Crystal structure of yeast V₁-ATPase in the autoinhibited state

Rebecca A Oot, Patricia M Kane, Edward A Berry & Stephan Wilkens*

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, V₁, is released from the membrane and its MgATPase activity silenced. The crystal structure of yeast V₁ 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 holo V-ATPase to a position at the bottom of V₁ 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; V₁-ATPase; vacuolar ATPase; X-ray crystallography

Subject Categories Membrane & Intracellular Transport; Structural Biology

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Introduction

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 (Forgac, 2007). V-ATPase function is essential for pH and ion homeostasis, protein trafficking, endocytosis, 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 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 & Manolison, 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 V₁-ATPase (A₁B₁C₁D₁E₁F₁G₁H₁) (Kitagawa et al., 2008) and the ~330-kDa membrane integral V₀ proton channel (a₁c₁c₉δ₁) (Powell et al., 2000; Zhao et al., 2015). ATP hydrolysis on the catalytic V₁ is coupled with proton pumping across the V₀ via a central rotor made of V₁ and V₀ subunits DFₑc₉c₉d₁. A peripheral stator complex composed of V₁ subunits E,G,H,C serves to stabilize the motor by binding to the N-terminal cytosolic domain (a₁c₁) of the membrane-anchored subunit of the V₀ (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 V₁ and membrane-bound V₀ (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, V₁ loses the ability to hydrolyze MgATP (Graf et al., 1996; Parra et al., 2000) and the V₀ 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 Vo (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 V1Vo 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. Crystals 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

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**Figure 1. Schematic of the bacterial and eukaryotic V-ATPases.**

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 (Vo). 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.
Activity silencing in yeast V₁-ATPase

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(NH₄)₂SO₄, 100 mM HEPES, pH 7.5, 50 mM SrCl₂), resulting in cion quality of the corresponding electron density. Crystallization N-termini of EG2 and EG3 that were not modeled due to insufficient crystal structures for subunits D,E,G, and H followed by search model (D) (Fig EV1B). Iterative manual placement of available crystal structures for subunits D,F, the base of subunit D and subunit H, which is unique to the eukaryotic 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 A₂B₃DF catalytic core is highly conserved between enzymes from prokaryotes to humans 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 (EGChead, representative of EG3) in two 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-terminals (EGCₜ sostin) 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 (Aₜ sostin) (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 (Hₜ sostin) while its C-terminus (Hₜ sostin) 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 A₂B₃DF 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 Vₐ in response to cellular signals, while keeping its inhibitory subunit bound via one of the peripheral stalks.

Table 1. Data collection and refinement statistics (molecular replacement).

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<td>39.72–6.211 (6.432–6.211)</td>
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⁴Two crystals.

ᵇValues in parentheses are for highest resolution shell.

³l/σl in the highest resolution shell after elliptical truncation = 1.51.

⁴See Appendix Table S1.

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 EG2 and EG3 that were not modeled due to insufficient quality of the corresponding electron density. Crystallization conditions were then further refined (8.25% PEG 8000, 250 mM (NH₄)₂SO₄, 100 mM HEPES, pH 7.5, 50 mM SrCl₂), resulting in slightly larger crystals that diffracted X-rays anisotropically to ~6.2 Å (Table 1). Diffraction data from two crystals were merged and subjected to elliptic truncation with resolution limits of 6.5, 6.2, and 6.7 Å (Appendix Table S1). This higher resolution (hereafter referred to 6.2–6.5 Å) dataset was used in MR employing the 7 Å structure as search model. The resulting electron density was of higher quality compared to the 7 Å data and allowed for modeling of subunit F, the base of subunit D and extension of 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
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 EG₁_

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,B₃ 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 (AB)₁−₃ according to which of the three peripheral stalks is bound to each B subunit. For example, (AB)₁ 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 (Aₘ) of the adjacent A/B pairs. While EG₁ and EG₂ are now near Aₘ from (AB)₁-3 and (AB)₂, respectively (see double headed arrows), only the bent EG₃ peripheral stalk is close enough to contact the corresponding Aₘ from (AB)₂ (see asterisk).
Catalytic hexamer and rotary shaft

As mentioned above, the catalytic core (A₃B₃DF) of V-type rotary ATPases is highly conserved, with primary sequence identities between bacterial, yeast, and mammalian A and B subunits of ~50–80%, respectively (Muench et al., 2011). Not surprisingly therefore, the structure of the ScV₁ catalytic core is overall very similar to its bacterial counterpart from *E. hirae* (EhV₁) with an rmsd of 2.3 Å. As in EhV₁, the ScV₁ 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 β-barrel, which together form a contiguous β-structure along the top of the molecule (Fig 3A). Alignment of the A subunits by their N-terminal β-barrels

![Diagram](https://example.com/diagram.png)

**Figure 3.** Catalytic A/B pairs and comparison of yeast and bacterial A₃B₃DF.
A The A₃B₃DF complex shown with the subunits unique to the eukaryotic V₁ 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 V₁, whereas A₃B₃DF represents the full complement of subunits in the prokaryotic V₁ complex. Notably, the prokaryotic V₁ 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 β-barrels, highlighting the change in conformation of C-terminal α-helical bundles associated with the closed ([AB]₁ and [AB]₂; dark and light gray, respectively) and open ([AB]₃; magenta) catalytic sites. Movement of this domain in response to nucleotide occupancy is thought to drive rotation of the central rotor during catalysis.
C ScV₁’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]₁ and [AB]₃) are non-equivalently closed.
D Alignment of the A₃B₃DF from ScV₁ and nucleotide-free EhV₁ by the catalytic hexamer. The A₃B₃DF from ScV₁ is shown in the same color scheme as in Fig 2, EhV₁ is in blue. The alignment shows the difference in position of the catalytic rotor subunit, D. Note that the C-terminus of subunit D (Dₘ) is longer in eukaryotic V₁-ATPase (see electron density contoured at 1.2σ in the box to the right).
E ScV₁ and EhV₁ are halted in different rotational positions. Alignment of the A₃B₃DF of ScV₁ and nucleotide-free EhV₁ (blue, with dashed lines drawn through the A subunit) by the central stalk subunit D, reveals that the two catalytic cores are halted in different rotational positions. The autoinhibited ScV₁ requires 40° rotation to overlay its catalytic hexamer with that of the EhV₁.
F Comparison of the D subunits of ScV₁ (red) and nucleotide-free EhV₁ (blue). HCT from ScV₁ is shown in yellow, and subunit F from EhV₁ is shown in dark blue.

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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 position of the D subunit (Fig 3D). Aligning the structure of auto-NCS-averaged and density-modified MR map showed clear electron density for subunit H in of particularly high quality, showing HCT bound to peripheral stalk EG1 and HNT in contact with the base of the catalytic core (Fig 4A). The interaction between HNT and EG1 is largely mediated by two α-helical segments in HNT (α14 and α17, comprising residues 237–255 and 284–296) and a short α-helical stretch in subunit E (residues 26–44) (Fig 4B). The contacts between HNT and the catalytic core are mediated by a loop in HNT (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 HNT interaction would only be available for binding in this rotational position (Fig 4D). Further, the site on the B subunit for the HNT 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 HNT 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 H_loop 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 HNT 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 ScHNT and HsHCT (HChim) to test whether HsHCT can silence MgATPase activity of ScV1 (Fig 5B). Interestingly, while HChim complemented the ScH deletion phenotype (Fig 5C), the chimeric subunit failed to silence MgATPase activity in the purified ScV1 sector (Fig 5D and E). This suggests that the region of HCT 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 ScV1 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 ScV1, 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 HsHCT (Fig 5B). While this loop mutant of ScH (ScHloop) 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 HCT loop being important for silencing in ScV1. In addition, the loop sequence in yeast HCT 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 ScV1 to holo ScV1,Vo

Comparison of the structure of the autoinhibited ScV1 with the recently reported cryo-EM structures of ScV1,Vo in three different

Figure 4. Inhibitory contacts of subunit H.

A ScV1–H subunit interactions. Region of electron density (contoured at 1.2 σ) outlined by the box in the side view of ScV1 shown to the right. The H subunit N-terminal domain (HNT) is bound to the N-terminal α-helices of peripheral stalk EG1 while its globular C-terminal domain (HCT) is bound at the base of the catalytic hexamer.

B Interaction between HNT and peripheral stalk EG1. 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 EG1. 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 ScV1 are mediated by a loop in HCT (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 ScV1 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.
Figure 5. Role of HCT in activity silencing.

A Alignment of the amino acid sequence of the yeast H subunit inhibitory loop region (shown in Fig 4C). The yeast (Sc) loop was aligned with those from insect (M. sexta), zebrafish (D. rerio), frog (X. trop.), mouse (Mus), bovine (B. taur), chimpanzee (P. trog), and human (Hum1, Hum2), illustrating the lack of sequence conservation in this region. Pink coloring denotes predicted α-helical secondary structure.

B H subunit mutations. Left panel, schematic representation of the H subunit mutations analyzed. Note that D410 is the only conserved residue in the inhibitory HCT loop. Right panel, overlay of the inhibitory HCT loop from yeast H (yellow) with homology models of the human H (HsH, purple) and yeast H subunit mutant Hmut (cyan).

C Growth phenotype of H subunit mutants. Deletion of V-ATPase subunits from yeast results in a conditional lethal phenotype (Vma1Δ). ATPase activity of H subunit mutants. The ScV1 sectors were purified from each strain that complemented the phenotype as well as from ScV1ΔH, for comparison of MgATPase activity. Average specific activities are shown plotted ± s.e.m. from two independent purifications. The ScV1 sector containing chromosomally encoded H subunit shows no detectable MgATPase activity (Parr et al, 2000; Zhang et al, 2003), whereas ScV1 containing plasmid-borne wild-type H (H WTpl) exhibits low levels of activity likely due to reduced expression from the low-copy plasmid. The HChim and HLoop mutants lead to a loss of activity silencing, displaying nearly the same activity as ScV1ΔH.

D SDS-PAGE and Western blot analysis of ScV1 preparations. Left panel, 15% SDS-PAGE of ScV1 purified from the subunit H mutant and WT strains. Note that H WT, is purified from a yeast strain deleted for the C subunit (used for crystallization). The remaining strains contain subunit C, which is co-purified with ScV1 in varying levels. Note that while H WT, ScV1, which is expressed from a strain deleted for subunit C, displays no detectable MgATPase activity, the H subunit mutants co-purified with the most C subunit display the least activity, suggesting that absence or presence of subunit C has no effect on MgATPase activity. Right panel, immunoblot probed with an antibody directed against the N-terminal Myc-tagged mutant H subunits expressed from a plasmid. Note that the levels of mutant H expression do not appear to vary from strain to strain.

E. SDS–PAGE and Western blot analysis of ScV1 preparations. Left panel, 15% SDS–PAGE of ScV1 purified from the subunit H mutant and WT strains. Note that H WT, is purified from a yeast strain deleted for the C subunit (used for crystallization). The remaining strains contain subunit C, which is co-purified with ScV1 in varying levels. Note that while H WT, ScV1, which is expressed from a strain deleted for subunit C, displays no detectable MgATPase activity, the H subunit mutants co-purified with the most C subunit display the least activity, suggesting that absence or presence of subunit C has no effect on MgATPase activity. Right panel, immunoblot probed with an antibody directed against the N-terminal Myc-tagged mutant H subunits expressed from a plasmid. Note that the levels of mutant H expression do not appear to vary from strain to strain.

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 the 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 ScV1V0 (Fig 6A–D), in line with their loss of binding partners upon enzyme disassembly (aCT for EG1 and EG2 and subunit C for EG2 and EG3). In ScV1, HNT and the N-terminal domain of EG1 move outwards towards the periphery of the V1 (Fig 6A and B, and Movie EV3). In addition, a striking change is seen in peripheral stalk EG3 that is bound to the C subunit in the assembled enzyme but binds to the C-terminal domain of an A subunit of closed catalytic site (AB)2 in ScV1 (Fig 6C and D; see also Fig 2D–F).

The most dramatic change is seen in the position of HCT, rotating ~150° from a position in V1V0 where its C-terminal α-helix is in contact with aNT to its position at the bottom of the catalytic hexamer in free ScV1, as described above (Fig 6C and E). Interestingly, this change in HCT conformation is accompanied by a repositioning of subunit D to accommodate HCT (Fig 6E). The apparent flexibility of HCT can be further illustrated upon comparison of the available structure of the isolated H subunit to that in...
ScV₁Vo and in ScV₁ (Fig 6F). Based on this comparison, H₂CT undergoes a 180° rotation from its position in isolated H to that in the autoinhibitory conformation in ScV₁. The alignment further suggests that H₂CT can adopt multiple low-energy conformations and that its flexibility is required for the inhibitory function in ScV₁ and for coupling ATP hydrolysis with proton transport in ScV₁Vo.

**Discussion**

**Activity silencing in ScV₁**

Previous studies have shown that subunit H, specifically H₂CT, is essential for both inhibition of MgATPase activity in free ScV₁ (Parra et al., 2000; Diab et al., 2009) and efficient coupling of ATP hydrolysis with proton transport.
hydrolysis with proton pumping in holo ScV1V0 (Ho et al, 1993; Liu et al., 2005; Flannery & Stevens, 2008). Exactly how H_CT performs these dual functions, however, was not understood. In EM reconstructions of holo V-ATPase (Zhang et al., 2008; Zhao et al., 2015), H_CT is seen in contact with a_NT whereas in the crystal structure of membrane-detached ScV1 presented here, H_CT 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 ScV1V0 to its inhibitory position in ScV1, H_CT must undergo a ∼150° rotation around the linker connecting H_CT and H_CT (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∆H is significantly reduced (Benlekib et al., 2012), indicating that H_CT’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 H_CT 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∆H 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 by affinity chromatography contains ~1.3 mol/mol ADP. ScV1∆H 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 Sch4 appears to be mediated by a loop in H_CT that is not conserved in other species, and while a chimera of yeast H_NT and human H_CT is able to complement the Vma− phenotype of a subunit H deletion, the human H_CT 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 ScV1V0 and implications for the mechanism of reversible disassociation**

Previous studies have shown that enzyme activity is required for regulated disassociation 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 disassociation (Muench et al., 2011). A comparison with the recent cryo-EM structures of ScV1V0 (Zhao et al., 2015) showed that autoinhibited ScV1 resembles the V1 conformation of state 2 (Appendix Table S3) with its open catalytic site and D_NT near EG1 and H_CT. State 2 must therefore represent the conformation where V1 can dissociate from V0. The comparison also reveals the distinct binding sites on H_CT for a_NT (in ScV1V0) and B/D_NT (in ScV1), each mediating, as mentioned above, a different function: energy coupling in ScV1V0 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 H_CT, they appear also to prevent both re-binding of V1 to V0 when disassociation 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 a_NT in vitro (Diab et al., 2009), a result that can be explained by the fact that the most C-terminal α-helix on H_CT that binds a_NT in V1V0 is hidden in V1 due to the 150° rotation of H_CT 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 ScV1V0, but as subunit C is released into the cytoplasm upon V-ATPase disassociation, 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 ScV1V0, 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 V1V0 and biochemical data allow for a more detailed picture of the mechanism of regulation by reversible disassociation. In holo V1V0, a_NT serves as membrane anchor for V1 subunits EG1, H_CT, EG2, and Cload. We have previously shown that the ternary a_NT-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 & Wilkens, 2012a). Recently, our laboratory has shown that enzyme disassociation involves a large movement of a_NT from its position near EG2 and Cload in V1V0, to a position near subunit d in free V0 (Cousin-Cardel et al., 2015). We speculate that once the signal is received targeting the low-affinity a_NT-EG2-Cload junction, a_NT changes conformation to bind subunit d, thereby breaking the interaction with H and allowing H_CT to rotate 150° to bind to and stabilize the open (AB)1 pair found in state 2 of ScV1V0 (Movie EV4). As the disassociation 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 $V_1V_0$ dissociation and silencing of free $V_1$ and $V_0$ 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 aNT (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. Ablative 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 × 0.1 × 0.1 mm. Crystals for the 6.2–6.5 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 $A_3B_3$ complex of nucleotide-free EhV1 (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 (4dl0) (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 (4rmd) (Balakrishna et al., 2015) and extension of the N-termini of peripheral stalks EG2 and EG3 (4dl0). 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 (Hd410A) 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 V$_{1}$, 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 V$_{1}$ΔH strain and the prSS316-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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