Functional reconstitution of vacuolar H\(^{+}\)-ATPase from V\(_{o}\) proton channel and mutant V\(_{1}\)-ATPase provides insight into the mechanism of reversible disassembly

Received for publication, January 16, 2019, and in revised form, February 10, 2019. Published, Papers in Press, February 21, 2019, DOI 10.1074/jbc.RA119.007577

Stuti Sharma\(^1\), Rebecca A. Oot, Md Murad Khan, and Stephan Wilkens\(^2\)

From the Department of Biochemistry & Molecular Biology, SUNY Upstate Medical University, Syracuse, New York 13210

Edited by Karen G. Fleming

The vacuolar H\(^{+}\)-ATPase (V-ATPase; V\(_{1}\)V\(_{o}\)-ATPase) is an ATP-dependent proton pump that acidifies subcellular compartments in all eukaryotic organisms. V-ATPase activity is regulated by reversible disassembly into autoinhibited V\(_{1}\)-ATPase and V\(_{o}\) proton channel subcomplexes, a process that is poorly understood on the molecular level. V-ATPase is a rotary motor, and recent structural analyses have revealed different rotary states for disassembled V\(_{1}\) and V\(_{o}\), a mismatch that is likely responsible for their inability to reconstitute into holo V-ATPase. Here, using the model organism Saccharomyces cerevisiae, we show that a key impediment for binding of V\(_{1}\) to V\(_{o}\) is the conformation of the inhibitory C-terminal domain of subunit H (H\(_{cT}\)). Using biolayer interferometry and biochemical analyses of purified mutant V\(_{1}\)-ATPase and V\(_{o}\) proton channel reconstituted into vacuolar lipid-containing nanodiscs, we further demonstrate that disruption of H\(_{cT}\)’s V\(_{1}\)-binding site facilitates assembly of a functionally coupled and stable V\(_{1}\)V\(_{o}\)-ATPase. Unlike WT, this mutant enzyme was resistant to MgATP hydrolysis-induced dissociation, further highlighting H\(_{cT}\)’s role in the mechanism of V-ATPase regulation. Our findings provide key insight into the molecular events underlying regulation of V-ATPase activity by reversible disassembly.

The vacuolar H\(^{+}\)-ATPase (V-ATPase; V\(_{1}\)V\(_{o}\)-ATPase) is an ATP-dependent proton pump found on the endomembrane system of all eukaryotic organisms. This multisubunit nano-motor acidifies subcellular compartments and, in certain specialized tissues, the extracellular space. V-ATPase is essential for vital cellular processes such as pH homeostasis, protein sorting, autophagy, endocytosis, mTOR, and Notch signaling, as well as bone remodeling, urine acidification, hormone secretion, and neurotransmitter release (1). Although complete loss of V-ATPase function is embryonic lethal in mammals, aberrant activity has been associated with widespread human diseases including renal tubular acidosis (2), osteoporosis (3), neurodegeneration (4), diabetes (5), male infertility (6), and cancer (7), making V-ATPase a potential drug target (8, 9). However, because of its essential nature, global inhibition of V-ATPase is not a therapeutic option. Instead, there is a need for targeted modulation of the enzyme’s activity, a goal that requires a detailed understanding of V-ATPase’s catalytic and regulatory mechanisms.

V-ATPase is composed of two subcomplexes, a cytosolic ATPase called V\(_{1}\), and a membrane integral proton channel termed V\(_{o}\) (Fig. 1A). In yeast, the subunit compositions for the V\(_{1}\) and V\(_{o}\) are A\(_{3}\)B\(_{3}\)(C)DE\(_{3}\)FG\(_{3}\)H and ac\(_{8}\)c\(_{8}\)def, respectively (10). The A and B subunits of V\(_{1}\) are arranged in a hexamer (A\(_{3}\)B\(_{3}\)), with three catalytic sites at alternating AB interfaces. Located within the hexamer and extending from it in the direction of the membrane is subunit D that, together with F, provides the functional link between V\(_{1}\) and V\(_{o}\). The V\(_{o}\) is constituted by subunit a that can be divided into cytosolic N-terminal and membrane-integral C-terminal domains (a\(_{NT}\) and a\(_{CT}\)), the c, c\(_{8}\) and c\(_{8}\) subunits (“proteolipids”) that form a ring (c-ring), and subunit d that connects the c-ring with V\(_{1}\) subunits D and F. V\(_{1}\) and V\(_{o}\) are held together by three heterodimers of subunits E and G (peripheral stalks EG1–3) that link the catalytic hexamer and the single copy C and H subunits to the membrane integral a subunit by binding to a\(_{NT}\).

V-ATPase is a rotary motor enzyme and employs a catalytic mechanism that is shared with the F-, A-, and A/V-type ATPases (11). In V-ATPase, ATP hydrolysis–driven rotation of the DF\(_{c}\)-ring central rotor is coupled to proton translocation at the interface of a\(_{CT}\) and the c-ring. During catalysis, the three peripheral stalks, in conjunction with C, H, and a\(_{NT}\), resist the rotary torque to keep the A\(_{3}\)B\(_{3}\) hexamer static against a\(_{CT}\) for efficient energy coupling. However, unlike F-, A-, and A/V-type enzymes, eukaryotic V-ATPase is regulated by a unique mechanism referred to as “reversible disassembly,” wherein V\(_{1}\) detaches from V\(_{o}\) in response to e.g. nutrient shortage (12–14) (Fig. 1B), with concomitant silencing of V\(_{1}\)’s MgATPase (15, 16), and V\(_{o}\)’s proton transport activities (17, 18). First described in yeast and insect, reversible disassembly is now emerging as an important and conserved regulatory mechanism, having...
been observed in mammalian systems as well (19–21). However, although we have some understanding of the process at the cellular level (22), less is known about the mechanism of reversible disassembly at the molecular scale. Biochemical studies in yeast have shown that the single copy C and H subunits, which are unique to eukaryotic V-ATPase, play key roles in enzyme regulation. Both C and H are two-domain proteins, with C composed of “foot” (Cfoot) and “head” (Chead), and H of N-terminal (HNT) and C-terminal (HCT) domains. H has a dual role because it is required for both coupling MgATPase to proton-pumping activities and for stabilizing the autoinhibited state of membrane detached V1; C functions to stabilize the V1–Vo interface in the holo enzyme but dissociates from the complex upon regulated disassembly. Recent structural studies have revealed that in assembled V1Vo, HCT is bound to aNT (23), an interaction required for energy coupling (24) (Fig. 1A). Upon disassembly of V1 from Vo, HCT undergoes a 150° rotation to wedge an inhibitory loop between the B subunit of an open catalytic site and subunits DF of the central rotor (16) (Fig. S1, A–D, red spheres). At the same time, aNT moves from its peripheral position near Cfoot and EG in V1Vo (Fig. 1A) toward a more central position in free Vo to bind subunit d (18, 25–27) (Fig. S1, E–G). Moreover, cryoEM models of three distinct rotary states of holo V-ATPase (states 1–3) (23), along with the structures of autoinhibited V1 (16) and Vo (25, 27), revealed that although V1 is halted in state 2, Vo adopted state 3 (10) (Fig. S1). We hypothesized that the mismatch of rotational states observed in autoinhibited V1 and Vo together with the large conformational changes of HCT and aNT that accompany enzyme dissociation, explain why V1 does not readily rebind free Vo under physiological conditions in vitro (10, 28), a safety mechanism that likely evolved to prevent spontaneous reassembly in vivo when the disassembled, inactive state is required.

We recently introduced biolayer interferometry (BLI) of purified V-ATPase in biotinylated and native lipid containing nanodiscs to analyze MgATP-dependent enzyme dissociation kinetics (29). Here, we have expanded on this approach to probe the interaction of V1 and Vo. Vo sector was reconstituted into vacuolar lipid containing nanodiscs (VoND) and immobilized on BLI sensors to screen V1 mutants for their ability to bind Vo. In line with available literature (28), WT V1 did not bind to VoND, presumably because of the “state mismatch” observed in the structures of free V1 and Vo (10). Previously, we generated a chimeric H subunit containing the yeast N-terminal and human C-terminal domains (Hchim) that does not inhibit free V1 because human HCT lacks an inhibitory loop that links an open catalytic site and the central rotor (16), and consequently, V1 containing Hchim is not restrained in any particular rotational state. We show that replacement of endogenous H in yeast V1 with Hchim (V1Hchim) (16) permits binding to VoND, and formation of a coupled holo V-ATPase (V1HchimVoND) with catalytic properties similar to the ones of the recently characterized WT V1VoND (29). However, V1HchimVoND was more resistant to ATP hydrolysis-induced disassembly compared with WT, highlighting the importance of HCT’s conformational switch in driving V-ATPase disassembly. The in vitro data presented here thus provide key insight into the molecular steps that accompany V-ATPase regulation by reversible disassembly.

**Results**

**Purification and characterization of native lipid nanodisc reconstituted Vo (VoND) and V1 mutants**

Vo was extracted from yeast vacuoles using the “reconstitution before purification” strategy as described for V1Vo (29) (Fig. 1C, panels i–iii). The resultant VoND complex consisted of Vo embed-
ded in endogenous vacuolar lipid containing nanodiscs encircled by biotinylated membrane scaffold protein (MSP; Fig. 1C, panel iii). The purified complex was monodisperse and contained all Vᵦ subunits plus MSP based on gel filtration and SDS-PAGE (Fig. 1, D and E). Examination of purified VᵦND using negative stain EM showed single particles of VᵦND with the typical size and appearance as described previously (26) (Fig. 1F).

Four different Vᵦ mutants were tested for their ability to bind VᵦND and form coupled holo V-ATPase (Fig. 2A): WT Vᵦ (containing subunits A₃B₃DE₃FG₃H), Vᵦ purified from a yeast strain deleted for subunit H (VᵦΔH), VᵦΔH reconstituted with recombinantly expressed WT H (VᵦHwt), and VᵦΔH reconstituted with chimeric H (VᵦHchim) (16). Although WT Vᵦ has no measurable MgATPase activity (Fig. 2B, dark green trace), VᵦΔH had an initial specific activity of 15.7 ± 1.7 units/mg, consistent with previous reports (16, 30) (Fig. 2B and C, blue trace and bar). Although MgATPase activity is measured in an ATP-regenerating system, the activity of VᵦΔH decreases over time as MgADP gets trapped in a closed catalytic site, leading to the MgADP-inhibited state (15). Although VᵦHwt has identical subunit composition to WT Vᵦ, VᵦHwt distinguishes itself from WT by having only ~0.4 instead of 1.3 mol/mol ADP in catalytic sites (16), and the complex therefore exhibits an initial MgATPase activity of ~4.95 ± 0.55 units/mg before becoming MgADP-inhibited (Fig. 2B and C, light green). VᵦΔH reconstituted with Hchim to yield VᵦHchim (Fig. 2, D–F) showed an initial MgATPase activity of 8.2 ± 0.4 units/mg that, as for the other Vᵦ mutants, declined over time because of MgADP inhibition (Fig. 2B and C, pink).

**Binding of C subunit to Vᵦ**

Experiments in yeast have shown that upon deletion of the gene encoding the C subunit, Vᵦ does not stably/functionally
associate with V_o. Moreover, biochemical analysis revealed that C_head binds isolated EG heterodimer with moderate affinity, whereas both C_foot and EG bind α_N weakly (31, 32). From these data we concluded that V_1 and V_o are held together by multiple weak interactions, resulting in an overall high-avidity interface, and that destabilization of one of these interactions by a cellular response to starvation would result in enzyme dissociation (10, 32). More recently we showed that although H_NT binds isolated EG with a K_d of ~0.2 μM, the affinity of the interaction is increased 40-fold when EG is part of V_1 (30). Moreover, when we analyzed binding of H (and H_NT) to V_1ΔH, we found that MgATP hydrolysis destabilized the V_1–H interaction, an effect likely caused by the cyclic conformational changes at the catalytic AB interfaces to which the EG heterodimers are bound (30). We therefore wished to determine whether C binding to EG on V_1 is also enhanced compared with isolated EG, and if so, (i) what the affinity of the interaction is, and (ii) whether the interaction is also destabilized during ATP hydrolysis. Because V_1 isolated from starving yeast has varying levels of substoichiometric amounts of C bound (15, 16, 33, 34), we purified V_1 from a yeast strain in which C was deleted (V_1ΔC) (16) to test for C binding. Using a BLI setup similar to the one we recently employed to analyze binding of H (and H_NT) to V_1ΔH (30), we found that V_1ΔC binds C with a K_d of ~0.7 nm (Fig. S4), indicating that one of the EG heterodimers bound to V_1 is in a conformation that is more favorable for C binding compared with the isolated heterodimer. However, to test whether C binding is also destabilized as a result of ATP hydrolysis, we could not use V_1ΔC because it contains the H subunit and so has no MgATPase activity, and we therefore used the catalytically active V_1H_chim instead. As seen for the V_1–H interaction, dissociation of V_1H_chim from MBP-C loaded sensors was greatly accelerated only when the sensors were dipped into wells containing MgATP. Fitting the subunit C release in MgATP to two exponentials revealed a fast off rate of ~0.012 ± 2.3 × 10^{-5} s^{-1} and a slower off rate of 5.1 × 10^{-4} ± 2 × 10^{-6} s^{-1}, values similar to those observed for MgATP hydrolysis induced subunit H release as reported earlier (30) (Fig. S5). This suggests that the in vivo dissociation of the C subunit from the vacuolar membrane that occurs as a result of starvation is a direct result of MgATP hydrolysis—induced conformational changes of the EG heterodimer that is bound to C_head in the assembled enzyme (EG3) (23, 35). In summary, as for subunit H, binding of C to EG is significantly enhanced when EG is part of V_1, and the V_1–C interaction is greatly destabilized upon ATP hydrolysis.

\[ V_1H_{chim} \text{ and } C \text{ subunit associate with } V_o \text{ ND to form coupled V-ATPase in vitro } \]

The ability of the V_1 mutants depicted in Fig. 2A to interact with V_o was tested using BLI. V_o ND reconstituted with biotinylated MSP was immobilized on streptavidin-coated BLI sensors (Fig. 3A, step 1), which were then dipped in wells containing V_1 mutants and subunit C (Fig. 3A, step 2). We found that of the four V_1 mutants, only V_1H_{chim} showed significant association with V_o ND (Fig. 3A, red trace). The sensors were then dipped in buffer to measure dissociation rates (Fig. 3A, step 3). However, no significant dissociation was observed, indicating stable assembly of V_1H_{chim} with V_o. Without C, none of the V_1 mutants showed significant binding (Fig. S3), consistent with studies in yeast that showed that deletion of C prevents assembly of V_1V_o (36). As a control, the sensors were then dipped in PreScission protease to cleave and release any remaining complex (Fig. 3A, step 4). Further, we conducted BLI experiments in which V_o ND-coated sensors were first dipped into wells containing a mAb (10D7) against α_N, which recognizes a cryptic epitope only available for binding in free V_o (12), before dipping the sensors into V_1H_{chim} plus C containing wells. Under these conditions, the observed on-rate (k_on) of V_1H_{chim} was significantly (~60-fold) reduced, indicating that the observed BLI signal upon dipping the sensors into V_1H_{chim} plus C was indeed due to binding of V_1H_{chim} to immobilized V_o ND (Fig. S2).

Whereas the BLI experiment showed slow, but stable, association of V_1H_{chim} and C with V_o ND, it was not clear whether functional V-ATPase was formed under these conditions. To address this question, we monitored MgATPase activity of a 1:1:2 mixture of V_1H_{chim}, V_o ND, and C that is sensitive to the V-ATPase specific inhibitor, concanamycin A (ConA), as a function of time (Fig. 3B). Although V_1H_{chim} has MgATPase activity on its own, ConA binds to the V_o complex and prevents c-ring rotation, so ATPase activity that is abolished by treatment with ConA is evidence of a functionally coupled V-ATPase complex. The experiment demonstrated that binding of V_1H_{chim} and C to V_o ND resulted in the formation of a coupled V_1H_{chim}V_o ND complex and that the reconstitution under these conditions was complete in ~2 h, with a final specific activity of 7.2 ± 1.09 units/mg, similar to what is reported for purified WT V_1V_o ND (6.7 ± 0.6 units/mg) (29) (Fig. 3B, inset, pink and blue bars, respectively). The ability of V_1H_{chim} to form a functional complex with V_o ND is consistent with the previous observation that H_{chim} can complement deletion of the native H subunit in yeast cells (16). ConA-sensitive ATPase activity was also measured with V_o ND, C, and V_1H_{chim} and produced only 0.26 ± 0.2 units/mg of coupled activity (Fig. 3B, inset, orange bar). Therefore, reconstitution of V_1H_{chim} with V_o and C is highly inefficient under these conditions, consistent with earlier in vitro studies (28) and the real-time BLI experiments presented here (Fig. 3A).

\[ V_1H_{chim}V_o ND \text{ is more stable in presence of MgATP compared with } V_1V_o ND \]

Using BLI, we have previously demonstrated that V_o dissociation from V_o is negligible under non-ATP hydrolyzing conditions but that in presence of MgATP, the complex undergoes spontaneous dissociation with an off-rate of 1 × 10^{-3} ± 3.3 × 10^{-6} s^{-1} (29). We conducted a similar experiment using V_1H_{chim}V_o ND, wherein after association of V_1H_{chim} and C with V_o ND on BLI sensors, we dipped the sensors in wells containing buffer or 1 mm MgATP. Significantly, unlike WT V_1V_o ND, V_1H_{chim}V_o ND showed very little to no dissociation in the presence of MgATP, indicating that the assembled V_1H_{chim}V_o ND complex is inherently more stable than the WT complex (Fig. 3C). The experiment thus highlights the importance of H_CT’s conformational switch (from energy coupling in the holo enzyme to autoinhibition of membrane detached V_1) in driving V-ATPase disassembly.
Figure 3. V₁Hchim and C associate with V₀ND to form coupled V₁V₀-ATPase. A, V₀ND was immobilized on streptavidin-coated BLI sensors via biotinylated MSP (step 1). Sensors were then dipped into 0.4 μM of V₁ mutants in presence of 1 μM C (association; step 2) followed by buffer (dissociation; step 3). Association with V₀ND was most efficient with V₁Hchim (red trace). Sensors were then dipped in PreScission protease to verify that the BLI signal was not due to nonspecific binding (step 4). Inset shows an enlarged view of the association and dissociation steps. B, equimolar amounts of V₁Hchim and V₀ND, and a 2-fold molar excess of C subunit were incubated at 22 °C, and the ConA-sensitive MgATPase activity was measured as a function of time. Each point represents the mean ± S.E. of two separate reconstitutions from two individual purifications. Inset, specific MgATPase activities of reconstituted V₁HchimV₀ND and V₁HwtV₀ND (± S.E. from two independent purifications) compared with purified V₁V₀ND (29). C, following association of the V₁HchimV₀ND complex, sensors were dipped in wells containing buffer (green) or buffer + 1 mM MgATP (blue) for dissociation rate measurement. The dissociation phase of WT V₁V₀ND in buffer (red) and buffer +1 mM MgATP (orange) is included for comparison (data from Ref. 29).
Functional reconstitution of yeast V-ATPase

Structural and functional characterization of \( V_1HCHIMV_0ND \) complex

To determine the efficiency of \( V_1HCHIMV_0ND \) complex formation, equimolar amounts of \( V_1HCHIM \) and \( V_0ND \) with a 2-fold molar excess of C were incubated for up to 16 h at 22 °C, and the reconstitution mixture was resolved by glycerol density gradient centrifugation. SDS-PAGE of the gradient fractions showed that the majority of \( V_1 \) and \( V_0 \) subunits co-migrated to fractions 7–10, similar to what was observed for purified WT \( V_1V_0ND \) (29), with the excess C subunit remaining in lighter fractions (Fig. 4A). Negative stain EM of the peak fractions showed single particles of \( V_1HCHIMV_0ND \), with the typical dumbbell-shaped appearance of holo V-ATPase reported previously (29, 37, 38) (Fig. 4B). A more detailed analysis indicated a good match between averages obtained by reference-free alignment and classification of a small data set of \( V_1HCHIMV_0ND \) and corresponding projections of a cryoEM map of yeast \( V_1V_0 \) (Protein Data Bank code 3J9U).

Discussion

In our experiments, only \( V_1HCHIM \) (in presence of C) shows significant binding to \( V_0 \). As mentioned earlier, human H\(_{CT}\) lacks the inhibitory loop found in yeast H\(_{CT}\) (16) (Fig. S1, A–D, red spheres), consistent with H\(_{CHIM}\)’s inability to inhibit \( V_1\Delta H\)’s MgATPase activity (Fig. 2, B and C). This lack of inhibition by H\(_{CHIM}\) is likely due to reduced binding of human H\(_{CT}\) to the open catalytic site at the bottom of the A\(_{2}B_{3}\) hexamer, with the remaining binding interaction between H\(_{CHIM}\) and \( V_1 \) being mediated by H\(_{NT}\)’s interaction with one of the EG peripheral stalks. Therefore, we conclude that for \( V_1 \) to reconstitute with \( V_0H_{CHIM} \) must be released from its inhibitory position on \( V_1 \), so that H\(_{CT}\) is available for binding to \( V_0 \)’s a\(_{NT}\). It is known that upon disassembly of \( V_1 \) from \( V_0 \), a\(_{NT}\) moves from a peripheral position in \( V_1 \) to a more central position in autoinhibited \( V_0 \), where it binds subunit d (18, 25–27) (Fig. S1, E–G). The observation that \( V_1H_{CHIM}C \) and autoinhibited \( V_0ND \) are sufficient to form a structurally and functionally coupled V-ATPase suggests that the release of H\(_{CT}\) from its autoinhibitory position on \( V_1 \) is necessary and sufficient for efficient reassembly of \( V_1 \) and \( V_0 \). Our finding is consistent with the fact that reassembly of \( V_1 \) with \( V_0 \) on vacuoles is not inhibited by ConA, an inhibitor of c-ring rotation in the \( V_0 \) sector (14).

H\(_{NT}\) and H\(_{CT}\) occupy specific binding sites on free \( V_1 \), with H\(_{NT}\) bound to EG1 and H\(_{CT}\) bound to the bottom of the A\(_{2}B_{3}\) hexamer, with its inhibitory loop wedged between the B subunit of an open catalytic site and the central stalk (Fig. S1, A–D) (16). The specific interaction of H\(_{CT}\) with an open catalytic site maintains inhibitory MgADP in the adjacent closed catalytic site, locking autoinhibited \( V_1 \) in rotational state 2. We have recently observed that transient MgATP hydrolysis on \( V_1H_{WT}\), which, unlike WT \( V_1 \), is not in the MgADP-inhibited state (Fig. 2B), lowers H’s affinity for \( V_1 \), and we reasoned that this destabilization of the \( V_1\rightarrow H \) interaction is caused by MgATP hydrolysis driven conformational changes at the catalytic sites and the central (DF) and peripheral stalks (EG1–3) (30). We propose that an allosteric structural change at the open catalytic site that is driven by release of inhibitory MgADP from the closed catalytic site in autoinhibited, WT \( V_1 \), by a yet unknown mechanism leads to the detachment of H\(_{CT}\) from its inhibitory position so that it can bind a\(_{NT}\) on \( V_0 \).

From studies in yeast it was shown that although C is required for binding of \( V_1 \) to \( V_0 \) on yeast vacuolar membranes (36), deletion of H allows assembly of a labile but inactive complex (39). The requirement of C for association of \( V_1 \) with \( V_0 \) is supported by our \textit{in vitro} BLI experiments, wherein none of the \( V_1 \) mutants reconstituted with \( V_0 \) in absence of C (Fig. S3). However, unlike \textit{in vivo}, the presence of C along with \( V_1\Delta H \), WT \( V_1 \), or \( V_1H_{CHIM} \) is not sufficient for reconstituting \( V_1V_0 \) \textit{in vitro}. This discrepancy is not due to a reduced affinity of C for
V₁, because we obtained a \( K_d \) of \( \approx 0.7 \) nm for the interaction between C and V₁ΔC (Fig. 5A). A high-affinity interaction between C and V₁ΔC is consistent with stoichiometric amounts of C remaining associated with purified V₁ΔH and V₁ (16), as well as the reported \( K_d \) of \( \approx 42 \) nm for the EG–C interaction (31). However, tight and stable binding of C to V₁ΔC would be inconsistent with the observed release of C into the cytosol upon disassembly of V₁ from V₀ (14), but, as shown here, ATP hydrolysis by V₁ leads to the rapid release of the V₁–C interaction, which likely explains why catalytically inactive enzyme does not disassemble upon glucose withdrawal (14). Although it has been reported that in the presence of the microtubule depolymerizing drug benomyl, C does not dissociate from V₁V₀ upon glucose removal (40), it is possible that C under these conditions quickly rebinds EG3 once V₁ is in the autoinhibited state. In addition, a direct interaction between C and tubulin has been observed (40, 41), suggesting the possibility that, upon disassembly, C is sequestered by microtubules, preventing its reassociation with V₁ and/or V₀.

Although reincorporation of C upon glucose addition does not require the microtubule network (40), efficient (re)assembly of holo V-ATPase requires a heterotrimeric chaperone complex referred to as RAVE (regulator of H⁺-ATPase of vacuolar and endosomal membranes). It has been proposed that upon receiving the signal for reassembly, RAVE recruits C and V₁ to V₀ on vacuolar membranes by directly interacting with C, EG (as part of V₁), and \( a_{NT} \) (as part of V₀) (42). Under the in vitro conditions employed here, it takes \( \approx 2 \) h for a 1:1:2 mixture of \( V₁/H₇chim/V₀/ND \), and C to complete reconstitution of \( V₁/H₇chim/V₀/ND \), a relatively slow process compared with the kinetics of reassembly observed in vivo (\( \approx 5 \) min) (43). It is possible that the RAVE complex, by increasing the proximity of V₁, C, and V₀, facilitates the otherwise low-affinity interactions at the V₁–V₀ interface (32), thereby accelerating reassembly.

From the here presented data, we conclude that the detachment of H₇CT from V₁ and the presence of C subunit are required for the reassembly of V₁ with V₀ (Fig. 5A–C). In our in vitro reconstitutions, the association between V₁ΔHₓchim and V₀/ND is driven by the H₇CT–\( a_{NT} \) interaction (Fig. 5B), but in vivo, the chain of events that leads to reassembly of autoinhibited V₁ and V₀ are probably different, because H₇CT is in its inhibitory conformation on V₁ (Fig. 5A). We propose that in yeast, upon receiving cellular signals, autoinhibited V₁ and C are first recruited to V₀ (Fig. 5D), a process that is likely the rate-limiting step for reassembly. Our reason for this hypothesis is that even with the requirements for reassembly being met in our in vitro reconstitution of V₁/H₇chim and C with V₀, the rate of reassembly was slow. In vivo, recruitment of V₁ and C to V₀ is facilitated and probably accelerated by the RAVE complex (42), but efficient (re)assembly in vivo requires additional factors such as the glycolytic enzymes aldolase (44) and phosphofructokinase (43), whose function in the process is currently not known. Once V₁ and C are recruited to V₀ at the vacuolar membrane, inhibitory MgADP is released upon opening of the closed catalytic site by a yet unknown mechanism (Fig. 5E). The release of inhibitory MgADP allows MgATP hydrolysis to resume, with concomitant conformational changes at the catalytic sites and rotation of the central stalk (DF), structural changes that result in detachment of H₇CT from V₁ (30) (Fig. 5F).

The proximity of \( a_{NT} \) to V₁-detached H₇CT facilitates the H₇CT–\( a_{NT} \) interaction, a requirement for coupling of V₁ to V₀ in the homo-enzyme (24). The H₇CT–\( a_{NT} \) interaction stabilizes the peripheral conformation of \( a_{NT} \) such that the Cfoot–EG2–\( a_{NT} \) ternary complex can be formed, thus completing functional (re)assembly (Fig. 5C).

V-ATPase regulation by reversible disassembly, originally discovered in lower eukaryotes, has been confirmed to be conserved in higher animals, including humans (19–21). Reconstitution of V₁ with V₀ has been investigated for mammalian V-ATPase from bovine brain clathrin-coated vesicles and in one study, chaotropically removed V₁ reassembled with V₀ on coated vesicle membranes upon dialysis, thereby regenerating \( \approx 80\% \) of the initial MgATPase activity (45). In another study, in vitro reconstitution of coated vesicle V-ATPase from V₁ and V₀ was shown to require the mammalian H subunit homolog SFD (sub-fifty-eight dimer) (46). However, in both cases, resulting V-ATPase complexes were not further characterized for subunit composition and structural integrity. Curiously, unlike yeast V₁, removal of SFD from bovine coated vesicle V₁ did not create a MgATP hydrolyzing V₁-ATPase, suggesting the presence of other regulatory mechanisms in mammalian V₁ (46). One of the likely reasons that few biochemical studies have focused upon the molecular mechanism of reversible disassembly in higher organisms is because mammalian V-ATPase is extraordinarily heterogeneous, with most subunits expressed as multiple isoforms or splice variants (including subunits H and a) (47), and to our knowledge, no in vitro system comparable to the one described here for the yeast V-ATPase has been reported for the mammalian enzyme. Yeast contains only one subunit with multiple isoforms (subunit a), and the two V-ATPase populations resulting from this single subunit difference appear to have different propensities to undergo dissociation, and only one of them requires the RAVE complex for (re)assembly (48, 49). It is likely that different isoform-containing enzymes in mammalian systems are subjected to differential regulatory mechanisms, resulting in variable propensities to dissociate. Because the human H₇CT does not silence yeast V₁ but does facilitate efficient functional coupling in V₁/V₀, it will be of interest to explore the mechanism of regulation by reversible disassembly as a function of subunit isoform composition of the mammalian system in greater detail by using the tools developed and presented here for the yeast enzyme.

**Experimental procedures**

**Strains**

The yeast strain SF838–5Aαα deleted for the vma2 gene (B subunit) vma2Δ::Nat was a kind gift from Dr. Patricia Kane, SUNY Upstate Medical University. A plasmid containing the FLAG tag with a KanMX6 marker, pFA6a-6xGL-FLAGkanMX6 was a gift from Dr. Mark Hochstrasser (50) (Addgene plasmid no. 20751). The primers vph1CTFlagFWD (gtc gtt gct agt gca gac tct tcc gct tca ggc GGA GGc Ggc GGG GGT GGa) and vph1CTFlagREV (cct gga tgt gga tgt cca tgt caa cgt tac ccc aag gca aat gat ggt cag tac tgg GAA TTC GAG CTC TAA TAA) were used to amplify the FLAG tag and
KanMX marker from pFA6a-6xGly-FLAG-kanMX6. The ~1.8-kb product was gel purified and used for homologous recombination to insert the FLAG-KanMX cassette in the C terminus of vph1 in the yeast strain SF838–5A vma2::Nat using the same primers as above. Colonies were selected for growth on YPD G418 plates, and the insertion of the FLAG tag at the C terminus of vph1 was confirmed by sequencing. The construction of chimeric H subunit (Hchim) encoding the N-terminal domain from *Saccharomyces cerevisiae* (residues 1–352) and the C-terminal domain (349–483) of the human H

**Figure 5. Model for reassembly of autoinhibited V₁ and V₀.** A–C, our *in vitro* experiments have shown that although WT H containing V₁ does not readily bind V₀ND (A), V₁Hchim spontaneously associates with V₀ND (B) to form a structurally and functionally coupled V-ATPase, albeit at a slow rate. C, *in vivo*, however, V₁ exists in the autoinhibited conformation (A), and the rate of assembly with V₀ is significantly faster (within 5 min). D–F, for *in vivo* (re)assembly, we propose that the following steps occur: step 1, recruitment of V₁ and subunit C to the vacuolar membrane (D); step 2, release of inhibitory MgADP (E); step 3, detachment of HCT from its inhibitory position on V₁; and step 4, HCT binding to aNT (F). For further details, see text.
subunit into the yeast pRS316 vector has been discussed in Ref. 16. From the pRS316 vector, using the primers MalChimF (TTA GCC GTT ACC GGG AGC AAC GAA GAT ATT AAT GGA C) and MalChimR (TTA CCA AAG CTT TTA GCT TCG GGC GGC AG), \( H_{\text{chim}} \) was amplified. The primers additionally introduced the restriction sites 5’ KpnI and 3’ HindIII, which were used to insert the amplicon into a pMal vector. The resultant vector encoded MBP-tagged \( H_{\text{chim}} \) separated by a PreScission protease cleavage site, as confirmed by sequencing.

**Purification of \( V_o \) and its reconstitution into endogenous vacuolar lipid**

\( V_o \) was purified from yeast vacuoles and reconstituted into endogenous vacuolar lipid containing nanodiscs as described for \( V_o \cdot V_{\text{ND}} \) in Ref. 29. The steps are briefly described as follows.

**Purification of biotinylated MSP**

Biotinylated MSP was purified as described in Ref. 29. Briefly, BL21 (DE3) cells were co-transformed with plasmids pHBPMS1E3D1 and pBirAcm (encoding the BirA gene). The cells were grown in rich broth supplemented with 0.1 mM D-biotin, 34 \( \mu \)g/ml chloramphenicol, and 30 \( \mu \)g/ml kanamycin to an \( A_{595} \) of \(-0.5 \) at 37 °C followed by induction using 0.5 mM isopropyl-\( \beta \)-d-thiogalactopyranoside for 3–4 h. Harvested cells were purified as described in Ref. 26. Briefly, the cells were lysed by sonicating three times for 30 s. The lysate was centrifuged at 13,000 \( \times \) g and passed over a nickel–nitrilotriacetic acid affinity column. The column was washed with 10 column volumes of the each of the three buffers: 40 mM Tris-HCl, 300 mM NaCl, and 1% Triton X-100, pH 8; 40 mM Tris-HCl, 300 mM NaCl, 50 mM sodium cholate, and 5 mM imidazole, pH 8; and 40 mM Tris-HCl, 300 mM NaCl, and 10 mM imidazole, pH 8. MSP was eluted with a 10–column volume gradient of the elution buffer (40 mM Tris-HCl, 300 mM NaCl, and 100 mM imidazole, pH 8). Purified biotinylated MSP was dialyzed into 25 mM sodium phosphate, pH 7.5, whereas the predicted charge of MBP is 3.5, whereas the predicted charge of MBP is +9 (Protein Calculator v3.4), allowing separation of the two proteins using cation exchange (carboxymethyl) chromatography. The cleaved MBP came off the carboxymethyl column in the flow through and wash steps, whereas pure \( H_{\text{chim}} \) was eluted in dialysis buffer supplemented with 100 mM NaCl. The preparation was subjected to a final step using a Superdex 200 1.6 \( \times \) 50-cm size-exclusion chromatography column.

**Functional reconstitution of yeast V-ATPase**

Three batches of purified vacuoles (12 liters each) were typically used for one extraction as described in Ref. 29. Briefly, thawed vacuoles were combined, supplemented with protease inhibitors, and solubilized using 1.2 mg of \( n \)-dodecyl-\( \beta \)-d-maltopyranoside/1 mg of vacuolar protein. To the detergent-solubilized sample, purified biotinylated MSP was added in a molar ratio of 1:50 (vacuolar protein:MSP). The mixture containing vacuolar protein, vacuolar lipids, and MSP was incubated at 4 °C for 1 h followed by detergent removal using beads. Reconstituted vacuolar membrane proteins in biotinylated and endogenous vacuolar lipid containing nanodiscs were subjected to anti-FLAG affinity chromatography to purify \( V_o \)-containing nanodiscs. The eluate from the FLAG column was then subjected to size-exclusion chromatography using a Superdex 200 1 \( \times \) 30-cm column. Peak fractions from gel filtration were combined and concentrated using a Vivaspin 100,000 molecular weight cutoff concentrator.

**Purification of the chimeric H subunit (\( H_{\text{chim}} \))**

*Escherichia coli* Rosetta2 (Novagen) cells expressing N-terminal MBP-tagged \( H_{\text{chim}} \) were grown to an \( A_{600} \) of \(-0.5 \) (in LB, 0.2% glucose, 50 \( \mu \)g/ml carbenicillin, and 34 \( \mu \)g/ml chloramphenicol), and expression was induced with 0.5 mM isopropyl-\( \beta \)-d-thiogalactopyranoside at 30 °C for 4 h. Protein was purified using amylose affinity chromatography, and the MBP tag was cleaved with PreScission protease as previously described (31). The pH of the cleavage product was adjusted to 7 by overnight dialysis in 25 mM sodium phosphate, pH 7, 0.5 mM EDTA, and 5 mM \( \beta \)-mercaptoethanol. At pH 7, \( H_{\text{chim}} \) has a predicted charge of +3.5, whereas the predicted charge of MBP is –9 (Protein Calculator v3.4), allowing separation of the two proteins using cation exchange (carboxymethyl) chromatography. The cleaved MBP came off the carboxymethyl column in the flow through and wash steps, whereas pure \( H_{\text{chim}} \) was eluted in dialysis buffer supplemented with 100 mM NaCl. The preparation was subjected to a final step using a Superdex 200 1.6 \( \times \) 50-cm size-exclusion chromatography column.
was concentrated and subjected to size-exclusion chromatography using a Superdex 200 1.6 × 50 cm column.

For preparation of $V_1^{H_{\text{chim}}}$ and $V_1^{H_{\text{wt}}}$, $V_1$ΔH eluted from the αFLAG column was incubated for 1 h at 4 °C with a ~5-fold molar excess of either $H_{\text{chim}}$ or $H_{\text{wt}}$ (purified as in Ref. 30) to form the $V_1^{H_{\text{chim}}}$ and $V_1^{H_{\text{wt}}}$ complexes, respectively. $V_1$ bound to $H_{\text{chim}}$ or $H_{\text{wt}}$ was then separated from the excess of $H_{\text{chim}}$ or $H_{\text{wt}}$ by size-exclusion chromatography using a Superdex 200 1.6 × 50 cm column. Subunit C was purified as previously described (31).

**Biolayer interferometry**

Interaction of $V_o$ with the purified $V_1$ mutants was screened using BLI, a light interference–based technique, similar to surface plasmon resonance. An Octet-RED system with streptavidin coated biosensors (Forte´Bio, SA biosensors, catalog no. 18-5019) were used for the experiments. All BLI experiments were conducted using 25 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5 mM EDTA, 1 mM β-mercaptoethanol, 0.5 mg/ml BSA, except for experiments analyzing $V_1^{H_{\text{chim}}}$ release from immobilized MBP-C, which required 10 mg/ml BSA because of an increased propensity of chimeric H containing $V_1$ to bind nonspecifically to the BLI sensors. The temperature was maintained at 22 °C, with each biosensor stirred in 0.2 ml of sample at 1000 rpm and a standard measurement rate of 5 s⁻¹. Streptavidin-coated biosensors were pretreated in BLI buffer and then dipped in wells containing 3 μg/ml of biotinylated $V_o$. A buffer control was included to show that none of the buffer components interacted with the sensors. Details of individual experiments have been described in the respective figure legends. The affinity of interaction between $V_o$ and MBP-C was measured using anti-mouse IgG Fc capturing biosensors (FortéBio, AMC biosensors catalog no. 18-5088) as described (30).

**ATPase activity assay**

MgATPase activity of purified $V_1$ mutants and reconstitution mixtures ($V_1$ mutants + $V_o$ND + subunit C) was measured using a coupled enzyme assay as described in Ref. 16. Briefly, 10 μg of the $V_1$ mutant was added to an assay mixture containing 1 mM MgCl₂, 5 mM ATP, 30 units/ml each of lactate dehydrogenase and pyruvate kinase, 0.5 mM NADH, 2 mM phosphoenolpyruvate, and 50 mM HEPES, pH 7.5, at 37 °C. The decrease of absorbance at 340 nm corresponding to the decline of NADH in the system was measured in the kinetics mode on a Varian Cary Bio100 spectrophotometer. In case of reconstitution mixtures, 20 μg of $V_1$ mutant with equimolar amounts of $V_o$ND, and a 2× molar excess of C subunit was added to an assay containing 4 mM MgCl₂.

**References**


