Crystal structure of yeast V$_1$-ATPase in the autoinhibited state

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Abstract

Vacuolar ATPases (V-ATPases) are essential proton pumps that acidify the lumen of subcellular organelles in all eukaryotic cells and the extracellular space in some tissues. V-ATPase activity is regulated by a unique mechanism referred to as reversible disassembly, wherein the soluble catalytic sector, V$_1$, is released from the membrane and its MgATPase activity silenced. The crystal structure of yeast V$_1$ presented here shows that activity silencing involves a large conformational change of subunit H, with its C-terminal domain rotating ~150° from a position near the membrane in holom V-ATPase to a position at the bottom of V$_1$ near an open catalytic site. Together with biochemical data, the structure supports a mechanistic model wherein subunit H inhibits ATPase activity by stabilizing an open catalytic site that results in tight binding of inhibitory ADP at another site.

Keywords autoinhibition; reversible disassembly; V$_1$-ATPase; vacuolar ATPase; X-ray crystallography

Introduction

The vacuolar H$^+$-ATPase (V-ATPase, V$_1$V$_o$-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 (Forgac, 2007). V-ATPase function is essential for pH and ion homeostasis, protein trafficking, endocytosis, TOR, 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 it pumps protons out of the cell, a process required for bone remodeling, urine acidification, and sperm maturation. While complete loss of V-ATPase function in animals is embryonic lethal (Inoue et al., 1999), partial loss or hyperactivity of the enzyme has been associated with a wide spectrum of human diseases including osteoporosis (Thudium et al., 2012), deafness (Karet et al., 1999), renal tubular acidosis (Smith et al., 2000), diabetes (Sun-Wada et al., 2006), infertility (Brown et al., 1997), neurodegeneration (Williamson & Hiesinger, 2010), and cancer (Sennoune et al., 2004), making V-ATPase a valuable drug target (Bowman & Bowman, 2005; Fais et al., 2007; Kartner & Manolson, 2014).

The V-ATPase couples ATP hydrolysis with the transport of protons across membranes using a rotary mechanism much like the related F- and A/V-type ATPases (Futai et al., 2012) (Fig 1A). In the V-ATPase from S. cerevisiae, a well-characterized model system for the enzyme from higher organisms, energy coupling requires the concerted action of fourteen different polypeptides that are organized into the ~640-kDa membrane extrinsic V$_1$-ATPase (A$_2$B$_6$CDE$_3$FG$_3$H) (Kitagawa et al., 2008) and the ~330-kDa membrane integral V$_o$ proton channel (a$_5$c$^d$e$^c$de) (Powell et al., 2000; Zhao et al., 2015). ATP hydrolysis on the catalytic V$_1$ is coupled with proton pumping across the V$_o$ via a central rotor made of V$_1$ and V$_o$ subunits DFC$^c$$^d$c$^d$. A peripheral stator complex composed of V$_1$ subunits E,G,H,C serves to stabilize the motor by binding to the N-terminal cytosolic domain (a$_5$e$_5$) of the membrane-anchored a subunit of the V$_o$ (Fig 1B).

As a major consumer of cellular energy, V-ATPase function must be tightly controlled. Regulation of enzyme activity is accomplished by a unique mechanism referred to as reversible dissociation, a condition under which the complex disassembles into cytoplasmic V$_1$ and membrane-bound V$_o$ (Kane, 1995; Sumner et al., 1995). As part of the process, the single-copy subunit C is released from the enzyme and re-incorporated during enzyme reassembly (Kane, 1995). Upon enzyme dissociation, V$_1$ loses the ability to hydrolyze MgATP (Graf et al., 1996; Parra et al., 2000) and the V$_o$ no longer conducts protons (Zhang et al., 1994), a phenomenon referred to as “activity silencing” (Fig 1C). Reversible dissociation of V-ATPase is well characterized in S. cerevisiae, but more recent data suggest that the mammalian enzyme is regulated by a similar process in some cell types. While yeast V-ATPase assembly is governed by environmental conditions such as nutrient availability (Parra & Kane, 1998), salinity, or pH (Diakov & Kane, 2010), the situation in animal cells appears to be more complicated in that next to glucose (Sautin et al., 2005), assembly can be triggered by amino acids (Stransky & Forgac, 2015), cell maturation (Trombetta et al., 2003), hormones (Voss et al., 2007), and growth factors (Xu et al., 2012).

An important role in regulating enzymatic activity in V-ATPase is played by the single-copy subunit H, a 54-kDa two-domain...
polypeptide found at the interface of V₁ and V₀ (Wilkens et al., 2004). Subunit H plays a dual role in enzyme function: While H is required for ATP hydrolysis and proton pumping in holo V-ATPase (Ho et al., 1993), the same subunit functions to inhibit MgATPase activity in membrane-detached yeast V₁, possibly in combination with product inhibition by ADP (Parra et al., 2000). The molecular mechanisms by which H functions in silencing MgATPase activity in free V₁ are not well understood, however, largely due to the lack of detailed structural information. Here, we present the crystal structure of the autoinhibited V₁ sector from S. cerevisiae (ScV₁) at 6.2–6.5 Å resolution. The structure shows that regulation by reversible dissociation involves a large movement of the C-terminal domain of H (HCT) from its position in V₁V₀ to a position at the bottom of the A₃B₃ hexamer in ScV₁. Together with accompanying biochemical data, the structure provides a mechanism of activity silencing in the membrane-detached ScV₁.

**Results**

**Crystallographic investigations of the autoinhibited ScV₁**

While subunit C is released into the cytoplasm during reversible enzyme disassembly (Kane, 1995) (Fig 1C), variable but typically substoichiometric levels of C have been seen to co-purify with ScV₁ (Zhang et al., 2003; Diab et al., 2009; Hildenbrand et al., 2010) (see also Fig 5E below). To ensure a homogeneous preparation for crystallogenesis, ScV₁ was therefore purified from a yeast strain deleted for the C subunit (Fig EV1A). Initial crystallization screening identified several conditions that resulted in small needle-shaped crystals. Crystal size and diffraction quality were gradually improved by refining a subset of the initial conditions together with additive screening. Crystals used for collecting initial datasets were obtained in 100 mM HEPES, pH 7.5, 150 mM ammonium sulfate, 12.5 mM magnesium or strontium chloride, and 9.5% PEG 8000 using vapor diffusion or a microfluidic device. A 7 Å resolution dataset collected from one ScV₁ crystal was used to start the structure determination by molecular replacement (MR) (Table 1). Since there is no crystal structure available for the eukaryotic V₁-ATPase, we employed the structure of the nucleotide-free A₃B₃ catalytic hexamer from the E. hirae sodium pumping V-type ATPase (EhA₃B₃) (Arai et al., 2013) for MR. The primary structures of E. hirae and yeast A and B subunits are highly similar (48 and 54% identity, respectively), indicating that the tertiary structures of the bacterial and eukaryotic catalytic subunits are conserved and that the bacterial A₃B₃ catalytic hexamer represents a suitable MR search model for solving the structure of the eukaryotic V₁. The MR with EhA₃B₃ revealed the presence of two ScV₁ sectors in the asymmetric unit (ASU) that were related by twofold non-crystallographic symmetry (NCS) (Appendix Fig S1). The density-modified and NCS-averaged MR map revealed electron density not only for A₃B₃, but also for ScV₁ subunits D,E,G,H that were either

![Figure 1. Schematic of the bacterial and eukaryotic V-ATPases.](image)

A Bacterial sodium pumping A/V-type ATPase from E. hirae (subunit nomenclature of the eukaryotic V-ATPase). Note that the bacterial A/V-type enzyme has only two peripheral stalks (EG₁ and EG₂) and no equivalents for the C and H subunits found exclusively in eukaryotic V-ATPase (see next panel).

B Eukaryotic proton pumping V-ATPase. The eukaryotic V-ATPase is a dedicated proton pump composed of a soluble catalytic sector (V₁) and a membrane integral proton translocating sector (V₀). The two functional sectors are linked via a peripheral stator that is formed from the N-terminal extension of the 100-kDa membrane integral subunit (aNT), three peripheral stalks (EG₁-3; shown in blue/orange), and subunits C (red) and H (yellow) that are unique to the eukaryotic enzyme.

C Reversible disassembly of eukaryotic V-ATPase. Cellular signals, such as starvation, lead to a dramatic structural rearrangement wherein the V₁ sector dissociates from V₀ and subunit C is released from the enzyme. While dissociated, both the V₁-ATPase and V₀ proton translocation domain are functionally silenced. This process is fully reversible and upon reassembly, enzymatic activity is restored.
not present in the bacterial complex (E,G,H) or not part of the MR search model (D) (Fig EV1B). Iterative manual placement of available crystal structures for subunits D,E,G, and H followed by refinement allowed for building of a largely complete model of the ScV1 sector except for subunit F, the base of subunit D and the N-termini of peripheral stalks EG2 and EG3 (Appendix Table S2). A stereo representation of the final electron density map is shown in Appendix Fig S2. Note that due to the limited resolution of the diffraction data, all polypeptides used for modeling were truncated at the Cβ-position. The more complete model obtained from the 6.2 to 6.5 Å dataset will be discussed in detail below.

Overall structure of ScV1

The structure presented here represents the highest resolution information available (to our knowledge) for the autoinhibited ScV1 sector and reveals the conformational changes associated with activity silencing and the loss of binding partners at the membrane. ScV1 can be divided into an A3B3DF catalytic core, three EG heterodimers that serve as peripheral stator stalks (hereafter referred to as peripheral stalks EG1-3) and subunit H, which is unique to the eukaryotic enzyme (Fig 2A). The A3B3DF catalytic core is highly conserved between enzymes from prokaryotes to human and contains the catalytic and non-catalytic interfaces between alternating A and B subunits arranged as a hexamer around a central cavity containing the central rotor composed of subunits D and F (see section “Catalytic hexamer and rotary shaft” below). Each of the three peripheral stalks is bound via its C-terminal domain (EC2-GC7) to the N-terminal β-barrel domain of the corresponding B subunit and crosses a non-catalytic A/B interface on its way toward the base of the hexamer (Fig 2B). However, while the stalks’ C-terminal domains are largely invariant, their N-termini (EC2-GC7) are in different conformations (Fig 2C and D and Appendix Fig S3A). Previously, we have solved the structure of the isolated peripheral stalk heterodimer bound to the C subunit head domain (EGChead, representative of EG3) in two conformations (Oot et al, 2012a). In that study, we provided evidence that the EG heterodimers contain two hinges and a partially disordered “bulge” region in subunit G that provide flexibility and that we speculated would play an important role in the mechanism of reversible disassembly (Appendix Fig S3A and B). In peripheral stalks EG1 and EG2, this segment of G is resolved as continuous tubular density, consistent with α-helical structure. In EG3, however, the density is patchy and flattened, indicating presence of the “bulge” structure as seen in isolated EGChead (Fig 2D and Appendix Fig S3C). Peripheral stalk EG3 is bound to the B subunit of a closed A/B pair and its N-terminal domain is bent inwards, wrapping around the C-terminus of the adjacent catalytic A subunit (ACT) (Fig 2D-F and Movie EV1; see also section “Comparison of autoinhibited ScV1 and ScV1Vo” below). This suggests that the bulge in subunit G may be present or absent depending upon binding partners and/or nucleotide bound state of the enzyme.

The regulatory H subunit can be seen bound to peripheral stalk EG1 via its N-terminal domain (HC7) while its C-terminus (HC7) is bound at the bottom of the catalytic hexamer near the rotary D subunit (Fig 2A and C). The interactions involving H are of particular interest, as this subunit has been shown to be essential for silencing the ATPase activity of the isolated V1 sector (see “Subunit H inhibitory interactions” below). While the interactions between the peripheral stalks and α267 and C are lost in isolated V1, their interactions with the hexamer and the inhibitory subunit H are preserved. The high affinity of the peripheral stalks for the catalytic hexamer and H, combined with the low affinity for the membrane, results in a V1 sector that can rapidly dissociate from the Vo in response to cellular signals, while keeping its inhibitory subunit bound via one of the peripheral stalks.
Figure 2. Overall structure of the autoinhibited V₁.

A Structure of autoinhibited ScV₁. The catalytic A (pink) and non-catalytic B (green) subunits are arranged as a heterohexamer around the central rotor subunits D (red) and F (cyan). The three peripheral stalks (EG heterodimers 1–3, blue and orange, respectively) are bound at the periphery of the B subunits. The inhibitory H subunit (yellow) is unique to the eukaryotic V-ATPase. Unless otherwise stated, the coloring scheme used here for the V₁ subunits will be used from here on.

B Interaction of the peripheral stalks with the catalytic hexamer. Each peripheral stalk crosses a non-catalytic A/B interface on its way from the top of the V₁ to the base of the catalytic core. Representative view of the interaction is shown for EG₃.

C Interaction of peripheral stalks EG₁ and 2 with the catalytic hexamer. The peripheral stalks are bound to the top of the B subunits via their C-termini (boxed). The electron density (contoured at 1.2σ) for the C-terminal domains of EG₁ and EG₂ is shown in the panels to the left and right.

D The structure of autoinhibited ScV₁ rotated 90° around the vertical from the view shown in panel (A) to highlight peripheral stalks EG₂ and EG₃. Note the bent appearance of EG₃ compared to EG₂ along with the presence of a partially unstructured region known as the "bulge" in subunit G (indicated with an arrow). The box shows electron density (contoured at 1.2σ) for the EG₃ N-termini contacting the C-terminal domain of the A subunit of a closed catalytic site (see asterisk). The horizontal lines on the structure indicate the sections shown in panels (E) (upper two lines) and (F) (lower two lines).

E Section through the ScV₁ electron density map showing interaction of the A,B₆ catalytic hexamer with the peripheral stalks (EG₁–3). View is rotated 90° from the view in (A), looking up the central rotor. The A/B pairs forming the three catalytic sites are designated (AB₁)-3 according to which of the three peripheral stalks is bound to each B subunit. For example, (AB₁) has peripheral stalk EG₁ bound to its B subunit. The black arrowheads indicate the non-catalytic A/B interfaces.

F Section through the ScV₁ electron density map at the level of the N-termini of the peripheral stalks. Note the change in position of the N-termini of the peripheral stalks, which have each crossed a non-catalytic interface (black arrowheads in E) and are now in proximity to the C-termini of the catalytic A subunits (Aₐ₅) of the adjacent A/B pairs. While EG₁ and EG₂ are now near Aₐ₅ from (AB₃) and (AB₁), respectively (see double headed arrows), only the bent EG₃ peripheral stalk is close enough to contact the corresponding Aₐ₅ from (AB₂) (see asterisk).
Catalytic hexamer and rotary shaft

As mentioned above, the catalytic core \((A_3B_3DF)\) of V-type rotary ATPases is highly conserved, with primary sequence identities between bacterial, yeast, and mammalian A and B subunits of \(\sim 50–80\%\), respectively (Muench et al., 2011). Not surprisingly therefore, the structure of the \(ScV_1\) catalytic core is overall very similar to its bacterial counterpart from \(E.\ hirae\) \((EhV_1)\) with an rmsd of 2.3 Å. As in \(EhV_1\), the \(ScV_1\) catalytic A and non-catalytic B subunits are arranged as a heterohexamer with their N- and C-termini found distal and proximal to the membrane, respectively (Fig 3A). The N-termini of both subunits are folded in a \(\beta\)-barrel, which together form a contiguous \(\beta\)-structure along the top of the molecule (Fig 3A). Alignment of the A subunits by their N-terminal \(\beta\)-barrels

**Figure 3.** Catalytic A/B pairs and comparison of yeast and bacterial A\(_3\)B\(_3\)DF.

A The A\(_3\)B\(_3\)DF complex shown with the subunits unique to the eukaryotic \(V_1\) removed, highlighting the core conserved catalytic complex labeled as "catalytic core". The three peripheral stalks and inhibitory subunit H are found only in the eukaryotic \(V_1\), whereas A\(_3\)B\(_3\)DF represents the full complement of subunits in the prokaryotic \(V_1\) complex. Notably, the prokaryotic \(V_1\) is an active ATP hydrolase (Arai et al., 2009). The orientation of the A and B subunits with respect to the membrane is indicated by the arrow on the left.

B Conformational differences in the catalytic A subunits. The catalytic A subunits are shown aligned by their N-terminal \(\beta\)-barrels, highlighting the change in conformation of C-terminal \(\alpha\)-helical bundles associated with the closed (\(AB\)\(_2\) and \(AB\)\(_3\); dark and light gray, respectively) and open (\(AB\)_1; magenta) catalytic sites. Movement of this domain in response to nucleotide occupancy is thought to drive rotation of the central rotor during catalysis.

C \(ScV_1\)'s catalytic hexamer contains one open and two closed catalytic sites. Side view of the three catalytic hexamer A/B pairs (shown in surface representation). Note that the two closed pairs (\(AB\)\(_2\) and \(AB\)\(_3\)) are non-equivalently closed.

D Alignment of the A\(_3\)B\(_3\)DF from \(ScV_1\) and nucleotide-free \(EhV_1\) by the catalytic hexamers. The A\(_3\)B\(_3\)DF from \(ScV_1\) is shown in the same color scheme as in Fig 2, \(EhV_1\) is in blue. The alignment shows the difference in position of the catalytic hexamer subunit, D. Note that the C-terminus of subunit D \(D_{CT}\) is longer in eukaryotic V-ATPase (see electron density contoured at 1.2 \(\sigma\) in the box to the right).

E \(ScV_1\) and \(EhV_1\) are halted in different rotational positions. Alignment of the A\(_3\)B\(_3\)DF of \(ScV_1\) and nucleotide-free \(EhV_1\) (blue, with dashed lines drawn through the A subunits) by the central stalk subunit D, reveals that the two catalytic cores are halted in different rotational positions. The autoinhibited \(ScV_1\) requires 40° rotation to overlay its catalytic hexamer with that of the \(EhV_1\).

F Comparison of the D subunits of \(ScV_1\) (red) and nucleotide-free \(EhV_1\) (blue). H\(_CT\) from \(ScV_1\) is shown in yellow, and subunit F from \(EhV_1\) is shown in dark blue.
illustrates that their C-terminal domains are in different conformations (Fig 3B), consistent with autoinhibited ScV1 containing one open and two closed catalytic sites as is evident in side views of the three A/B pairs (Fig 3C). From here on, the three catalytic sites will be designated (AB)1-3, depending on which of the peripheral stalks EG1-3 is bound to its B subunit, and following this nomenclature, the open catalytic site is (AB)1 and the two closed ones (AB)2 and (AB)3 (Figs 3C and 2E and F). Note that of the two closed catalytic sites, (AB)2 appears more closed than (AB)3 (Fig 3B and C). The presence of two non-equivalently closed sites has been also observed in the EhV1 (Arai et al, 2013) and in the cryo-EM maps of holo eukaryotic V-ATPase (Zhao et al, 2015) (see Discussion section below for more detail).

The central rotor subunit D is an elongated coiled-coil folded as a hairpin, with the N-terminus (DNT) located within and the longer C-terminus (DCT) passing entirely through the A3B3 hexamer (Fig 3A). The ~90-Å-long DNT helix penetrates the hexamer up to the level of the phosphate binding or P-loops that are involved in nucleotide binding (Fig 3A). The ~143-Å-long DCT protrudes from the top of the hexamer by ~10 Å, a feature unique to the eukaryotic enzyme (highlighted by the box in Fig 3D). Subunit F is bound to D below the catalytic hexamer via a conserved C-terminal α-helix (FCT; Fig 3A) in a manner similar to the prokaryotic enzyme (Arai et al, 2013). While density for the FCT α-helix was observed in both of the ScV1 structures solved, only the higher resolution structure allowed modeling of the F subunit N-terminal domain, suggesting some flexibility or mobility of the subunit.

Comparing the crystal structures of yeast (ScV1) and bacterial (EhV1) catalytic cores reveals considerable differences regarding the position of the D subunit (Fig 3D). Aligning the structure of autoinhibited ScV1 to available structures of nucleotide-free and AMP-PNP-bound EhV1 (Arai et al, 2013) using the D subunit as reference illustrates that the eukaryotic V1 is halted in a different rotational position (Fig 3E). The largest angular difference observed (40°) was to nucleotide-free EhV1, a noteworthy observation as nucleotide-free EhaA8 was used as MR search model to solve the structure of ScV1. Besides the difference in rotational position of the D subunit in ScV1 compared to EhV1, the base of the central rotor is bent in the direction of the interaction between the FCT and a B subunit from the hexamer in E. hirae, whereas in ScV1, the base of the central rotor appears to be more straight and shifted toward the C-terminal domain of the H subunit (Fig 3F). Interestingly, a more straight central rotor is also seen in the ADP-bound catalytic core from the T. thermophilaus A/V-ATPase (TtV1; Appendix Fig S4) (Nagamatsu et al, 2013), suggesting that the conformation of the DF subcomplex may be influenced by the nucleotide occupancy of the two closed catalytic sites of the complex.

Subunit H inhibitory interactions

Subunit H was the first component of eukaryotic V-ATPase whose structure was solved by X-ray crystallography (Sagermann et al, 2001). Subunit H is a two-domain polypeptide with a larger N-terminal domain composed of α-helical HEAT repeats (HNT, residues 1–351) connected to a smaller globular α-helical C-terminal domain (HCT, 356–478) by a flexible linker (Fig 4A). While the NCS-averaged and density-modified MR map showed clear electron density for HNT and HCT (Fig EV1B), the two domains of H had to be modeled separately because of the considerable conformational change required for HCT to assume its inhibitory position in ScV1 (discussed in “Comparison of autoinhibited ScV1 and ScV1Vo” below). The electron density for subunit H is of particularly high quality, showing HCT bound to peripheral stalk EG1 and HCT in contact with the base of the catalytic core (Fig 4A). The interaction between HCT and EG1 is largely mediated by two α-helical segments in HCT (α-14 and α-17, comprising residues 237–255 and 284–296) and a short α-helical stretch in subunit E (residues 26–44) (Fig 4B). The contacts between HCT and the catalytic core are mediated by a loop in HCT (residues 408–414) and two short segments in the C-terminal domain of the B subunit of the open catalytic site (AB)1 (residues around Ile427 and Glu471) together with two α-helical turns in DNT (residues 38–45) (Fig 4C). During catalysis, conformational changes in the catalytic hexamer (Fig 3B and C) drive rotation of the central DF rotor (Movie EV2). As the D subunit forms part of the rotor, the site on DNT for the HCT interaction would only be available for binding in this rotational position (Fig 4D). Further, the site on the B subunit for the HCT interaction is only exposed and available for binding in the open conformation of the (AB)1 pair (Movie EV2). From its inhibitory interactions, it appears that part of the mechanism of HCT inhibition is to stabilize the open conformation of the (AB)1 pair specifically, which would have the additional effect of stabilizing the adjacent closed conformations of the (AB)2 and (AB)3 catalytic sites due to the highly cooperative nature of the catalytic sites in rotary ATPases. As we are able to resolve this interaction in the crystal structure, this must be a specific stopping point associated with inhibition of the enzyme.

Mutational analysis of HCT

The inhibitory interactions between subunit H and the catalytic core are mediated by a loop in HCT (Fig 4C), and surprisingly, while the overall structure and mechanism of eukaryotic V-ATPase, including its unique mode of regulation, are highly conserved, the loop in HCT that is seen in contact with subunits B and D, is not (Fig 5A). To test the possible role of the loop in activity silencing, we performed mutagenesis experiments together with ATPase activity and growth complementation assays. A schematic representation of the mutations is shown in Fig 5B along with a structural alignment of the loop region from autoinhibited V1 to homology models of the human V-ATPase H subunit (HsH) and the Hloop mutant. While the loop seen in ScH is not present in HsH, it had been shown previously that overexpression of HsH complemented deletion of H in yeast (Lu et al, 1998). However, we found that expression in yeast of the two splice variants of HsH (HsH1 and HsH2) from a low-copy plasmid failed to complement the deletion Vma− phenotype likely due to reduced affinity of HsH for ScV1 (Fig 5C). In separate experiments, however, recombinant HsH splice variants were able to bind ScV1 isolated from a yeast strain deleted for subunit H (ScV1ΔH) in pulldown assays (Appendix Fig S5A and B), but subsequent activity measurements to test whether HsH isoforms inhibit ScV1ΔH’s MgATPase activity were inconclusive.

Experiments in yeast had shown that while HCT expressed from a plasmid is co-purified with ScV1, HCT is not (Diab et al, 2009), indicating that the majority of the binding energy between ScV1 and
H is provided by the interaction between HNT and EG1. We therefore generated a chimeric H subunit construct containing ScH NT and HsH CT (HChim) to test whether HsH CT can silence MgATPase activity of ScV 1 (Fig 5B). Interestingly, while H Chim complemented the ScH deletion phenotype (Fig 5C), the chimeric subunit failed to silence MgATPase activity in the purified ScV 1 sector (Fig 5D and E). This suggests that the region of H CT that is involved in energy coupling in the holoenzyme is distinct from the portion that functions in activity silencing in isolated V1.

Sequence comparison between ScH and HsH revealed that while overall identity is only 19%, conservation in the C-terminal domains is significantly higher (30% identity, 52% similarity). Homology modeling of HsH using ScH as a template predicted that the loop seen in contact with B and D subunits in ScV 1 is shorter by three residues and the flanking α-helices are shortened about one turn (Fig 5B, right panel and Appendix Fig S5C). To rule out that regions other than the inhibitory loop are playing a role in activity silencing in ScV 1, the α-helices flanking the loop in ScH were each shortened by one turn (Δ405–408, Δ413–418) to match the local shape of the loop in HsH CT (Fig 5B). While this loop mutant of ScH (ScH Loop) complemented the growth phenotype of the subunit H deletion (Fig 5C), it did not silence MgATPase activity, displaying a specific activity (~9.8 μmol/min/mg) at a level similar to ScV1,ΔH (~12 μmol/min/mg) (Fig 5D). This finding agrees with the results from the chimeric H subunit above and is in line with the H CT loop being important for silencing in ScV 1. In addition, the loop sequence in yeast H CT contains a single aspartic acid residue that is present at an equivalent position in all other organisms analyzed (Fig 5A and B). Mutation of this aspartic residue to an alanine (D410A) complemented the deletion phenotype in yeast (Fig 5C) but had little effect on activity silencing when compared to the wild-type H subunit expressed from a plasmid (Fig 5D). Together, these data indicate that the non-conserved loop is essential for silencing isolated ScV1, that mutagenesis of the only conserved residue in this region is not sufficient to negate function, and that the dual function of subunit H in activity silencing and energy coupling involves distinct regions in ScHCT.

**Comparison of autoinhibited ScV 1 to holo ScV 1V 6**

Comparison of the structure of the autoinhibited ScV 1 with the recently reported cryo-EM structures of ScV 1V 6 in three different

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**Figure 4. Inhibitory contacts of subunit H.**

A ScV 1–H subunit interactions. Region of electron density (contoured at 1.2 σ) outlined by the box in the side view of ScV 1 shown to the right. The H subunit N-terminal domain (HNT) is bound to the N-terminal α-helices of peripheral stalk EG 1 while its globular C-terminal domain (HCT) is bound at the base of the catalytic hexamer.

B Interaction between HNT and peripheral stalk EG 1. View of HNT as in panel (A) rotated 110° along the horizontal, showing the α-helical HEAT repeats of HNT forming a pocket for binding the N-termini of peripheral stalk EG 1. HNT’s α-helices forming the pocket are labeled as in Sagermann et al (2001). Pro197 on HNT (red sphere) has been previously shown to cross-link subunit E (Jefferies & Forgac, 2008).

C Interaction between HCT and the base of the catalytic hexamer. The H subunit inhibitory contacts at the base of the ScV 1 are mediated by a loop in H CT (highlighted in magenta) that is seen to contact two short segments of the C-terminal domain of the subunit B from the open catalytic site (AB)1 (highlighted in purple) and two turns of the N-terminal α-helix of the central rotor subunit D (highlighted in blue).

D HCT binds to the open (AB)1 pair. View of ScV 1 looking along the central rotor subunit D toward the cytoplasm, showing the position of the H subunit binding at the interface of the open A/B pair (AB)1. The catalytic hexamer is shown in surface representation, and the central rotor, peripheral stalks, and subunit H are shown as ribbons.
states (Zhao et al., 2015) revealed that autoinhibited ScV1 most closely matches the conformation of the ATPase sector of the “state 2” conformation (Appendix Table S3). However, alignment of the two structures revealed significant conformational differences in all V1 subunits except the A3B3 hexamer (Fig 6 and Movie EV3). While the C-terminal domains of the peripheral stalks are largely invariant, their N-terminal domains are in different conformations in ScV1 and ScV1Vo (Fig 6A–B and Movie EV3). Interestingly, this change in HCT conformation is accompanied by a repositioning of subunit D to accommodate HCT (Fig 6E). The most dramatic change is seen in the position of H CT, rotating ~150° from a position in V1Vo where its C-terminal α-helix is in contact with ACT to its position at the bottom of the catalytic hexamer in free ScV1 as described above (Fig 6C and D; see also Fig 2D–F).

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ScV\textsubscript{1}Vo and in ScV\textsubscript{1} (Fig 6F). Based on this comparison, H\textsubscript{CT} undergoes a 180° rotation from its position in isolated H to that in the autoinhibitory conformation in ScV\textsubscript{1}. The alignment further suggests that H\textsubscript{CT} can adopt multiple low-energy conformations and that its flexibility is required for the inhibitory function in ScV\textsubscript{1} and for coupling ATP hydrolysis with proton transport in ScV\textsubscript{1}Vo.

**Discussion**

**Activity silencing in ScV\textsubscript{1}**

Previous studies have shown that subunit H, specifically H\textsubscript{CT}, is essential for both inhibition of MgATPase activity in free ScV\textsubscript{1} (Parra et al., 2000; Diab et al., 2009) and efficient coupling of ATP
hydrolysis with proton pumping in holo ScV₁,V₀ (Ho et al, 1993; Liu et al, 2005; Flannery & Stevens, 2008). Exactly how Hₐₜ performs these dual functions, however, was not understood. In EM reconstructions of holo V-ATPase (Zhang et al, 2008; Zhao et al, 2015), Hₐₜ is seen in contact with aₜ and whereas in the crystal structure of membrane-detached ScV₁ presented here, Hₐₜ binds (and thereby links) the B subunit of the open catalytic site and the central rotor subunit D. Remarkably, to transition from its activating position in ScV₁,V₀ to its inhibitory position in ScV₁, Hₐₜ must undergo a ~150° rotation around the linker connecting Hₐₜ and Hₜₐ (Movie EV2). It has been shown that when the flexibility of the linker is impaired, subunit H’s ability to inhibit MgATPase activity of ScV₁,ΔH is significantly reduced (Benlekbir et al, 2012), indicating that Hₐₜ’s interaction at the bottom of V₁ is indeed responsible for activity silencing. Photochemical cross-linking on the other hand suggested that activity silencing maybe due to a direct interaction of Hₐₜ with the rotor subunit F (Jefferies & Forgac, 2008); however, for reasons that are unclear, this interaction is not observed in the structure of autoinhibited ScV₁ presented here.

From kinetic profiles of Mg or CaATPase activity assays of ScV₁,ΔH or ScV₁, respectively, it has been proposed that ADP inhibition may contribute to activity silencing independent of subunit H (Parra et al, 2000). Interestingly, while ScV₁ purified using ammonium sulfate precipitation and ion exchange chromatography contained no bound nucleotide (Parra et al, 2000), the ScV₁ as purified here by affinity chromatography contains ~1.3 mol/mol ADP. ScV₁,ΔH on the other hand retained only ~0.4 mol/mol of the nucleotide, indicating that subunit H, by stabilizing an open catalytic site near EG₁, leads to increased affinity for inhibitory ADP at another site, which in turn results in activity silencing. Since there are two non-equivalently closed catalytic sites in ScV₁ (Fig 3B and C), it is likely that one of these sites is completely filled and the other partially occupied with nucleotide. The presence of strong positive difference density near the P-loop in (AB)2 indicates that the inhibitory nucleotide is bound in the closed catalytic site near EG₂ and while we cannot assign the identity of the nucleotide at this resolution, our biochemical measurements indicate that it is likely to be ADP (Appendix Fig S6). Remarkably, the N-terminal domain of EG₃ is seen to wrap around the C-terminal domain of the A subunit of (AB)2 and it is possible that this interaction, seen only in membrane-detached V₁, provides yet another mechanism to stabilize the closed conformation of (AB)2 for efficient trapping of inhibitory ADP. Taken together, the data suggest that activity silencing is not caused by a single high-affinity protein–protein interaction, but is rather the result of several interactions, which must be readily reversible to allow for efficient enzyme reassembly. Interestingly, inhibition of ScV₁ by ScH appears to be mediated by a loop in Hₐₜ that is not conserved in other species, and while a chimera of yeast HNT and human Hₐₜ is able to complement the Vma⁻ phenotype of a subunit H deletion, the human Hₐₜ does not function in silencing ScV₁’s MgATPase activity. Whether mammalian subunit H plays a role in activity silencing similar to yeast H remains to be determined.

Comparison of ScV₁ and ScV₁,V₀ and implications for the mechanism of reversible dissociation

Previous studies have shown that enzyme activity is required for regulated dissociation but not for reassembly or V-ATPase biogenesis (Parra & Kane, 1998). As this has been shown to be the case even in enzymes that can bind nucleotide, but not hydrolyze it, it has been proposed that the V-ATPase must adopt a specific conformation during rotary catalysis that favors enzyme dissociation (Muench et al, 2011). A comparison with the recent cryo-EM structures of ScV₁,V₀ (Zhao et al, 2015) showed that auto-inhibited ScV₁ resembles the V₁ conformation of state 2 (Appendix Table S3) with its open catalytic site and Dₕₜ near EG₁ and H₤ₐ. State 2 must therefore represent the conformation where V₁ can dissociate from V₀. The comparison also reveals the distinct binding sites on Hₐₜ for aₜ (in ScV₁,V₀) and B/NT (in ScV₁), each mediating, as mentioned above, a different function: energy coupling in ScV₁,V₀ and silencing in ScV₁, consistent with the biochemical assays presented here involving chimeric and loop mutant H subunits. Besides the different and opposing function mediated by each of these two distinct binding sites on Hₐₜ, they appear also to prevent both re-binding of V₁ to V₀ when dissociation is favored and prohibit inhibitory interactions from forming when turnover and assembly is required (Movie EV3). For example, it has been shown that ScV₁ does not bind aNT in vitro (Diab et al, 2009), a result that can be explained by the fact that the most C-terminal α-helix on Hₐₜ that binds aₜ in V₁,V₀ is hidden in V₁ due to the 150° rotation of Hₐₜ during disassembly. Unintended reassembly may also be prevented by the conformational change of peripheral stalk EG₃ that occurs as a result of enzyme disassembly. EG₃ is bound to Cload in ScV₁,V₀, but as subunit C is released into the cytoplasm upon V-ATPase dissociation, the bent conformation of EG₃ seen in ScV₁ may help to prevent rebinding of C, which is required for functional association of V₁ with V₀. Indeed, V₁ purified from yeast cytoplasm or insect midgut has variable levels of C subunit bound, ranging from non-detectable (Graf et al, 1996; Parra et al, 2000) to more significant amounts (Zhang et al, 2003; Diab et al, 2009; Hildenbrand et al, 2010), consistent with EG₃ adopting a conformation unfavorable for Cload binding. Together, the differences in conformations of ScV₁ versus ScV₁,V₀ not only allow silencing of ScV₁’s MgATPase activity but may also serve to prevent functional re-association of V₁ to V₀ when the disassembled state is required.

The structure presented here of ScV₁ along with the available structures of V₁,V₀ and biochemical data allow for a more detailed picture of the mechanism of regulation by reversible dissociation. In holo V₁,V₀, aNT serves as membrane anchor for V₁ subunits EG₁, Hₐₜ, EG₂, and Cload. We have previously shown that the ternary aNT-EG₂-Cload junction is formed by low-affinity interactions and we proposed that targeting of one of these interactions by an as yet unknown process would initiate enzyme disassembly (Oot & Wijlens, 2012b). Recently, our laboratory has shown that enzyme dissociation involves a large movement of aNT from its position near EG₂ and Cload in V₁,V₀ to a position near subunit d in free V₀ (Couso-Cardel et al, 2015). We speculate that once the signal is received targeting the low-affinity aNT-EG₂-Cload junction, aNT changes conformation to bind subunit d, thereby breaking the interaction with H and allowing Hₐₜ to rotate 150° to bind to and stabilize the open (AB)1 pair found in state 2 of ScV₁,V₀ (Movie EV4). As the dissociation signal may present itself to the enzyme in any of the three (or more) catalytic states of the enzyme, it seems likely that once state 2 is reached, the conformational...
changes associated with V1V0 dissociation and silencing of free V1 and V0 are more favorable than maintaining the holoenzyme. The above-mentioned requirement for ATP hydrolysis in dissociation may serve multiple purposes: (i) achieving state 2 in which (AB)1 and DNT are in proximity to subunit H, (ii) generating the torque needed to break remaining interactions with the membrane sector, and (iii) leaving inhibitory MgADP in catalytic site (AB)2. The model of enzyme regulation proposed here thus couples the dissociation event with activity silencing directly. As regulated disassembly is fully reversible, the enzyme must come together again in a rapid and efficient manner when its function is required. While still unresolved, the pathways involving re-association have been shown to be distinct from those involved in dissociation. The dramatic conformational changes in ScV1 and V0 subunits as a result of enzyme dissociation may explain the reported inefficiency of direct rebinding of ScV1 to the membrane in vitro, requiring extreme conditions such as low pH (Parra & Kane, 1996), and may account for the requirement of a dedicated chaperone known as the RAVE complex (Seol et al., 2001; Smardon et al., 2002) for reassembly in vivo. The exact function of the RAVE complex is currently unknown, but there is evidence that the chaperone displays several distinct binding sites for subunits C, EG, and DNT (Smardon et al., 2015), a subset of the very same subunits that change conformation during regulated enzyme disassembly as described above.

In conclusion, eukaryotic V-ATPase is a dynamic molecular machine. The structural model of ScV1, presented here highlights the structural changes occurring in the V1-ATPase sector as a result of activity silencing and reversible disassembly. Ablation V-ATPase activity has been recognized as being responsible for, or associated with many devastating human conditions. Activity silencing and the mechanism of reversible dissociation may thus offer a way to modulate enzyme activity by, for example, the design of molecules that either promote or inhibit the process. These kinds of studies would benefit from higher resolution information, efforts that are ongoing in our laboratory.

Materials and Methods

Protein purification

ScV1 used for crystallization was affinity purified from a yeast strain deleted for the genes encoding subunits C (VMA5) and G (VMA10) (vma5Δ::Nat, vma10Δ::URA3) and transformed with a pRS315 vector containing N-terminally FLAG tagged G subunit (Zhang et al., 2003). ScV1AH and ScV1 containing subunit H constructs expressed from a pRS316 plasmid were affinity purified as above, also using a plasmidborne G subunit carrying an N-terminal FLAG tag. Human subunit H variants HsH1 and HsH2 were expressed in E. coli as N-terminal GST fusions. A detailed description of the ScV1 and subunit H purifications can be found in the Appendix Supplementary Methods.

Crystallogenesis, data collection, phasing, and refinement

Crystals for the 7-Å dataset were obtained in a microfluidic plate (Microlytics High Throughput Plate) in 9.5% PEG 8000, 150 mM (NH4)2SO4, 100 mM HEPES pH 7.5 and 12.5 mM MgCl2 using ~2 weeks and grew to maximum dimensions of 0.3 x 0.3 x 0.1 mm. Crystals for the 6.2–6.3 Å dataset were obtained using the hanging drop vapor diffusion setup at 10 mg/ml ScV1 in 8.25% PEG 8000, 250 mM (NH4)2SO4, 100 mM HEPES pH 7.5, and 50 mM SrCl2. Since diffraction of the slightly better crystals (visible spots to 6.2 Å) was anisotropic, the dataset was submitted to the anisotropy server at UCLA (Strong et al., 2006) and elliptically truncated to 6.5, 6.2, and 6.7 Å as suggested by the server. Completeness of the dataset after truncation is shown in Appendix Table S1. The structure from the 7 Å dataset was solved by molecular replacement using the A3B3 complex of nucleotide-free EbV1 (3vr5) (Arai et al., 2013). The resulting electron density map allowed modeling of the N- and C-terminal domains of subunit H (1ho8) (Sagermann et al., 2001) and the N- and C-terminal α-helices of subunit D (3vr5). Cycles of refinement, density modification, and NCS averaging allowed modeling of subunits E and G (4dll0) (Oot et al., 2012a). The refined model was then used to solve the structure of the 6.2–6.5 Å dataset. The resulting map allowed placement of the base of subunit D and subunit F (4rn9) (Balakrishna et al., 2015) and extension of the N-termini of peripheral stalks EG2 and EG3 (4dll0). For both structures, side chains were not modeled due to the limited resolution of the data. Details of the structure determination and refinement can be found in the Appendix Supplementary Methods. A stereo representation of the final electron density map is shown in Appendix Fig S2.

H subunit constructs and site-directed mutagenesis

A yeast strain deleted for the genes encoding subunits H (VMA13) and G (VMA10) (vma13Δ::KanMX, vma10Δ::Nat) (Diab et al., 2009) was transformed with a pRS315 plasmid containing N-terminally FLAG tagged G subunit and a pRS316 plasmid containing untagged human H isoforms (HsH1, HsH2), myc-tagged chimeric H subunit (Hchim), helix truncated yeast H (Hloop) as well as D410A (HΔ410A) mutants. Transformants were selected on double dropout (SD –Ura, –Leu) medium and tested for complementation of the Vma– phenotype by growth at pH 7.5 in the presence of 60 mM Ca2++. A detailed description of the yeast and human subunit H constructs can be found in the Appendix Supplementary Methods.

ATPase activity assays and endogenous nucleotide content

ATPase activity of the purified ScV1 sectors was measured in an ATP regenerating assay as described (Zhang et al., 2003; Diab et al., 2009). Endogenous nucleotide content of ScV1 and ScV1AH were measured using the ATP Bioluminescence Assay Kit CLSII (Roche) on a BioTek Synergy HT Luminometer. ATPase and nucleotide content measurements are described in detail in the Appendix Supplementary Methods.

Other methods

Figures and movies were generated in UCSF Chimera (Yang et al., 2012).
Accession codes

Protein Data Bank: Structure factors and atomic coordinates for the 7 and 6.2–6.5 Å resolution ScV1 structures have been deposited with accession codes 5BW9 and 5D80, respectively.

Expanded View for this article is available online.

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Author contributions

RAO and SW collected/processed the diffraction data, solved, and refined the structure and wrote the manuscript. RAO purified and optimized crystallization of V12, made the H subunit mutant constructs, performed the ATPase and nucleotide assays. PMK generated the subunit G, subunit C deletion yeast strain used for ScV1 purification, provided the V12ΔH strain and the pRS316-mycVMA13 plasmid in which the H subunit mutations were constructed. EAB provided technical advice with data analysis and structure refinement.

Conflict of interest

The authors declare that they have no conflict of interest.

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