MgATP hydrolysis destabilizes the interaction between subunit H and yeast V1-ATPase, highlighting H’s role in V-ATPase regulation by reversible disassembly

Received for publication, March 16, 2018, and in revised form, April 22, 2018. Published, Papers in Press, May 12, 2018, DOI 10.1074/jbc.RA118.002951

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Edited by Karen G. Fleming

Vacuolar H+-ATPases (V-ATPases; V1V0-ATPases) are rotary-motor proton pumps that acidify intracellular compartments and, in some tissues, the extracellular space. V-ATPase is regulated by reversible disassembly into autoinhibited V1-ATPase and V0 proton channel sectors. An important player in V-ATPase regulation is subunit H, which binds at the interface of V1 and V0. H is required for MgATPase activity in holo-V-ATPase but also for stabilizing the MgADP-inhibited state in membrane-detached V1. However, how H fulfills these two functions is poorly understood. To characterize the H–V1 inter- action and its role in reversible disassembly, we determined binding affinities of full-length H and its N-terminal domain (HNT) for an isolated heterodimer of subunits E and G (EG), the N-terminal domain of subunit α (αNT), and V1 lacking subunit H (V1ΔH). Using isothermal titration calorimetry (ITC) and biolayer interferometry (BLI), we show that HNT binds EG with moderate affinity, that full-length H binds αNT weakly, and that both H and HNT bind V1ΔH with high affinity. We also found that only one molecule of HNT binds V1ΔH with high affinity, suggesting conformational asymmetry of the three EG heterodimers in V1ΔH. Moreover, MgATP hydrolysis–driven conformational changes in V1 destabilized the interaction of H or HNT with V1ΔH, suggesting an interplay between MgADP inhibition and subunit H. Our observation that H binding is affected by MgATP hydrolysis in V1 points to H’s role in the mechanism of reversible disassembly.

Vacuolar H+-ATPases (V-ATPases; V1V0-ATPases) are ATP-dependent proton pumps present in all eukaryotic cells.

This work was supported by National Institutes of Health Grant GM058600 and a Bridge Grant from SUNY Upstate Medical University (to S.W.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

The V-ATPase shares a similar architecture and catalytic mechanism with the F-ATP synthase such that it consists of a water-soluble ATP-hydrolyzing machine (V1) and a membrane-integral proton channel (V0), which are structurally and functionally coupled via a central stalk and multiple peripheral stalks (12–14). The subunit composition of the V-ATPase from yeast is A8B8CDE4FG3H for the cytosolic V1 (15) and A8C6C’e’d’e def for the membrane-integral V0 (16, 17). The subunit architecture of the V-ATPase has been studied by electron microscopy (EM) and several low- to intermediate-resolution reconstructions of bovine, yeast, and insect V-ATPase are available, which, together with X-ray crystal structures of individual subunits and subcomplexes of yeast V-ATPase and bacterial homologs, have provided a detailed model of the subunit architecture of the eukaryotic V-ATPase complex (17–22) (see Fig. 1A).

V-ATPase is a rotary-motor enzyme (14). ATP hydrolysis in the catalytic A8B3 hexamer is coupled to rotation of the proteolipid ring (c6c’c") via a central rotor made of subunits D, F, and d with concurrent proton translocation at the interface of the proteolipid ring and the C-terminal domain of subunit α (αCT). During rotary catalysis, the motor is stabilized by a peripheral stator complex consisting of three peripheral stalks constituted by heterodimers of subunits E and G (hereafter referred to as EG1–3) that connect the A8B3 hexamer to the N-terminal domain of the membrane-bound a subunit (αNT) via the single-copy “collar” subunits H and C (19, 21) (see Fig. 1A). These intermediates (referred to as rotational states 1–3), in which the central rotor is spaced 120° relative to the catalytic hexamer and subunit a, have been visualized in the yeast enzyme by cryo-EM (21).
Subunit H interactions at the V₁–V₀ interface

Figure 2. Binding interactions of subunit H in holo-V₁V₀ and autoinhibited V₁–ATPase. A, in V₁V₀, (Protein Data Bank code 3J9U) (21), HNT (dark green) is bound to EG1 (blue/orange), and HCT (light green) is in contact with aNT (purple). B, in membrane-detached and autoinhibited V₁, (Protein Data Bank code 5DBI) (22), the contact between HNT and EG1 is preserved, but HCT undergoes a ∼150° rotation to bind the bottom of the A₈B₃ hexamer and the rotor subunit D. The large conformational change in HCT is depicted by the positions of the C-terminal α-helix (magenta) and the inhibitory loop (red) in holo-V₁V₀ versus autoinhibited V₁–ATPase.

Results

Expression, purification, and biophysical characterization of H, HNT, HCT, and aNT (1–372)

To understand the role of the V₁–V₀ interface in the mechanism of reversible disassembly, our laboratory has previously characterized the interactions among Chead, Cfoot, EG, and aNT (33, 34). Interactions involving subunit H, however, are yet to be quantified. Pulldown and yeast two-hybrid assays have shown that H is able to bind the N-terminal region of subunit E (40). In addition, EM and crystal structures of V₁V₀ and V₁, respectively, show HNT bound to one of the three EG peripheral stalks (EG1; see Fig. 2, A and B), whereas HCT is seen to either rest on the coiled-coil middle domain of aNT (in V₁V₀; Fig. 2A) or at the bottom of the A₈B₃ hexamer (in autoinhibited V₁) (Fig. 2B) (21, 22). To analyze the interactions of H within the enzyme in more detail, we expressed H, HNT, and HCT separately and quantified their interactions with purified EG, aNT, and V₁ lacking subunit H (V₁ΔH) using isothermal titration calorimetry (ITC) and biolayer interferometry (BLI).

We found that HNT binds no more than one of the three EGs on V₁ΔH and that the affinity of this interaction is ~40-fold higher than that between HNT and isolated EG, suggesting that HNT prefers a particular conformation of EG on V₁. We further found that full-length H interacts with V₁ΔH with an ~70-fold higher affinity than HNT, indicative of a significant contribution of HCT to the binding energy. Furthermore, we show that MgATP hydrolysis–driven conformational changes in the catalytic A/B pairs, the central rotor (DF), and the peripheral stalks (EG) destabilize the V₁–H interaction until inhibitory MgADP is trapped in a catalytic site. The findings are discussed in context of the mechanism of V-ATPase regulation by reversible disassembly.

Figure 1. Schematic of yeast V-ATPase regulation by reversible disassembly. A, subunit architecture of holo-V-ATPase. The V₁ and V₀ sectors are assembled and form an active enzyme, B, upon glucose deprivation, the V₁ and V₀ sectors disengage, and the activity of both sectors is silenced. The subunits of the peripheral stator that form the V₁–V₀ interface are shown in color. prox., proximal; dist., distal.

Unlike the related F-ATP synthase, eukaryotic V-ATPase is regulated by a unique mechanism referred to as reversible disassembly wherein, upon receiving cellular signals, V₁ dissociates from V₀, and the activity of both sectors is silenced (22–26) (see Fig. 1B). Reversible disassembly was first observed in yeast (27) and insects (28), but the process has recently also been observed in higher animals including human (29–32). Studies in yeast have shown that, during enzyme disassembly, subunit C is released into the cytosol by an unknown mechanism and reincorporated during reassembly (27).

Interactions involving subunit H, HNT, and HCT suggested that, although HNT is required for MgATPase activity in V₁V₀, HCT has a dual function in that it is required for both coupling of V₁’s ATPase activity to proton pumping in V₁V₀ (37) and inhibition of MgATPase activity in membrane-detached V₁ (39). The dual role and functional separation of HNT and HCT along with differences in regulatory function compared with C are not well understood and prompted the analyses of the interactions of H, HNT, and HCT with its binding partners in V₁ and V₀. We therefore used recombinant H, HNT, and HCT for quantification of their interactions with purified EG, aNT, and V₁ lacking subunit H (V₁ΔH) using isothermal titration calorimetry (ITC) and biolayer interferometry (BLI).
coli as N-terminal fusions with maltose-binding protein (MBP). After amylose affinity capture, fusions were cleaved, and MBP was removed by ion exchange and size exclusion chromatography, resulting in purified subunits and subunit domains (Fig. 3A). All proteins eluted near their expected molecular masses on size exclusion chromatography except αNT(1–372), which exists in a dimer–monomer equilibrium as described previously (26, 34) (Fig. 3B and C). Consistent with available structural information, circular dichroism (CD) spectroscopy revealed a high degree of α-helical secondary structure, suggesting proper folding of the recombinant polypeptides (Fig. 3D).

**Interaction of HNT with EG**

We previously established that binding of C (or Chead) to isolated EG occurs with high affinity and that the interaction greatly stabilizes EG (33). To further characterize the interactions at the V1–Vo interface, we set out to determine the affinity of the HNT–EG interaction using ITC. Titration of HNT into EG was exothermic, and the binding curve was fit to a single-binding-site model, revealing a $K_d$ of the interaction of 187 nM. The binding enthalpy ($\Delta H$) and entropy ($\Delta S$) were $-8 \text{ kcal/mol}$ and 2.5 cal/(mol·K), respectively, with a concomitant free energy change ($\Delta G$) of $-36 \text{ kcal/mol}$ (Fig. 4A). Consistent with the ITC titration, size exclusion chromatography of a mixture of EG and an excess of HNT resulted in the formation of a ternary HNT–EG complex (Fig. 4B, C), and taken together, the data show that HNT forms a stable complex with the EG heterodimer. Previously, we found that the EG's N-terminal right-handed coiled coil is thermally labile with a $T_m$ of $25 \degree C$ (Fig. 4D, blue trace) (29) and that the $T_m$ of EG is increased by about 10 °C upon complex formation with Chead (33). To test whether HNT binding has a similar stabilizing effect on EG, thermal unfolding of the individual proteins and EGHNT complex was monitored by recording the CD signal at 222 nm as a function of temperature. The data show that isolated HNT unfolds with an apparent $T_m$ of $63 \degree C$ (Fig. 4D, green trace). The thermal unfolding curve of the EGHNT complex showed two transitions, one at $25 \degree C$ and one at $64 \degree C$, suggesting that the stability of EG is not increased upon HNT binding (Fig. 4D, black trace). Moreover, as also shown previously (33), isolated EG heterodimer dissociates during native agarose gel electrophoresis, whereas in the presence of Chead the three proteins migrate as a heterotrimeric complex in the electric field. However, consistent with the thermal unfolding data, a complex of EGHNT did not comigrate on the native gel but ran as three separate species (Fig. 4E). Therefore, although both Chead and HNT form a
Figure 4. Interaction of HNT with the EG heterodimer and H with αNT. 

A, isothermal titration calorimetry of the interaction between HNT and EG. HNT was titrated into EG (top panel, lower trace) or buffer only (top panel, top trace), and the heat associated with the interaction was measured at 10 °C in 20 mM Tris-HCl, 0.5 mM EDTA, 1 mM TCEP, pH 8. The area under the peaks in the top panel was integrated and plotted as kcal/mol of HNT as a function of binding stoichiometry in the bottom panel. These data were fit using a one-site binding model resulting in a $K_d$ of 187 nM with $\Delta H$ and $\Delta S$ values of $-8$ kcal/mol and $2.5$ cal/(mol·K), respectively, resulting in a $\Delta G$ of the interaction of $-36$ kcal/mol. A representative of three separate titrations is shown. 

B, the ITC cell content was subjected to gel filtration on a 1.6 × 50-cm Superdex 200 column (black trace). The individual gel filtration elution profiles of HNT and EG using the same column are shown in green and blue, respectively. C, SDS-PAGE of gel filtration fractions from the ITC cell content. EGHNT elutes at higher molecular mass with excess HNT in a well separated peak. The shift of the EGHNT peak (compared with EG or HNT) toward higher molecular weight indicates complex formation. The inset in B shows the EGHNT peak fraction (fraction 55) from the SDS-PAGE gel of the ITC cell content shown in C. D, thermal unfolding of HNT, EG, and EGHNT monitored by recording the CD signal at 222 nm with increasing temperature. HNT shows highly cooperative unfolding with an apparent $T_m$ of $63$ °C (green). EG has an apparent $T_m$ of $-25$ °C (data taken from Ref. 33). The EGHNT complex shows two unfolding transitions with $T_m$ values similar to the those observed for the individual proteins, suggesting that HNT binding to EG does not stabilize the EG heterodimer. E, native agarose gel electrophoresis of EG, HNT, and a mixture of equimolar amounts of HNT and EG (total 60 μg) were loaded. Unlike binding of Chead to EG (33), binding of HNT does not appear to stabilize EG under these conditions. F, isothermal titration calorimetry of the interaction between αNT and H. H was titrated into αNT (top panel, lower trace) or buffer (top panel, top trace), and the heat associated with the interaction was measured at 10 °C in 20 mM Tris-HCl, 0.5 mM EDTA, 1 mM TCEP, pH 8. Subtracting the heat of dilution of titration of H into buffer revealed an endothermic binding reaction. Fitting the data (bottom panel) to a one-site binding model with a fixed $n = 1$ allowed an estimate of the $K_d$ of $-130$ μM. A representative titration from two repeats is shown. mAU, milliabsorbance units.
Subunit H interactions at the V₁–V₀ interface

stable complex with EG, the nature of the two interactions are strikingly different.

Interaction of H with α₅NT

Prior work from our laboratory has shown that the EG2–α₅NT–Cfoot junction at the V₁–V₀ interface (Fig. 1A) is formed by multiple low-affinity interactions, and we reasoned that the sum of these interactions provides a high-avidity binding site between V₁ and V₀ that could be targeted for regulated disassembly (34). Another interaction that is seen in EM reconstructions of the intact V-ATPase, and that must be broken and reformed during reversible disassembly, is between H and α₅NT (Figs. 1A and 2A). To estimate the affinity between H and α₅NT, we performed ITC experiments by titrating H into α₅NT(1–372) (Fig. 4F). Subtracting the heat generated from diluting H into buffer from the heat generated from titrating H into α₅NT supports our existing model that V₁ binds Vo via several single-copy subunits in the V₁ complex (for example subunit D), indicating that no more than one copy of HNT bound to V₁α₅NT. The V₁(ΔH)HNT complex was concentrated, and approximately equal amounts of V₁ΔH and V₁(ΔH)HNT were resolved using SDS-PAGE. The staining of HNT in the purified V₁(ΔH)HNT complex was similar to that of single-copy subunits in the V₁ complex (for example subunit D), indicating that no more than one copy of HNT bound to V₁ΔH (Fig. 5E). Therefore, although there are three EGs in V₁ΔH, only one of these is in a conformation that is able to bind HNT with high affinity, highlighting the conformational asymmetry of the peripheral stalks.

Interaction of V₁ΔH with H, HNT, and HCT characterized by BLI

Previous experiments showed that H remains bound to V₁ even at the low concentrations used in enzyme assays (e.g. ~15 nM) (22, 25, 39) and under the conditions of electrospray ionization used for native MS (15). Although the data so far have shown that the affinity of HNT for EG as measured using ITC is moderately high, the observed K₅₆ values of ~0.2 μM (Fig. 4A) could not explain the above observations, which means that the interaction of H with V₁ has to be much stronger (39). We therefore wished to determine the affinity of full-length H as well as HNT and HCT for V₁ΔH. The interaction between V₁ΔH and MBP-tagged H, HNT, and HCT was quantified using BLI. MBP-tagged proteins were immobilized on anti-mouse Fe capture (AMC) biosensors using an anti-MBP antibody, and the rate of association and dissociation of V₁ΔH was measured hereafter. The slow dissociation of MBP-tagged H, HNT, and HCT from the anti-MBP antibodies was subtracted from the V₁ΔH dissociation rates for analysis of the kinetic data. BLI experiments for measuring association and dissociation kinetics between V₁ΔH and MBP-H/HNT were conducted at five different V₁ΔH concentrations, and the resulting association and dissociation curves were fit to a global single-site binding model (Fig. 5, A and B). Analysis of the data for MBP-H and MBP-HNT revealed K₅₆ values of ~65 pm (Fig. 5A) and ~4.5 nm (Fig. 5B), respectively. We also tested the binding of V₁ΔH to MBP-H₉七星, but we were not able to determine a K₅₆ as there was no measurable association at low V₁ΔH concentrations (~100 nm), and higher V₁ΔH concentrations (e.g. 1 μM) resulted in nonspecific binding to the BLI sensors (data not shown). Overall, the interaction of HNT with EG as part of V₁ΔH was ~40-fold tighter when compared with the interaction between HNT and isolated EG (as measured using ITC; Fig. 4A), suggesting that the conformation of EG on V₁ΔH is more favorable for HNT binding than the conformation(s) of isolated EG. In addition, although we could not detect an interaction between HCT and V₁ΔH under the conditions of BLI, a ~70-fold higher affinity of V₁ΔH for H as compared with HNT suggests a significant contribution of HCT to the V₁–H interaction. From our ITC and BLI experiments, we infer that the binding interaction between HNT and EG allows HCT to switch conformations so that it can either bind α₅NT (in V₁V₀) or subunits B and D (in V₁ΔH) to efficiently carry out its dual role in reversible disassembly.

V₁ΔH binds no more than one HNT

Because V₁ΔH contains three EG heterodimers, we wished to determine whether all three or only one of the EGs can bind HNT. Purified V₁ΔH was mixed with a 5-fold molar excess of HNT followed by size exclusion chromatography. Under these conditions, some HNT coeluted with V₁ΔH with the excess HNT eluting from the column as a separate, lower molecular weight peak (Fig. 5, C and D). The V₁(ΔH)HNT complex was concentrated, and approximately equal amounts of V₁ΔH and V₁(ΔH)HNT were resolved using SDS-PAGE. The staining of HNT in the purified V₁(ΔH)HNT complex was similar to that of single-copy subunits in the V₁ complex (for example subunit D), indicating that no more than one copy of HNT bound to V₁ΔH (Fig. 5E). Therefore, although there are three EGs in V₁ΔH, only one of these is in a conformation that is able to bind HNT with high affinity, highlighting the conformational asymmetry of the peripheral stalks.

The interaction of H with V₁ΔH is destabilized upon MgATP hydrolysis

The preference of HNT for one of three EGs suggested that the asymmetry of the peripheral stalks originates in the catalytic core (A₈B₈DF) of V₁. Upon MgATP hydrolysis, however, the conformational changes of the catalytic sites from open to loose to tight drive counterclockwise rotation of the central rotor along with cyclic structural changes in the peripheral stalks from EG1 to EG3 to EG2 (41). In addition, based on the structure and nucleotide occupancy of the autoinhibited V₁ sector, our laboratory suggested that HCT inhibits V₁-ATPase activity by preferentially binding to an open catalytic site, consequently maintaining inhibitory MgADP in the adjacent closed catalytic site (22). Taken together, HNT’s role in MgADP inhibition, indicated a potential interplay between the nucleotide occupancy of the catalytic sites and the interaction of V₁ΔH with H. To probe the effect of nucleotides on the interaction of H with V₁ΔH, we again used BLI. V₁ΔH was bound to immobilized MBP-H, and the sensor was then dipped in wells containing buffer or buffer with 1 mM MgATP, MgADP + P, or MgAMPPNP. Interestingly, in the presence of MgATP, a biphasic dissociation curve was observed with an initial dissociation rate that was ~6 times faster than the rate in buffer alone (Fig. 6, A and B). However, only ~25% of the bound V₁ΔH dissociated with a fast rate with the remaining 75% coming off the sensor at a rate similar to the dissociation rate in buffer (Fig. 6, A and B). In contrast, a relatively slower rate of
dissociation was observed in the presence of MgADP + P_i and MgAMPPNP.

The destabilization of the V_1–H interaction upon MgATP hydrolysis came as a surprise to us as the H subunit is known to inhibit V_1-ATPase activity (22, 25, 39). To confirm that the fast dissociation of V_1/H was specifically due to MgATP binding to V_1's catalytic sites, we conducted a similar BLI experiment using V_1/H treated with N-ethylmaleimide (NEM). It is known that NEM modification of a catalytic-site cysteine residue prevents binding of nucleotides (42). NEM-treated and untreated V_1/H were bound to MBP-H immobilized on sensors and then dipped in wells containing MgATP, MgADP/P_i, or buffer (Fig. 6, C and D). We found that NEM-treated V_1/H no longer showed a fast dissociation rate when dipped in MgATP-containing wells, suggesting that MgATP binding to the catalytic sites caused destabilization of the V_1–H interaction. However, if the above mentioned fast dissociation rate was a result of only MgATP binding, but not hydrolysis, we should have observed fast dissociation in the presence of MgAMPPNP, the nonhydrolyzable ATP analog. MgAMPPNP, however, had no effect on the V_1/H dissociation rate (Fig. 6A, green trace). Taken together, the BLI experiments with NEM-modified V_1/H and in the presence of MgAMPPNP suggest that it is MgATP binding and hydrolysis that destabilize the V_1–H interaction. Because both HNT and HCT contribute to the interaction of H with V_1/H, we also measured the off-rate of V_1/H from immobilized MBP-HNT (Fig. 6, E and F).

To verify that V_1/H bound to H was capable of transient turnover, we purified V_1/H, incubated it with an excess of H, and resolved the mixture using size exclusion chromatography (Fig. S2). We found that V_1/H reconstituted with H (V_1/H) showed ~4.9 ± 0.55 units/mg of MgATPase activity, which was ~30% of the activity of V_1/H (15.75 ± 1.7 units/mg) (Fig. 6G and Ref. 22). Considering the high-affinity interaction between V_1/H and H with a K_d of ~65 pm, we expected stoichiometric

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**Figure 5. Interaction of H and HNT with V_1/H.** A, the affinity of the interaction between H and V_1/H was determined using BLI at 22 °C in BLI buffer. Association and dissociation sensorgrams from five different concentrations of V_1/H (0.1, 0.3, 1, 3, and 9 nM) are shown in blue. The data were globally fit (traces in red) to reveal a K_d of ~65 pm. B, a similar experiment was conducted to analyze the interaction between HNT and V_1/H. HNT was dipped in 10, 20, 40, 80, and 160 nM V_1/H followed by buffer to generate association and dissociation curves, respectively (blue). The data were globally fit (red traces) to reveal a K_d of ~4.5 nM. C, V_1/H was incubated with a 5-fold excess of HNT, and the mixture was resolved on a 1.6 × 50-cm Superdex 200 column. D, SDS-PAGE of gel filtration fractions. The higher molecular weight peak showed V_1/H in complex with HNT and the lower molecular weight peak corresponding to excess HNT. E, approximately equimolar amounts of V_1/H and V_1/H in complex with HNT were resolved by SDS-PAGE. The staining intensity of the HNT compared with the single-copy subunit D band in the V_1/H/HNT complex suggests that V_1/H binds no more than one copy of HNT with high affinity. mAU, milliabsorbance units.
amounts of H in the V₁(H)H reconstituted complex. However, to exclude the possibility that the observed MgATPase activity in the V₁(H)H complex was due to substoichiometric binding of H, we performed a pulldown experiment in which a 10-fold molar excess of MBP-H bound to amylose resin was used to capture V₁(H) (Fig. S3). Although some MBP-H and V₁(H) appeared in the supernatant and washes of the amylose resin, most of the MBP-H eluted in 10 mM maltose along with stoichiometrically bound V₁(H). Elution fraction E1 (Fig. S3A) exhibited significant MgATPase activity (Fig. S3B), indicating that a stoichiometric complex of V₁(H) with MBP-H was capable of hydrolyzing MgATP.

A consistent feature of the dissociation curve of V₁(H) from H in MgATP was its biphasic nature (Fig. 6A, orange trace), indicating that MgATP hydrolysis–dependent destabilization was transient. We found that, by using different concentrations of MgATP during dissociation, we were able to regulate the fast phase of the dissociation rate and consequently the duration of destabilization (Fig. 7). Not only does this experiment confirm MgATP hydrolysis as being the cause of V₁–H destabilization,
Subunit H interactions at the V₁–Vₒ interface

MgADP in a catalytic site, a phenomenon termed MgADP inhibition are two domain proteins, and we previously found that Chead binds MgADP in the absence of MgATP (10 μM, purple; 100 μM, red; 1 mM, orange; 4 mM, pink). A representative from two independent experiments is shown. B, off-rates determined by fitting the sensorgrams in A to dual-exponential equations.

Figure 7. Modulation of the dissociation rate of V₁–H from H by varying the concentration of MgATP. A, sensorgrams of V₁–H dissociation from immobilized H subunit in the absence (blue) and presence of MgATP (10 μM, purple; 100 μM, red; 1 mM, orange; 4 mM, pink). A representative from two independent experiments is shown. B, off-rates determined by fitting the sensorgrams in A to dual-exponential equations.

it also explains why the destabilization is transient. Using P₁ release–based ATPase assays, it has been shown that MgATPase activity of V₁–H subsides rapidly with time (25). This rapid decrease in activity, which is also observed in an ATP-regenerating assay (Fig. S4), has been attributed to the trapping of MgADP in a catalytic site, a phenomenon termed MgADP inhibition. We have observed that MgADP inhibition of V₁–H is more efficient under high Mg²⁺ (and by extension high MgATP) concentrations and that decreasing the initial concentration of MgATP results in delayed MgADP inhibition (Fig. S4). In the BLI experiment shown in Fig. 7, decreasing the concentration of MgATP to 100 μM (Fig. 7, red trace) and consequently decreasing the rate of MgATP hydrolysis led to a delay in the culmination of the fast dissociation phase. Taken together, these data suggest that MgATP hydrolysis causes transient destabilization of the V₁–H interaction until MgADP inhibition sets in.

Discussion

V₁-ATPase is regulated by reversible disassembly, a process that involves the breakage and reformation of several protein–protein interactions at the interface of V₁ and Vₒ. These interactions are mediated by the central rotor of V₁ (DF) binding to Vₒ’s subunit d and the three peripheral stalks (EG1–3) that link the collar subunits H and C to aNT (see Fig. 2A). Both H and C are two domain proteins, and we previously found that Chead binds EG with high affinity, whereas Cfoot and EG bind aNT weakly. Here, we analyzed binding of H and aNT to isolated EG, aNT, and V₁ΔH purified from yeast. We found that the majority of the binding energy between H and V₁ is contributed by the interaction between HNT and EG and that binding of HNT to EG is much stronger when EG is part of V₁ compared with isolated EG. However, only one copy of HNT binds V₁ΔH with high affinity, indicating that the three EGs on V₁ are in different conformations and that only one of these conformations (EG1) is competent for H binding. The three different conformations of the EGs are evident from the crystal structure of autoinhibited V₁ (22) as well as the cryo-EM structures of V₁Vo (20, 21). The observation that HNT binds only EG1 as part of V₁ with high affinity suggests that HNT’s preference is a result, and not the cause, of the conformational asymmetry of the peripheral stalks, which most likely originates in the catalytic core of V₁ (A₁B₁DF). Although we were not able to detect an interaction between isolated HCT and V₁ΔH, the observation that intact H binds V₁ΔH with significantly higher affinity compared with HNT suggests that the contact between HCT and A₁B₁DF seen in the V₁ crystal structure (22) contributes to the avidity of the V₁–H interaction. In addition, much like the Cfoot–HNT–EG low-affinity (but high-avidity) ternary junction, we found that H and aNT interact weakly. Taken together, the data support and extend our earlier findings that V₁ and Vₒ are held together by multiple weak interactions that allow rapid breaking and reforming in response to cellular needs.

MgATP hydrolysis–dependent destabilization of the V₁–H interaction

Studies in yeast have shown that membrane-detached V₁ has no measurable MgATPase activity, a property of WT V₁ that has been attributed to the presence of the inhibitory H subunit (25). Therefore, our BLI data showing that the V₁–H interaction is destabilized in the presence of MgATP came as a surprise as we did not expect V₁ΔH to be catalytically active. In contrast, previous biochemical studies had shown that V₁ΔH retained ~20% MgATPase activity upon addition of an excess of recombinant H, an observation that, at the time, was attributed to substoichiometric binding of H (39). However, using pulldown assays, we here show that a stoichiometric complex of V₁ΔH with H has indeed transient MgATPase activity, indicating that WT V₁ isolated from yeast is not equivalent to recombinant V₁ΔH. One striking difference between V₁ and V₁ΔH is that V₁ contains ~1.3 mol/mol of tightly bound ADP, whereas V₁ΔH, and by extension V₁Δ(H)H, has only ~0.4 mol/mol of ADP (22). This suggests that V₁’s ATPase activity is inhibited by tightly bound ADP and that the lack of ADP in V₁Δ(H)H allows transient MgATP hydrolysis with the associated conformational changes leading to destabilization of the V₁–H interaction on the BLI sensor.

MgADP inhibition is a conserved feature of the catalytic headpiece of rotary ATPases wherein, under ATP-regenerating conditions, the rate of MgATP hydrolysis decreases due to retention of tightly bound MgADP at a closed catalytic site. The MgADP–inhibited state is a conformation off-pathway from the catalytic cycle and associated with a structural change in the catalytic site (43). MgADP inhibition has been observed in both the F₁–ATPase (e.g. F₁ from bovine heart (44) and Bacillus PS3 (45)) and the cytosolic A₁/V₁ sector from Thermus thermophilus (46). Parra et al. (25) reported a decrease in MgATPase
activity of purified yeast \( V_1 \Delta H \) using \( P_i \) release assays, and we have observed a similar decrease in MgATPase activity of \( V_1 \Delta H \) using an ATP-regenerating assay system (22) (Fig. S4).

**Interplay of MgADP inhibition with the \( V_1 \)−H interaction**

The structure of autoinhibited \( V_1 \) revealed an inhibitory loop in \( H_{CT} \) (amino acids 408 – 414) that mediates important contacts with \( V_1 \). First, \( H_{CT} \) binds to the C-terminal domain of subunit B, thereby stabilizing the corresponding catalytic A–B interface in its open conformation. Second, it interacts with two \( \alpha \)-helical turns in the central rotor subunit D (residues 38 – 45) (22) (Fig. 2B). At any given rotational state of \( V_1 \), only one of the catalytic sites is in the open state with the two \( \alpha \)-helical turns from the central rotor facing the open site (22, 47). It is therefore evident that \( H_{CT} \) preferentially binds to the open catalytic site with the central rotor in a particular conformation. Our data suggest that the peripheral stalks exhibit a conformational asymmetry, which most likely originates from the conformations of the catalytic core of the enzyme \( (A_3B_3DF) \). \( H_{NT} \) preferentially interacts with \( EG1 \), the peripheral stalk associated with the open catalytic site. Under the conditions of our BLI experiments, when \( V_1 \Delta H \) is bound to subunit H on the sensor, both \( H_{NT} \) and \( H_{CT} \) are associated with their binding partners at the open catalytic site, resulting in overall tight binding of H as evident from the observed slow dissociation of \( V_1 \Delta H \) from the BLI sensor (Fig. 8A). When sensors containing \( V_1 \Delta H \) bound to MBP-H are dipped into MgATP, the nucleotide binds to the open catalytic site and is hydrolyzed. Subsequent MgATP hydrolysis results in central stalk rotation, which destabilizes the interaction with \( H_{CT} \). The conformational changes are also propagated to the peripheral stalks, resulting in destabilization, and ultimately breaking, of the interaction between \( H_{NT} \) and \( EG1 \) (Fig. 8B). However, MgATP hydrolysis on \( V_1(\Delta H)H \) is transient and stops once inhibitory MgADP gets trapped in a tight catalytic site. All the \( V_1(\Delta H)H \) complexes that withstood transient MgATP hydrolysis are now bound with high affinity because the binding site for \( H_{CT} \) is restored once the MgADP-inhibited conformation is obtained (Fig. 8C). Therefore, we conclude that the lack of inhibitory MgADP in \( V_1(\Delta H)H \) allows transient MgATP hydrolysis and destabilization of the \( V_1 \)–H interaction and that high-affinity binding of H is restored once inhibitory MgADP is trapped in a catalytic site.

Considering the here observed MgATP hydrolysis-dependent destabilization of the interaction between H (and \( H_{NT} \)) with \( V_1 \) raises the question: how is this interaction maintained in \( V_1V_\circ \) during rotational catalysis? Between the three rotational states of the enzyme observed by cryo-EM (21), minor conformational differences are observed for the peripheral stalk bound to H (EG1 in \( V_1V_\circ \)). In \( V_1V_\circ \), besides providing binding sites for both \( H_{NT} \) and \( H_{CT} \), \( a_{NT} \) also interacts with and probably stabilizes the N termini of \( EG1 \) (Figs. 1A and 2A). The N-terminal region of the peripheral stalks have been described as unstable and flexible based on experiments conducted with isolated EG (33) and EG as part of \( A_3/V_1 \) (48) and as seen in the \( V_1 \) crystal structure (22). With \( EG1 \)’s N termini unsupported, as in membrane-detached \( V_1 \), expected conformational changes associated with rotary catalysis would be larger than those observed in \( V_1V_\circ \). Hence, although multiple interactions with \( a_{NT} \) maintain the conformation of \( EG1 \) in \( V_1V_\circ \), the lack of these interactions in \( V_1 \) enable rotary catalysis—driven conformational changes in \( EG1 \) with concomitant destabilization of the \( H_{NT} \)–\( EG1 \) interaction.

**Implications for the mechanism of reversible disassembly**

Experiments conducted with yeast spheroplasts, isolated vacuoles, and purified \( V_1V_\circ \) have established that efficient disassembly of V-ATPase requires a catalytically active complex (49 – 51). From a structural comparison with the three rotational states of \( V_1V_\circ \), we previously noted that upon disassembly of the holoenzyme autoinhibited \( V_1 \) and \( V_\circ \) end up in dif-
different rotational states: $V_1$ in state 2 and $V_o$ in state 3 (22, 52, 53). This suggests that the MgATPase activity that is necessary for efficient disassembly serves to generate the rotational state mismatch associated with enzyme dissociation, a mismatch that likely functions to prevent rebinding of $V_1$ to $V_o$ under cellular conditions that favor the disassembled state. It is well established that enzyme dissociation is accompanied by a release of subunit C into the cytosol, and it is possible that the energy from ATP hydrolysis also serves to break the high-affinity EGCh head interaction (33). Live cell imaging has captured $V_1$ on vacuolar membranes while C is released into the cytosol upon glucose removal, suggesting that C release may be one of the initial steps of disassembly. Our data suggest that, due to catalysis-driven conformational changes in EG, membrane-detached V1 sector is incapable of binding H in a stable conformation while MgATP is being hydrolyzed at the catalytic sites. Therefore, it is possible that $V_1$ detaches from $V_o$ on vacuolar membranes after its MgATPase activity is completely silenced. The specificity of HNT and HCT for their binding sites on V1 supports a model wherein once $V_1$ is MgADP-inhibited in rotational state 2 the proximity of the open catalytic site and central rotor favors HNT’s conformational switch to its inhibitory position on $V_1$. At the same time, by binding and stabilizing the open catalytic site, HCT facilitates the trapping of MgADP in the adjacent closed catalytic site (22). Hence, inhibitory MgADP and subunit H synergize by stabilizing each other to ensure that free $V_1$ remains in the autoinhibited state to prevent wasteful ATP hydrolysis when V-ATPase’s proton-pumping activity is down-regulated.

The autoinhibited state of $V_1$ (with MgADP in a closed catalytic site stabilized by HCT, bound to an open catalytic site) is likely a low-energy state of $V_1$. For reassembly to occur, $V_1$ needs to be “reactivated” to allow HCT to switch from its binding site on $V_1$ to bind $a_{NT}$ in $V_1V_o$. Based on our observations in this study, we speculate that release of inhibitory MgADP and subsequent MgATP binding/hydrolysis induce structural changes in $V_1$ that detach HCT, making it available to bind $a_{NT}$, in turn coupling $V_1$ to $V_o$. What then causes the required release of inhibitory MgADP from cytosolic $V_1$? Although this mechanism is currently not understood, it is possible that one of the protein factors that have been shown to be required for efficient reassembly, such as the regulator of the ATPase of vacuolar and gDonaldal membranes (RAVE) complex (54) or aldolase (55), plays a role in the release of inhibitory ADP, thereby allowing HCT to assume its binding site on $a_{NT}$ and restore MgATP hydrolysis−driven proton pumping.

**Experimental procedures**

**Materials and methods**

Plasmids encoding subunit H and its C-terminal domain (HCT, residues 352−478) N-terminally tagged with a Precission protease-cleavable maltose-binding protein (MBP-H and MBP-HCT encoded by a pMalP-Pase vector derived from pMAL-c2E), a yeast strain deleted for subunits H and G (39), and a pRS315 vector containing FLAG-tagged subunit G (56) were kindly gifts from Dr. Patricia Kane, SUNY Upstate Medical University.

**Plasmid construction**

The plasmid expressing the N-terminal domain of subunit H (HNT; residues 1−354) was made using the above MBP-H pMalP-Pase vector as a template for QuikChange mutagenesis to delete the nucleotide sequence coding for amino acids 355−478 using the following primers: HNT−354 F, GGA AAT CCT AGA AAA CGA GTA TCA AAG GCT TGG CAC TGG CCG TCG TTT TAC AAC GTC G; HNT−354 R, GAC GGC CAC TGC CAA GCT TTT AGG TCA ATT CTT GGT ACT CTT TTA CAA GTA TTT CGT TG. The construction of the pMalP-Pase plasmid encoding N-terminally MBP-tagged $a_{NT}$ (1−372) has been described (26).

**Expression and purification of recombinant V-ATPase subunits**

V-ATPase subunit constructs HNT, EG, HCT, H, and $a_{NT}$ were expressed in *E. coli* strain Rosetta2, grown to mid-log phase in rich broth (LB Miller plus 0.2% glucose) supplemented with ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (except for expression of EG where 1 mM isopropyl β-D-thiogalactopyranoside was used). Expression was induced at 30 °C for 6 h for HNT, 20 °C for 6 h for H, 20 °C for ~16 h for $a_{NT}$, 25 °C for ~16 h for $HCT$, and 30 °C for 6 h for EG. Cells were harvested by centrifugation, resuspended in amylose column buffer (CB; 20 mM Tris-HCl, 1 mM EDTA, pH 7.4), and stored at ~20 °C until use. For purification, cells were treated with DNase (67 μg/ml), lysozyme (840 μg/ml), and PMSF (1 mM) before lysis by sonication. The lysate was then cleared by centrifugation at 12,000 × g for 30 min, the supernatant was diluted 1:4 with CB and applied to an amylose inclusion chromatography using a Superdex S75 column (1.6 cm) for H and HCT and a Superdex 200 column of the same size for EG heterodimer. EG heterodimer was purified as described (33).

**Purification of $V_1$**

$V_1$-ATPase lacking subunit H was purified as described (22). Briefly, the yeast strain deleted for the genes encoding subunits H and G was transformed with a pRS315 plasmid encoding subunit G with an N-terminal FLAG tag (56). The cells were grown in synthetic defined medium lacking Leu to an OD of ~4.0 and harvested by centrifugation, and the cell pellets were resuspended in TBSE (25 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5 mM EDTA) and stored at ~80 °C until use. Thawed cells...
were supplemented with 5 mM β-mercaptoethanol, 1 mM PMSF, and 2 μg/ml each of pepstatin and leupeptin before lysis by 15 passes through a microfluidizer with intermittent cooling on ice. The lysate was centrifuged at 4000 × g for 25 min, and the resultant supernatant was centrifuged again at 13,000 × g for 40 min. The cleared lysate was applied to a 5-ml FLAG column (Sigma) topped with Sephadex G50 and pre-equilibrated in TBSE. The column was washed with 10 column volumes of TBSE and eluted using 0.1 mg/ml FLAG peptide. V₁ΔH-containing fractions were pooled, concentrated, and resolved using a Superdex 200 1.6 × 50-cm column attached to an ÄKTA FPLC (GE Healthcare). Fractions were analyzed by SDS-PAGE and concentrated to 10 mg/ml, and the activity of the complex was measured using a coupled enzyme assay as described below (22).

**CD spectroscopy**

CD spectra were recorded on an Aviv 202 spectropolarimeter using a 2-mm–path length cuvette. CD spectra were recorded between 250 and 195 nm in 25 mM sodium phosphate, pH 7, and protein stability was monitored by recording the CD signal at 222 nm as a function of temperature. For cysteine-containing proteins, 0.3–1 mM TCEP was included in the buffer. Protein concentrations of H, HNT, HCT, and αNT were 2, 2.25, 9.2, and 2.36 μM, respectively. The far-UV CD spectrum of 6.7 μM HNT–EG complex was obtained with protein dissolved in 20 mM Tris-HCl, 1 mM TCEP. CD spectra were recorded on an Aviv 202 spectropolarimeter to monitor protein–protein binding and dissociation for the complex was measured using a coupled enzyme assay as described below (22).

**Isothermal titration calorimetry**

The thermodynamic parameters of the interaction between HNT and EG and between HCT and αNT were determined using a Microcal VP-ITC isothermal titration calorimeter. The interaction of HNT and EG was monitored by titrating a stock of 0.278 mM HNT into 0.0315 mM EG in 20 mM Tris-HCl, 0.5 mM EDTA, 1 mM TCEP, pH 8, at 10 °C. A total of 30 injections with 5% saturation per injection was carried out. The average value of signal postsaturation (last eight titration points) was subtracted from the HNT into EG titration. Complex formation between αNT and H was analyzed by titrating 0.3 mM H into 0.017 mM αNT in 20 mM Tris-HCl, 0.5 mM EDTA, 1 mM TCEP, pH 8. A blank titration of H into buffer was subtracted from the αNT into H titration using the curve fit option in OriginLab. ITC data were analyzed using VP-ITC programs in OriginLab.

**Biolayer interferometry**

BLI was used to measure the association and dissociation kinetics of interaction between V₁ΔH and MBP-tagged H, HNT, and HCT. An Octed-RED system and AMC–coated sensors (FortéBio, AMC biosensors, catalogue number 18-5088) were used to monitor protein–protein binding and dissociation for determination of binding affinities. Anti-MBP antibody (New England Biolabs) at 1 μg/ml was immobilized on the AMC biosensors. The anti-MBP antibody formed the bait for MBP-tagged H, HNT, and HCT (used at 5 μg/ml). Biosensors with immobilized H, HNT, or HCT were dipped in varying concentrations of V₁ΔH followed by buffer to measure association and dissociation rates. BLI buffer (20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.5 mg/ml BSA) was used in all BLI experiments to reduce nonspecific binding to the biosensors except for experiments in the presence of nucleotides where the EDTA concentration was reduced to 0.5 mM. All steps were done at 22 °C with each biosensor agitated in 0.2 ml of sample at 1000 rpm and a standard measurement rate of 5 s⁻¹. Control experiments were performed to check for any nonspecific binding interaction between the antibodies and the proteins used. Reference sensors were used in each experiment with immobilized MBP-H/HNT/HCT but no V₁ΔH. FortéBio data analysis software (version 6.4) was used for subtraction of reference sensors, Savitzky-Golay filtering, and global fitting of the kinetic rates of V₁ΔH binding with H, HNT, or HCT.

**ATPase activity assays**

MgATPase activity of V₁, V₁ΔH, and V₁(ΔH)H was measured using an ATP-regenerating assay as described (22). Briefly, 10 μg of the V₁ 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, 50 mM HEPES, pH 7.5, at 37 °C. The decrease of absorbance at 340 nm was measured in the kinetics mode on a Varian Cary Bio100 spectrophotometer.

**Native gel electrophoresis**

For native gel electrophoresis, purified V-ATPase subunits and subcomplexes were resolved using 2% agarose gels in 20 mM bis-Tris-acetic acid, pH 6, 1 mM TCEP. Gels were resolved for 1 h at 100 V, fixed in 25% iso-propanol, 10% acetic acid for 30 minutes, rinsed in 95% ethanol, and dried on a slab dryer for 2 h at 80 °C. The dried gel was stained with Coomassie G and destained in fixing solution.

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