I) Crystal structure of the proteolipid ring from *E. hirae* (EhK10; 2bl2)[53]. (J) CryoEM map of ScV_1V_o (emd-6284)[45] with fitted coordinate models of individual subunits and subcomplexes (3j9t).[45] The overall model was generated from individual crystal structures of yeast subunits and homology models generated by threading yeast primary sequences into crystal structures of bacterial A-ATPase subunits.

counterparts. The highest resolution information currently available for the intact enzyme has been obtained by cryoEM of the yeast V-ATPase.[45] Three-dimensional reconstruction and classification of a large dataset of individual molecular projections allowed generation of maps of the enzyme in three distinct rotational states, which are distinguished by the three rotational positions of the DF*d* rotor within the catalytic A_3B_3 hexamer. The analysis showed "state 1" to be most populated, resulting in a map at 6.9 Å resolution [Fig. 2(J)]. To generate a pseudo atomic model of holo V-ATPase [Fig. 2(J)], the individual crystal structures listed above [Fig. 2(A–I)] were placed into the map, and the individual subunits' structures were flexibly fit guided by the cryoEM map density. Note that there is no high-resolution structure available for the membrane embedded C-terminal domain of subunit a as part of holo V-ATPase (but see below for the recent 3.9 Å cryoEM map of autoinhibited ScV_o).

The V-ATPase in animal cells

The comparison of the early low-resolution EM studies of bovine brain V-ATPase[56] with images of the yeast enzyme[57,58] showed that the basic architecture of the V-ATPase complex is conserved from lower eukaryotes to mammals. As mentioned above, in yeast only subunit *a* is expressed as two organelle specific isoforms, Vph1p and Stv1p.[25] Although subunits *c*, *c′*, and *c″* are sometimes called proteolipid isoforms, all three variants of the *c* subunits are essential for enzyme function in yeast[59] (as of today, *c′* has not been identified in higher animals). In the animal V-ATPase, on the other hand, most of the subunits are expressed as multiple isoforms, some of which are organelle or tissue specific.[24] Most notable

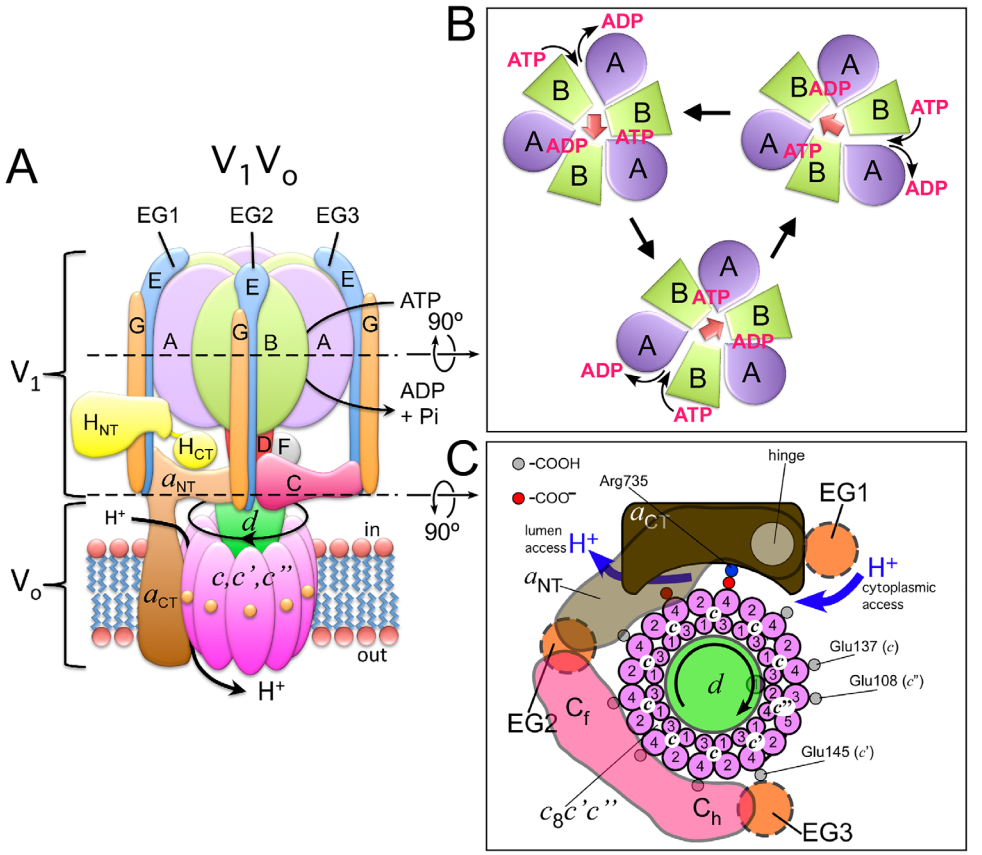

Figure 3. V-ATPase mechanism. (A) V-ATPase consists of two motors, a V_1-ATPase and a V_o proton channel. (B) ATP hydrolysis at three of the six AB interfaces drives counterclockwise rotation of the central DF rotor (A_3B_3 seen towards the cytosol). (C) Rotation of the central DF rotor drives rotation of the proteolipid ring past the essential arginine residue in a_{CT}. Protons enter a cytoplasmic half-channel and, after being carried 360° on lipid exposed glutamate residues on the *c* subunits, are released into the lumenal half-channel.

are the four isoforms of subunit *a* with isoform a_1 found in neurons, a_2 in endothelia, a_3 in osteoclast ruffled membrane and pancreatic β cells, and a_4 in the kidney. However, isoform distribution is not strictly defined, making it very challenging, if not impossible, to isolate and purify animal V-ATPases with a homogeneous isoform composition.

V-ATPase Mechanism

The V-ATPase, like all members of the rotary ATPase family, contains two motors: a V_1 motor that converts the chemical energy of ATP hydrolysis into mechanical energy (torque of central DF rotor), and a V_o proton turbine that converts mechanical energy into the potential energy store of an ion gradient[28–30] [Fig. 3(A)]. Conformational changes powered by ATP hydrolysis at the three catalytic sites on the A_3B_3 hexamer drive rotation of the central DF rotor [Fig. 3(B)], which in turn, via its connection to subunit *d*, rotates the c-ring past the membrane integral a_{CT} for proton translocation across the lipid bilayer [Fig. 3(C)]. During catalysis, the three peripheral stalks (EG1-3), in conjunction with subunits C, H, and a_{NT}, function to resist the torque of rotation, thus keeping the A_3B_3 hexamer static against the proton channel to allow for the rotation to be productive [Fig. 3(A)].

From biochemical and biophysical work on related bacterial enzymes, we have some understanding of the molecular mechanism of ATP hydrolysis and the concomitant conformational changes on the A_3B_3 catalytic hexamer that drive rotation of the central DF rotor. For each ATP hydrolyzed, the central rotor rotates 120° counter-clockwise (when looking towards the cytoplasm), driven by conformational changes that occur in the A_3B_3 hexamer upon ATP binding, hydrolysis, and product release. Interestingly, while in F-ATP synthase each 120° rotation occurs in substeps of ~80° (ATP binding/release) and 40° (ADP/Pi binding/release),[60] no substeps have been observed for bacterial V-like ATPase from, for example *T. thermophilus*[61] or *E. hirae*[62] as of today.

As previously mentioned, the central DF rotor, via its connection to subunit *d*, rotates the *c*-ring past the membrane integral a_{CT} to pump protons across the lipid bilayer. Each *c* subunit contains an essential glutamic acid residue mid-membrane that, after getting protonated in an aqueous half channel accessible from the cytoplasmic side, is able to enter the lipid bilayer and following a close to 360°

rotation of the c-ring, delivers its proton to an aqueous half-channel accessible from the lumen of the organelle or the outside of the cell [Fig. 3(C)]. A strictly conserved arginine residue in a_{CT} located at the interface of a_{CT} and the *c*-ring (Arg735 in yeast[63]) serves to stabilize the essential glutamic acid residues of the *c* subunits while in the deprotonated state between lumen and cytoplasmic half channels. Besides the essential Arg735, site-directed mutagenesis experiments in yeast V-ATPase have uncovered several charged and polar residues in a_{CT} that are essential for proton pumping.[64] How these residues work together to guide the protons from the cytoplasmic to the lumen or cellular exterior, however, is not known, largely because of lack of high-resolution structural information for the V-ATPase's (or, for that matter, any of the rotary ATPases') membrane sector, as well as the dynamic nature of the process.

V-ATPase Regulation

Breaking up...

V-ATPase hydrolyzes up to ~300 ATP·(sec^{-1})[65] and as a major consumer of cellular energy[‡], its MgATPase activity must be tightly controlled. In eukaryotic organisms, the enzyme's proton pumping activity is regulated by reversible dissociation (or disassembly), a process resulting in free, water soluble cytoplasmic ATPase (V_1) and membrane bound proton channel (V_o) sectors. In yeast, reversible dissociation is driven by environmental factors such as nutrient availability,[31,32] salinity,[66] and extracellular pH.[67] In higher animals, reversible disassembly or stimulated assembly (without prior disassembly) occurs in response to nutrients,[33,35] cell maturation,[34] hormones,[68] and growth factors.[6] Upon enzyme dissociation, the activity of both sectors is silenced, resulting in a V_1 that has no MgATPase activity[69–71] and a V_o that does not catalyze passive proton translocation.[72,73] From studies in yeast and insect, we have some information as to the molecular mechanism of regulated disassembly. Upon initiating disassembly by, for example glucose withdrawal in yeast, the C subunit dissociates from the vacuolar membrane[31] and is not part of either V_1 or V_o when these complexes are purified subsequent to enzyme disassembly.[40,69,70,73] It is also known that inhibition of ATP hydrolysis by, for example mutation of residues involved in catalysis, nucleotide binding, or proton transport, or by binding of specific drugs that block rotation, results in "hyper-assembly",[74,75] which indicates that disassembly occurs while the enzyme is in a high-energy conformation during rotary catalysis. Similar results were obtained from *in vitro* experiments conducted with detergent solubilized insect V-ATPase in which MgATP, but not MgADP or the nonhydrolyzable MgAMPPNP, resulted in the formation of free V_1 and V_o sectors.[76] Thus, while some aspects of enzyme disassembly are known, there is still no clear understanding of the nature of the signal that initiates the process, though several possibilities have been proposed, including involvement of protein kinase A (either upstream or downstream of V-ATPase), ATP to ADP ratio, and cytoplasmic pH to name only a few.[75,77–79] Recent structural models of autoinhibited ScV_1 and ScV_o summarized in the following paragraphs revealed some of the conformational changes that occur as a result of holo enzyme dissociation.

Structural features of autoinhibited ScV_1. As mentioned above, membrane detached V_1 isolated from yeast or insect midgut has no measurable MgATPase activity, though the complex can hydrolyze ATP in presence of Ca^{2+} (given the absence of magnesium ions).[40,69,70] In yeast, deletion of subunit H allows purification of a $V_1\Delta H$ complex that displays robust MgATPase activity.[70,71,80] Interestingly, deletion of subunit H still allowed assembly of the resulting $V_1\Delta H$ on the membrane, but the resulting complex has no measurable MgATPase or proton pumping activities.[81] Biochemical work in yeast showed that it is H_{CT} that is responsible for MgATPase inhibition.[80,82] However, even $V_1\Delta H$'s ATPase activity is short lived because of trapping of the complex in the so-called MgADP inhibited state.[70,71] The MgADP inhibited state is an off-catalytic pathway conformation that is also found in other related rotary ATPase catalytic sectors.[83] The role of subunit H in activity silencing has recently been illuminated in the 6.2 Å crystal structure of autoinhibited ScV_1 [Fig. 4(A), right panel]. A comparison of the ScV_1 crystal structure with the cryoEM models of holo V-ATPase [Fig. 4(B)] revealed that autoinhibition is accompanied by a large conformational change of the C-terminal domain of subunit H (H_{CT}) [Fig. 4(A), left panel]. Upon release from the membrane, H_{CT} moves and rotates 150° from its binding site on a_{NT} in ScV_1V_o to a position at the bottom of the A_3B_3 hexamer in autoinhibited ScV_1 to contact the central rotor subunit D and the B subunit of the open catalytic site [Fig. 4(A)]. The structural and accompanying biochemical studies also showed that while $V_1\Delta H$ contained only ~0.4 moles/mol ADP, autoinhibited ScV_1 had ~1.3 ADP molecules per complex, consistent with ScV_1 being in the MgADP inhibited state, a conformation likely stabilized by H_{CT}'s interaction with the central rotor and the open catalytic site. Curiously, the loop in H_{CT} that was identified as being responsible for the subunit's ability to inhibit MgATPase activity is not present in higher

[‡]Specific activities of up to 18 μmoles·min^{-1}·mg^{-1} have been reported for the enzyme from, for example yeast,[65] corresponding to the hydrolysis of ~300 ATP·sec^{-1}.

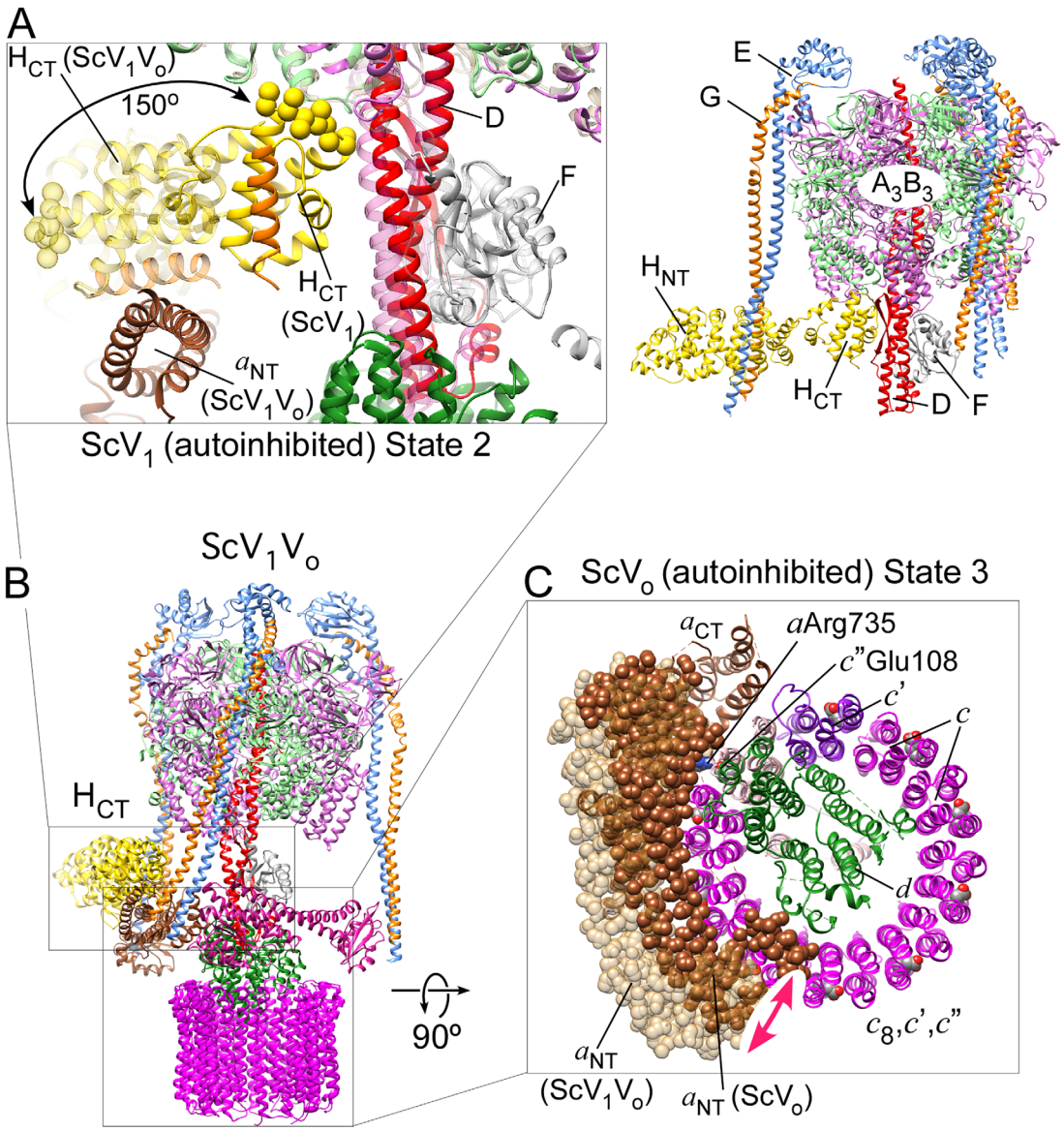

Figure 4. V-ATPase conformational changes accompanying reversible disassembly. (A) Conformational changes in the auto-inhibited ScV_1-ATPase (5d80).[71] Upon holo enzyme dissociation, the C-terminus of subunit H (H_{CT}) is released from its binding site on a_{NT} (in V_1V_o) and rotates 150° to bind at the bottom of the A_3B_3 hexamer (in V_1). H_{CT} binds to the B subunit of the open catalytic site, thereby stabilizing inhibitory ADP in another site.[71] The inhibitory loop is in spacefill and the α helix that binds a_{NT} in V_1V_o is in orange. (B) CryoEM model of holo ScV_1V_o[45] (3j9u). (C) Conformational changes in the V_o proton channel (5tj5)[26]. Upon release of V_1-ATPase from the membrane, the N-terminus of subunit *a* (a_{NT}) moves from its ternary interface with C_{foot} and EG2 to form a new binding interface with subunit *d*[26,73,88] and the *c*-ring is positioned so that Glu108 of isoform *c″* is in contact with the essential Arg735 in a_{CT}.

animals including human[71] and it remains to be seen whether mammalian subunit H is inhibitory, or whether autoinhibition of mammalian V_1 is solely because of trapping of MgADP.

Structural features of autoinhibited ScV_o. Unlike F-ATPase F_o membrane sector, which allows passive proton conductance when detached from F_1,[84], free V_o does not catalyze downhill translocation of protons along an electrochemical gradient.[72,73,85] The autoinhibition of free V_o allows co-existence of free V-ATPase membrane sectors next to assembled holo enzyme complexes as has been observed in yeast and higher animals.[75] Early negative stain EM images of bovine V_o showed an interaction of a_{NT} with subunit *d*, an observation that lead to the proposal that blockage of passive proton transport is because of the interaction between rotor and stator. Subsequent studies in yeast confirmed the a_{NT}–*d* interaction, however, selective removal of either a_{NT} or subunit *d* did not relieve the inhibition,[73,86] suggesting that structural features of the *c*-ring or a_{CT} may be responsible for the process. Early genetic screens in yeast revealed the presence of multiple

proteolipid isoforms referred to as subunit *c*, *c′*, and *c″*. As mentioned above, the *c* and *c′* proteolipid isoforms have each four TMH while the *c″* isoform has five. It was noted that while the essential glutamic acid residues in *c* and *c′* are in TM helix 4 (Glu137 and Glu145, respectively), the corresponding residue in *c″* is in helix 3 (Glu108).[59] This led to the speculation that the resulting asymmetry in the spacing of the proton transporting glutamic acid residues at the periphery of the *c*-ring may be the cause of autoinhibition as the large gap in carboxyls at the interface of *c″* and *c′* might not be able to move past a_{CT}'s Arg735, thereby preventing the reversal of the direction of rotation that would be required *for* passive proton transport.[73] Recently, Mazhab-Jafari *et al*. reported a cryoEM reconstruction of autoinhibited ScV_o, allowing a first glimpse at the interactions between residues of the a_{CT} stator and the *c*-ring rotor within the membrane[26] [Fig. 4(C)]. As first seen in the related F-ATP synthase from *Polytomella* sp.,[87] a_{CT} contains two long α helices that cross the lipid bilayer at an angle of 30° with respect to the plane of the membrane and contact the periphery of the c-ring mid-membrane. In V-ATPase, these two α helices contain some of the charged and polar residues identified in the site-directed mutagenesis experiments mentioned above,[64] including the essential Arg735. While the EM model of the autoinhibited ScV_o showed a cavity on the cytoplasmic side likely to represent the aqueous access for protons to enter the membrane before they bind the glutamates on the proteolipids, no such cavity was identified on the lumenal side of the complex.[26] The cryoEM model of autoinhibited yeast V_o also confirmed the earlier proposal in that Glu108 of *c″* was seen to be in contact with Arg735[26] [Fig. 4(C)]. However, whether the asymmetry in the glutamic acid spacing is the sole reason for V_o's autoinhibition remains to be seen.

… and making up

The comparison between ScV_1 and holo ScV_1V_o mentioned above also revealed that subunit H and MgADP facilitated autoinhibition halts ScV_1 in state 2 based on the position of the central DF rotor[71] [Fig. 4(A,B)]. The corresponding comparison of the ScV_o sectors based on the position of the proteolipid ring or the *d* subunit showed that autoinhibited ScV_o is arrested in state 3.[26,88] These observations lead to the proposal that the resulting mismatch between state 2 V_1 and state 3 V_o conformations may serve to prevent unintended reassembly of the complex when its proton pumping activity is not required.[88] Consequently, before reassembly can take place, the conformational mismatch must be overcome. Bringing V_1 and V_o into matching rotational states can either happen by converting V_o from state 3 to state 2 or by converting V_1 from state 2 to state 3. Since autoinhibited V_o is arrested in state 3 despite a transmembrane proton gradient, it is more likely that subunit H stabilized MgADP inhibition of V_1 is relieved to allow hydrolysis of at least one ATP molecule with concomitant rotation of the central rotor from state 2 to state 3 to match V_o's rotational state. What could be the mechanism for relieving the ADP inhibition? From studies in yeast, it is known that assembly, be it the initial assembly following biosynthesis, or the re-assembly following reversible disassembly, occurs in absence of turnover but requires presence of the "regulator of H^+-ATPases of vacuolar and endosomal membranes" (RAVE),[89,90] a heterotrimeric chaperone complex that has been shown to promote assembly by binding to the V_1, the V_o, and the C subunit.[91,92] Curiously, for reasons that are currently not understood, the RAVE complex seems not to be required for the assembly of Stv1 containing V-ATPase found in the Golgi.[93] Besides the RAVE complex, several enzymes of the glycolytic pathway have been shown to be required for efficient V-ATPase assembly, most notably aldolase[94] and phosphofructokinase-1.[95] However, the mechanism by which RAVE and any of these enzymes promote the assembled state is not known.

The V_1–V_o structural interface—a predetermined breaking point. Reversible disassembly requires breaking (and re-forming) of several protein–protein interactions at the V_1–V_o interface [Fig. 5(A)]. The affinities of many of these interactions have been determined with biophysical tools such as isothermal titration calorimetry and optical methods (surface plasmon resonance and biolayer interferometry) using recombinant subunits or subcomplexes of the yeast[73,88,96,97] and human[98] V-ATPase. From the interactions that need to be broken during disassembly of the yeast enzyme (EG3-C_{head}; EG2-$a_{NT(dist)}$; EG1-$a_{NT(prox)}$; C_{foot}-$a_{NT(dist)}$; H_{CT}-a_{NT}; DF-*d*), all have affinities in the micromolar range except EG3-C_{head}, which has a K_d of ~42 n*M*.[96] It has been proposed that the junction of a_{NT} with EG2 and C_{foot} is stabilized through the high avidity of the multiple weak interactions involved and that breaking one of these weak interactions could destabilize the V_1–V_o interface and initiate dissociation of the complex.[97] A possible role in this process could be played by EG3. The SAXS solution and X-ray crystal structures of the ScEGC[42] and $ScEGC_{head}$[49] subcomplexes suggested that EG3 may be under strain in holo V-ATPase, and it has been proposed that this "spring-loading" of EG3 that occurs upon assembly of the complex could play a role in the disassembly process by preventing re-association of C_{foot} with $a_{NT(distal)}$ once the interaction is broken at the initiation of the process [Fig. 5(B), blue arrow].

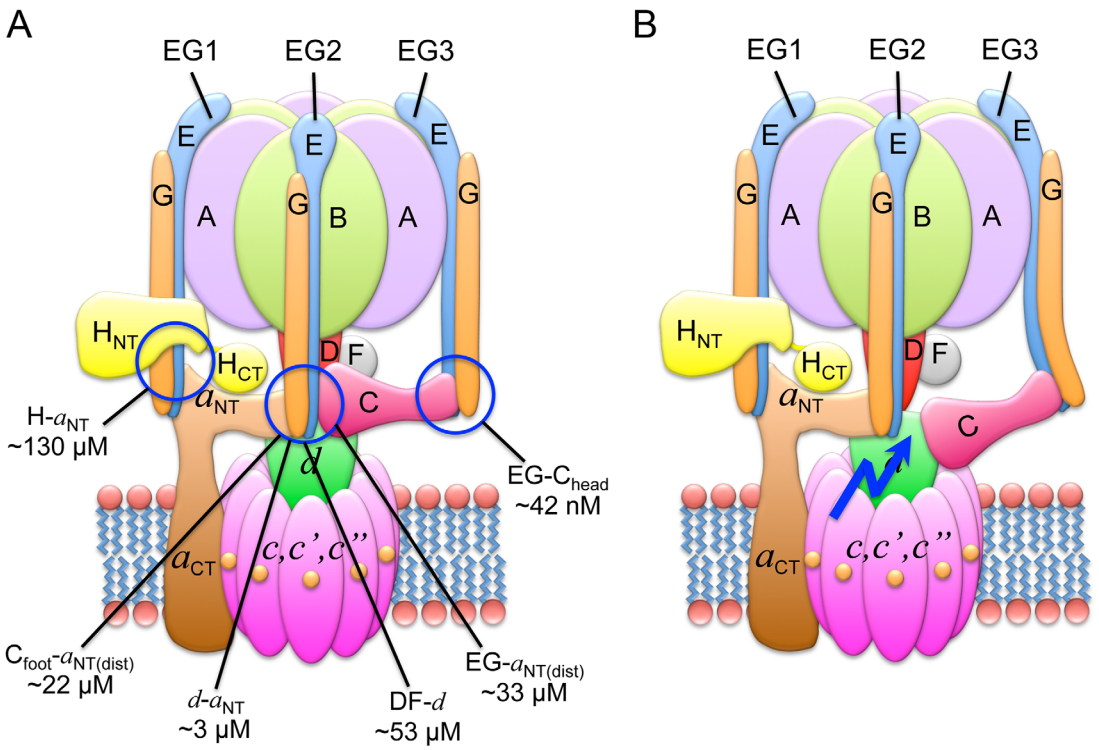

Figure 5. Interactions at the V_1–V_o interface. (A) The affinities of the individual interactions have been measured using isothermal titration calorimetry of recombinant yeast V-ATPase subunits.[48,96,97] (B) Spring-loading of EG3. It has been suggested that the tension in EG3 would prevent re-forming of the EG2–$a_{NT(distal)}$–C_{foot} ternary interface after one of these interactions is broken to initiate disassembly.[49]

Mechanistic model for V-ATPase dissociation. From recent structural and biochemical work, we now know what some of the conformational changes are that take place during regulated V-ATPase disassembly. From the observations that autoinhibited V_o is arrested in rotational state 3, the same state that was the least populated in the cryoEM analysis of the holo enzyme,[45] it has been speculated that disassembly occurs when V-ATPase goes through this state during turnover.[26] In our current working model [Fig. 6, Supporting Information Fig. S1], we therefore assume that disassembly starts when holo V-ATPase is in the catalytic dwell in state 3 [Fig. 6(A)]. Once the (as yet unknown) signal for disassembly is received, the ternary interface EG2–$a_{NT(distal)}$–C_{foot} is broken, allowing a_{NT} to bind to subunit *d* [Fig. 6(B)]. This in turn breaks the H_{CT}–a_{NT} interaction and frees up H_{CT}. Since ScV_1 is found to be arrested in state 2, DF has to rotate two 120° steps at the expense of the hydrolysis of two MgATP molecules [Fig. 6(C, D)]. As V_o remains in state 3, this would require uncoupling of the V_1 central DF rotor from the *d* subunit. Once V_1 is in state 2, MgADP inhibition sets in and A_3B_3DF presents a high-affinity binding site for H_{CT} [Fig. 6(E)]. Note that subunit C is released during this process, possibly because of the conformational changes in EG3 induced by the two steps of ATP hydrolysis. Since most of the weak interactions are now broken, the complex is destabilized to a point so that disassembly could occur [Fig. 6(F)]. It should be pointed out that the timing of the structural changes, a_{NT} binding to *d* and H_{CT} binding to A_3B_3DF [Fig. 6(G, H)] has not been firmly established. Furthermore, whether the final dissociation step [Fig. 6(F)] occurs right away or whether the weak affinity of some of the residual interactions keeps the V_1 transiently attached to the membrane *in vivo* has been debated based on *in vivo* fluorescence and FRET imaging.[99]

Conclusions and the "To-Do" List

While our understanding of the structure and regulatory mechanism of the vacuolar ATPase is better than ever before, more information leads to new questions and there is still much work to be done. Most pressing is the need for high-resolution structural information for the active holo V-ATPase and its autoinhibited V_1 and V_o sectors, information that will be essential for our understanding of the proton-pumping and regulatory mechanisms of the enzyme. However, V-ATPase is a dynamic nano-motor that races through many conformations during its catalytic and regulatory cycle, and structural information alone will not suffice to uncover V-ATPase's many remaining secrets.

Currently, the highest resolution information available is for the autoinhibited V_o from yeast.

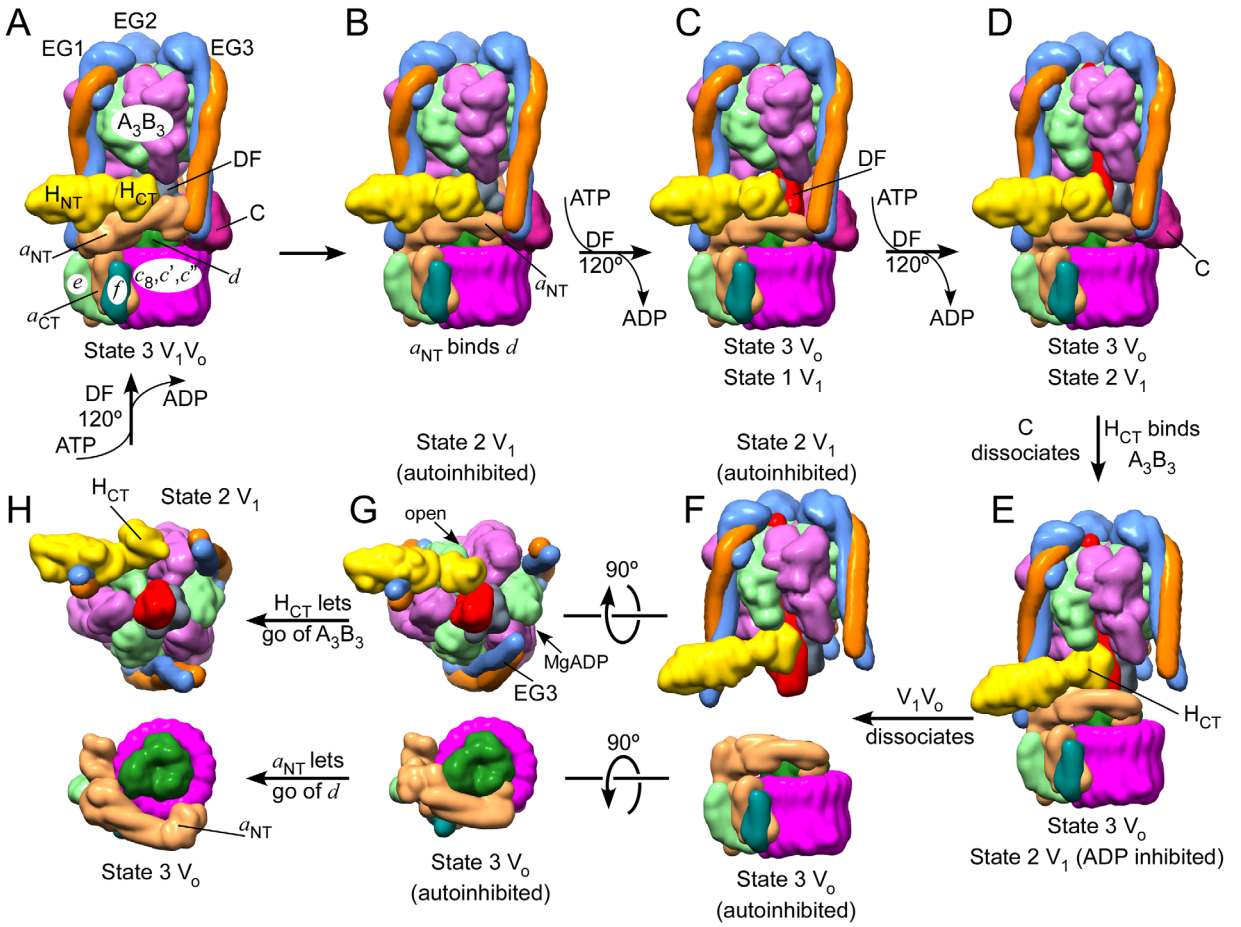

Figure 6. Model of the structural changes along the steps of holo V-ATPase disassembly. (A) It has been proposed that disassembly is initiated in state 3 V_1V_o.[26] (B) The ternary a_{NT}–EG2–Cfoot interaction is destabilized, allowing a_{NT} to bind subunit *d*. (C,D) Two uncoupled catalytic steps convert V_1 into the state 2 conformation. (E) Subunit C dissociates and H_{CT} rotates 150° to bind at the open catalytic site. V_1 is now in state 2 and autoinhibited and V_o is in state 3, also autoinhibited. (F) V_1V_o dissociates. (G) Views towards to bottom of autoinhibited V_1, illustrating H_{CT}'s binding site on A_3B_3DF, and towards the top of V_o, showing the interaction of a_{NT} with subunit *d*. (H) Reassembly must involve release of H_{CT} and a_{NT} from their autoinhibitory positions and one catalytic turnover of V_1 to return both sectors into matching state 3 conformations. Note that the starting conformation of V_1V_o and the order of the steps are not firmly established. See also Supporting Information Fig. S1 for an animation of the process.

However, whether the interactions between a_{CT} and the *c*-ring seen in V_o are the same as in state 3 holo V_1V_o, and how the ring interacts with a_{CT}'s essential arginine in the remaining states 1 and 2 remains to be seen. Also unresolved is the question whether V_o autoinhibition is caused solely by the asymmetric spacing of the proton carrying glutamates in the *c*-ring, or whether the conformational change in a_{NT} upon enzyme disassembly is transmitted to a_{CT} to help block the proton channel?

Static structures must be complemented with functional studies to resolve remaining mechanistic questions, including, how does the proteolipid ring interact with residues in a_{CT} during rotation, and how do these interactions result in net transport of protons? Does the asymmetry in the angular steps of the proton carrying glutamates in the *c*-ring serve primarily for autoinhibition of free V_o, or does the asymmetry also function to inhibit reverse rotation in holo V_1V_o? What is the molecular signal for enzyme dissociation, and how does this signal cause breaking of protein–protein interactions at the V_1–V_o interface, in particular the high-affinity interaction between subunit C and peripheral stalk EG3? Where does subunit C go upon release from V_1 and V_o, and does the binding and/or release of C involve post-translational modifications? What is the order of the functional events (e.g. MgADP inhibition) and structural changes (movements of H_{CT} and EG3 in V_1 and a_{NT} in V_o) that occur during enzyme dissociation? Is MgADP inhibition a prerequisite for enzyme disassembly? Conversely, what is the signal (or signals) that lead to reassembly, and what role do assembly chaperones such as the RAVE complex play in the process? Do the assembly chaperones merely serve to bring the components back together, or does the RAVE complex also function to overcome the conformational mismatch between state 2 V_1 and state 3 V_o?

Furthermore, there is mounting evidence that V_o, possibly via its proteolipid ring, participates in cellular functions other than proton pumping such as membrane fusion and neuronal communication.[100–102] However, the significance of these so-called "noncanonical" functions of the free V_o are only beginning to emerge. Finally, aberrant V-ATPase function is involved in many widespread human diseases. Therefore, the ultimate question is how we can apply our present knowledge and insights from future structural and functional studies to aid in the design of therapeutics to fight against human disease, with one attractive possibility being the targeting of reversible disassembly of the human V-ATPase in an isoform and tissue specific manner.

Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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