Subunit Interactions and Requirements for Inhibition of the Yeast V<sub>1</sub>-ATPase<sup>1,3</sup>

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Disassembly of the yeast V-ATPase into cytosolic V<sub>1</sub> and membrane V<sub>0</sub> sectors inactivates MgATPase activity of the V<sub>1</sub>-ATPase. This inactivation requires the V<sub>1</sub> H subunit (Parra, K. J., Keenan, K. L., and Kane, P. M. (2000) J. Biol. Chem. 275, 21761–21767), but its mechanism is not fully understood. The H subunit has two domains. Interactions of each domain with V<sub>1</sub> and V<sub>0</sub> subunits were identified by two-hybrid assay. The B subunit of the V<sub>1</sub> catalytic headgroup interacted with the H subunit N-terminal domain (H-NT), and the C-terminal domain (H-CT) interacted with V<sub>1</sub> subunits B, E (peripheral stalk), and D (central stalk), and the cytosolic N-terminal domain of V<sub>0</sub> subunit Vph1p. V<sub>1</sub>-ATPase complexes from yeast expressing H-NT are expressed and purified H-CT domain inhibits MgATPase activity, cytosolic pH homeostasis, and resistance to multiple organelle acidification, V-ATPases are linked to cellular func-

V-ATPases are ubiquitous proton pumps responsible for compartment acidification in all eukaryotic cells (1, 2). These pumps couple hydrolysis of cytosolic ATP to proton transport into the lysosome/vacuole, endosomes, Golgi apparatus, clathrin-coated vesicles, and synaptic vesicles. Through their role in organelle acidification, V-ATPases are linked to cellular functions as diverse as protein sorting and targeting, zymogen activation, cytosolic pH homeostasis, and resistance to multiple types of stress (3). They are also recruited to the plasma membrane of certain cells, where they catalyze proton export (4, 5).

V-ATPases are evolutionarily related to ATP synthases of bacteria and mitochondria and consist of two multisubunit complexes, V<sub>1</sub> and V<sub>0</sub>, which contain the sites for ATP hydrolysis and proton transport, respectively. Like the ATP synthase (F-ATPase), V-ATPases utilize a rotational catalytic mecha-
nism. ATP binding and hydrolysis in the three catalytic subunits of the V<sub>1</sub> sector generate sequential conformational changes that drive rotation of a central stalk (6–8). The central stalk subunits are connected to a ring of proteolipid subunits in the V<sub>0</sub> sector that bind protons to be transported. The actual transport is believed to occur at the interface of the proteolipids and V<sub>0</sub> subunit a. Rotational catalysis will be productive in proton transport only if V<sub>0</sub> subunit a is held stationary, whereas the proteolipid ring rotates (8). This “stator function” resides in a single peripheral stalk in F-ATPases (9, 10), but is distributed among up to three peripheral stalks in V-ATPases (11–13). The peripheral stator stalks link V<sub>0</sub> subunit a to the catalytic headgroup and ensures that there is rotation of the central stalk complex relative to the V<sub>0</sub> a subunit and catalytic headgroup.

Eukaryotic V-ATPases are highly conserved in both their overall structure and the sequences of individual subunits. Although homologs of most subunits of eukaryotic V-ATPases are present in archaeobacterial V-ATPases (also known as A-ATPases), the C and H subunits are unique to eukaryotes. Both subunits have been localized at the interface of the V<sub>1</sub> and V<sub>0</sub> sectors, suggesting that they are positioned to play a critical role in structural and functional interaction between the two sectors (14–16). The yeast C and H subunits are the only eukaryotic V-ATPase subunits for which X-ray crystal structures are available (17, 18). The structure of the C subunit revealed an elongated “dumbbell-shaped” molecule, with foot, head, and neck domains (18). The structure of the H subunit indicated two domains. The N-terminal 348 amino acids fold into a series of HEAT repeats and are connected by a 4-amino acid linker to a C-terminal domain containing amino acids 352–478 (17). These two domains have partially separable functions in the context of the assembled V-ATPase (19). Complexes containing only the N-terminal domain of the H subunit (H-NT)<sup>2</sup> supported some ATP hydrolysis but little or no proton pumping in isolated vacuolar vesicles (19, 20). The C-terminal domain (H-CT) assembled with the rest of the V-ATPase in the absence of intact subunit H, but supported neither ATPase nor proton pumping activity (19). However, co-expression of the H-NT and H-CT domains results in assembly of both sectors with the V-ATPase and allows increased ATP-driven proton

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pumping in isolated vacuolar vesicles. These results suggest that the H-NT and H-CT domains play distinct and complementary roles even when the two domains are not covalently attached.

In addition to their role as dedicated proton pumps, eukaryotic V-ATPases are also distinguished from F-ATPases and archaeal V-ATPases in their regulation. Eukaryotic V-ATPases are regulated in part by reversible disassembly of the V₁ complex from the V₀ complex (1, 21, 22). In yeast, disassembly of previously assembled complexes occurs in response to glucose deprivation, and reassembly is rapidly induced by glucose read-
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VPH1 was amplified with VPH1 5′-2H and VPH1 3′-2H. All primer sequences are listed in supplemental Table 1.

Two-hybrid Assays—Pairs of pACT and pAS-1 plasmids were introduced into yeast strain pJ69-4A (MATa trpl-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80A LYS2::GAL1-HIS3 GAL2-ade2 met2::GAL7-lacZ (34)) by cotransformation, and transformants were selected on supplemented minimal medium lacking tryptophan and leucine (SC-trp,-leu). Transformants were then grown in liquid medium, serially diluted, and spotted onto: 1) SC-trp,-leu plates (control); 2) SC-trp,-leu,-his plates containing 2.5 mM aminotriazole (to select for expression of the HIS3 gene as a marker of two-hybrid interaction); 3) SC-trp,-leu,-adenine (to select for expression of the ADE2 gene as a marker of two-hybrid interaction); and 4) SC-trp,-leu,-his,-ade (to select for expression of both markers). All of the interactions shown in Fig. 1 were observed on plates 2–4.

Expression and Affinity Purification of MBP- and FLAG-tagged Proteins and Complexes—For bacterial expression of MBP-tagged subunit H and the H-CT fragment, the expression constructs described above were transformed into E. coli BL21 pLysE and plated on LB + chloramphenicol (34 μg/ml) + ampicillin (100 μg/ml). MBP-tagged Vph1-NT was transformed into E. coli BL21 and transformants were selected on LB + ampicillin. Transformed cells were inoculated into LB liquid medium with 125 μg/ml ampicillin and grown overnight at 30 °C. 10 ml of the culture was added to 1 liter of LB containing ampicillin, supplemented with 2 g of glucose. The culture was allowed to grow at 37 °C until it reached A600 of 0.6–0.8, at which point isopropyl-β-D-galactopyranoside was added to 300 μM. Cells expressing H and H-CT were then induced for 2 h at 37 °C, whereas the cells expressing Vph1-NT were induced at 30 °C for 6 h. After induction, cells were pelleted by centrifugation, resuspended in TBSE buffer (20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5 mM EDTA), and frozen at −20 °C until use. For Vph1-NT purification, the final suspension was adjusted to 5 mM β-mercaptoethanol. For purification, frozen cell pellets were thawed, and the suspension was sonicated 3 times for 25 s on ice. Lysed cells were centrifuged at 15,000 × g in a J-20 Beckman rotor for 30 min at 4 °C. The supernatant was passed over 1 ml of amylase resin (New England Biolabs) once at the rate of 0.5 ml/min or less. The amylase resin was washed with at least 20 column volumes of cold TBSE and then eluted with 5 fractions of 1 ml of TBSE with 100 mM maltose. 5 ml of eluant was concentrated in a Viva Spin 30-kDa cut off centrifugal filter device (Sartorius) to 300 μl or less. The concentrated sample was then further purified on a SEC250 gel filtration column (Bio-Rad) equilibrated with TBSE. Fractions of the predicted molecular mass of monomeric Vph1-NT were collected, pooled, and the protein concentration was determined from absorption at 280 nm using an extinction coefficient obtained from ProtParam Program (ExPaSy). If the experiment required Vph1-NT to be detached from MBP, then 20 μl of Prescision protease at 3 mg/ml was added and left overnight at 4 °C after the concentration step, but before injection into the fast protein liquid chromatography gel filtration column. MBP-tagged H and H-CT were purified similarly except that no β-mercaptoethanol was added to the initial suspension, and the MBP-tagged protein was combined with 1 ml of amylase beads per 50 ml of crude cell extract. The mixture was incubated at 4 °C for 1–2 h with gentle rocking, then the beads were washed three times with 20 ml of TBSE followed by elution in TBSE containing 100 mM maltose. There was no further purification of the eluted proteins; they were used directly, either with or without protease cleavage.

V₁ complexes were affinity purified from yeast cells via FLAG-tagged G subunit as described (13) with the following modifications. Cells were not incubated with zymolyase before lysis in the microfluidizer. After ammonium sulfate precipitation and desalting, the cytosolic fraction was centrifuged in a TLA-100 ultracentrifuge for 15 min at 52,000 × g before application of the supernatant to the anti-FLAG M2 affinity column. V₁ complexes were eluted with FLAG peptide and used directly in most experiments. An additional gel filtration step using a Sephadex G-250 column on a Bio-Rad BioLogic Duo-Flow fast protein liquid chromatography system was added after affinity purification in a few experiments, as indicated in the figure legends. To test binding of H-CT to V₁ pre-bound to anti-FLAG beads (Fig. 4C), V₁ complexes were mixed with anti-FLAG beads and incubated for 1 h at 4 °C (~125 μg of V₁ was mixed with 100 μl of beads to give a final volume of 220 μl in TBSE). Purified MBP-H-CT was then added to the mixture (~9 μg from an 0.1 mg/ml solution). This mixture was incubated for 2–3 additional hours at 4 °C, then the beads were pelleted by centrifugation, rapidly washed twice to remove unbound MBP-H-CT, and the bound material was eluted and subjected to SDS-PAGE and immunoblotting. For experiments with MgATP, 2 mM ATP and 2 mM MgCl₂ were added with the MBP-H-CT.

Enzyme and Binding Assays—ATP hydrolysis activity was measured by a coupled enzyme assay at 37 °C as described (35). V₁(-H) complexes were incubated with expressed MBP-H or MBP-H-CT for 1 h in the indicated ratios before addition to the ATPase assay mixture. V₁ containing H-NT (V₁(-H)/H-NT) was incubated and assayed in the same manner. NEM-sensitive ATPase activity (V₁-ATPase activity) was determined by incubating V₁ samples for 20 min on ice with 50 μM NEM prior to assay (19).

Immunoprecipitation of the FLAG-tagged proteins was used to assess binding of the bacterially expressed proteins to the purified V₁(-H) or V₁(-H)/H-NT. 100 μl of anti-FLAG-agarose beads suspended in TBSE were added to a mixture of one part V₁ and five parts expressed protein (MBP-H, MBP-H-CT, or the cleaved proteins) to give a final volume of 300 μl. The mixtures were incubated at 4 °C with gentle shaking for 3 h. The resin was washed three times with TBSE to remove nonspecific binding. 100 μg/ml FLAG peptide was used to elute V₁ and its binding partners from the beads and eluted protein were concentrated by trichloroacetic acid precipitation. Trichloroacetic acid pellets were solubilized with cracking buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% SDS, 5% β-mercaptoethanol). Samples were separated by SDS-PAGE followed by Coomassie staining. For immunoblotting, samples were transferred to a nitrocellulose membrane. The blots were probed with anti-MBP (1:100,000) and anti-Myc monoclonal antibodies. A colorimetric assay was used for detection after incubation with an anti-mouse secondary antibody linked to alkaline phosphatase.
For immunoprecipitation of Vph1-NT, 250 μl of cultured supernatant containing monoclonal antibody 10D7 (36) was added to a 200-μl mixture of Vph1pNT and a partner protein that had been allowed to stand for ∼3 h on ice. The mixture of the proteins and the antibody was shaken overnight at 4 °C. The next day, 100 μl of a 50% (v/v) Protein A-Sepharose suspension (Sigma) in phosphate-buffered saline buffer (137 mM NaCl, 2.6 mM KCl, 12 mM sodium phosphate, containing 0.2% gelatin) was added to the mixture and incubated on ice for 2 h with occasional shaking. Protein A-Sepharose was gently pelleted by centrifugation and washed twice with the same buffer. The samples were eluted by directly adding cracking buffer to the pellets.

RESULTS

Interactions of the N- and C-terminal Domains of Subunit H with $V_1$ and $V_0$ Subunits—The crystal structure of yeast subunit H indicated that it was comprised of two domains, one consisting of the N-terminal 348 amino acids of the protein (H-NT) and the second consisting of amino acids 352–478 (H-CT) (17). To address the interactions of the two domains with $V_1$ and $V_0$, we introduced each H subunit domain into a two-hybrid vector, and tested its interaction with several $V_1$ subunits and the cytosolic N-terminal domain of $V_0$ subunit a. In this version of the two-hybrid assay, all combinations should grow on supplemented minimal medium lacking leucine and tryptophan, because these two nutritional markers are encoded on the two-hybrid plasmids. As we have seen previously, some combinations of plasmids partially inhibit growth even under the permissive (+His) conditions, possibly reflecting dominant negative interactions as a result of subunit overexpression (13, 27, 37). Two-hybrid interactions are identified by their ability to support growth on medium that also lacks histidine, which requires reconstitution of a transcription factor capable of inducing the HIS3 gene through interaction of the partners (34). As shown in Fig. 1, H-NT displayed an interaction only with the $V_1$ B subunit. H-CT interacted with a number of subunits, including the $V_1$ B, D, and E subunits, and the $V_0$ a-NT domain. Interactions with subunit B of the catalytic headgroup, subunit E, of the peripheral stalks, and the a-NT were expected from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was unexpected. This interaction could from previous results. Interaction with the D subunit, of the central rotor stalk was un

![FIGURE 1. Two-hybrid assay for interactions of H-NT and H-CT with other V-ATPase subunits.](Image)

![H-NT](Image)

![H-CT](Image)

with $V_1$(-H), we visualized both whole cell lysates and the purified $V_1$ complexes by immunoblotting to detect the Myc epitope present on H-CT. As shown in Fig. 2, A and B, the Myc-tagged H-CT was present in the whole cell lysates, but not in the purified $V_1$ sectors. These results indicate that H-CT does not co-purify with $V_1$(-H) complexes.

In contrast, the immunoblots in Fig. 2, A and D, show that Myc-tagged H-NT was present in both the whole cell lysate and the purified $V_1$(-H)/H-NT complexes. Coomassie staining of the isolated $V_1$ complexes also supports co-purification of the H-NT with established $V_1$ subunits present in the assembled $V_1$ complexes containing subunit H (Fig. 2C). Comparison of staining in the region corresponding to H-NT to the staining of subunit D, which is also believed to be a single copy subunit, suggests that H-NT levels are substoichiometric. We measured NEM-sensitive MgATPase activity in the isolated $V_1$(-H)/H-NT complexes. The average NEM-sensitive ATPase activity in these complexes was 0.49 ± 0.04 μmol/min/mg protein (mean ± S.E., n = 3), whereas the average $V_1$ activity from strains lacking any subunit H was 1.92 ± 0.57 μmol/min/mg (n = 5). (NEM is a specific inhibitor of the $V_1$ ATPase that binds at the catalytic site.) These results indicate that the H-NT domain can be co-purified with $V_1$, and that MgATPase activity of $V_1$(-H) complexes is partially inhibited by the presence of H-NT. These results are summarized in Table 1.
Inhibitory Activity of the Expressed H-CT Domain—The absence of H-CT from isolated V₁ sectors could reflect either poor binding to V₁ sectors or preferential binding to other partners in the cell. To assess the ability of the H-CT domain to bind V₁(-H) and inhibit V₁-ATPase activity, we first expressed both the intact H subunit and H-CT as N-terminal MBP fusions in E. coli, and purified the proteins by amylose affinity chromatography. As shown in Fig. 3A, both proteins were obtained in good purity, although some fragments were present with the MBP-H fusion even without addition of protease. Cleavage with protease generated MBP and untagged H or H-CT. We next assessed the ability of both H and H-CT to bind and inhibit V₁. As shown in Fig. 3B, the expressed, intact H subunit efficiently inhibited MgATPase activity in V₁(-H) complexes, even when present at only a 2-fold molar excess over V₁(-H). Somewhat more surprisingly, the H-CT inhibited almost as efficiently, although the level of inhibition reached was never as high (average of 80% inhibition for intact H and 62% for H-CT at 5-fold excess). There was no difference in inhibition by MBP-tagged H and H-CT versus H and H-CT that had been proteolytically cleaved from MBP. We also expressed and purified MBP alone and tested for inhibition of V₁-ATPase activity. There was no inhibition by MBP alone (data not shown).

When expressed together in yeast, the H-NT and H-CT domains show increased activation of ATP-driven proton transport by the V-ATPase in vacuolar vesicles over either domain alone (19). We next tested whether the H-CT would give further inhibition of ATPase activity in V₁(-H)/H-NT. We found that addition of H-CT to H-NT-containing V₁ complexes inhibited

![FIGURE 2. Isolation of V₁ sectors from yeast strains containing H-CT and H-NT. A, immunoblot of whole cell lysates from vma13Δ cells, which completely lack subunit H (V₁(-H)), vma13Δ cells expressing Myc-tagged H-NT from a plasmid (V₁(-H)/H-NT), and vma13Δ cells expressing Myc-tagged H-CT (V₁(-H)/H-CT). Lysates were separated by SDS-PAGE and transferred to nitrocellulose, and Myc-tagged proteins were detected with anti-Myc monoclonal antibody. B, V₁ sectors were purified from vma13Δ cells expressing Myc-tagged H-CT as described under “Experimental Procedures,” and visualized by Coomassie Blue staining (left) or immunoblot using anti-Myc antibody (right). C and D, V₁ sectors were isolated from vma13Δ cells (V₁(-H)) and vma13Δ cells containing a plasmid-borne H-NT (V₁(-H)/H-NT). Isolated V₁ sectors from both strains were separated by SDS-PAGE and visualized by Coomassie Blue staining in C, and by anti-Myc immunoblot in D. The Myc-tagged H-NT runs at ~40 kDa. The position of molecular mass standards are shown on the left of all panels. Note that in panels B–D, V₁ was purified by anti-FLAG affinity chromatography without a final gel filtration step. Under these conditions, varying amounts of a band running near 95 kDa are present in the preparation, along with the expected V₁ subunits indicated.

| TABLE 1 Summary of V₁-ATPase inhibition experiments |
|-----------------------------------------------------|
| V₁ complex expressed in yeast | Bacterially expressed (H subunit added) | H subunit bound? | % V₁(-H) (ATPase activity) |
|-------------------------------|------------------------------------------|-----------------|--------------------------|
| V₁(-H)                        | None                                     | None            | 100                      |
| V₁(-H)/H-NT                   | None                                     | H-NT co-isolates with V1 | 26           |
| V₁(-H)/H-CT                   | None                                     | H binds tightly through repurification | 20           |
| V₁(-H)                        | Full-length H                            | Very little detected after repurification | 38           |
| V₁(-H)                        | H-CT                                    | More H-CT retained after repurification | ND*          |
| V₁(-H)/H-NT                   | H-CT                                    | H subunit partially displaces H-NT | 5 (20)*       |
| V₁(-H)/H-NT                   | Full-length H                            | ND              | 14 (55)*                 |

* ND, not determined.  
* Activity relative to isolated V₁(-H)/H-NT complexes (from Fig. 3) is shown in parentheses. To compare the overall activity and inhibition of these complexes to V₁(-H) complexes, the % activity in the V₁(-H)/H-NT complexes as isolated (26%) was multiplied by the proportion of the activity remaining after addition of expressed H or H-CT.
an average of 55% of the MgATPase activity present in these complexes (Fig. 3C). The uncleaved MBP-H-CT inhibited both $V_1(\cdot H)$ and $V_1(\cdot H)/H$-NT complexes to a level comparable with the cleaved H-CT. This result suggests that steric hindrance between the MBP and H-NT does not significantly affect inhibition. We also tested whether the expressed, intact H subunit could further inhibit $V_1(\cdot H)/H$-NT complexes. As shown in Fig. 3C, the intact H subunit did inhibit $V_1(\cdot H)/H$-NT containing complexes as efficiently as it inhibited $V_1(\cdot H)$ complexes. These results are summarized in Table 1.

Inhibition by intact H in the presence of H-NT was somewhat surprising, but one explanation might be displacement of H-NT from $V_1(\cdot H)/H$-NT complexes by the intact H-CT. We tested this by incubating $V_1(\cdot H)/H$-NT complexes with intact and expressed H subunits. As shown in Fig. 3D, the intact H partially displaced H-NT, so that less Myc-tagged H-NT is present after re-isolation of $V_1$ complexes. This suggests that H-NT binds to $V_1$ less tightly than the intact H subunit and thus can be displaced.

To address whether H-CT was easily removed from $V_1$, possibly accounting for the loss of H-CT expressed in yeast during $V_1$ isolation, we incubated $V_1(\cdot H)$ complexes with expressed MBP-H subunit or MBP-H-CT under the same pre-incubation conditions as for the inhibition experiments, re-isolated $V_1$, and assessed binding of the expressed subunits. Intact MBP-H subunit was isolated in near-stoichiometric amounts with $V_1$, as suggested by the prominent band at ~95 kDa in the Coomassie-stained gel (Fig. 4A), and confirmed by MBP immunoblot in Fig. 4B. MBP-tagged H-CT, which inhibited ATPase activity as well as the cleaved H-CT, was not present in the re-isolated $V_1$. This result suggests that although the inhibition of $V_1$-ATPase activity by H-CT indicates that it must be able to bind, there may be a relatively low affinity/high off-rate that could account for its absence in $V_1$ isolated from yeast cells expressing H-CT. To further address this issue, we concen-
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V₁(-H) complexes were isolated from yeast cells by pre-binding to anti-FLAG beads, and then incubated with MBP-tagged H subunit, followed by rapid washing to elute unbound MBP-H-CT. A small amount of MBP-H-CT was present at 19.6 μM. Coomassie stained gel of the reisolated V₁ complexes is shown. B, an immunoblot with anti-MBP antibody is shown to support identification of MBP-H and the absence of MBP-H-CT after reisolation of complexes as described in Fig. 3D. Note that V₁ complexes lacking subunit H were initially isolated by both FLAG affinity chromatography and gel filtration to reduce co-purification of the 95-kDa impurity because it runs near MBP-H. C, V₁ complexes were pre-bound to anti-FLAG beads, then incubated with MBP-H-CT (with or without MgATP present) as described under "Experimental Procedures." After two washes to remove unbound MBP-H-CT, the bound material was eluted, and subjected to SDS-PAGE and immunoblotting. The immunoblot was probed with a combination of anti-MBP antibody and monoclonal antibody (8B1) against the V₁ A subunit. The indicated MBP fusion proteins were loaded singly (MBP-Vph1-NT, MBP-H-CT, or MBP-H) or incubated together for 3 h at 4 °C followed by immunoprecipitation with anti-Vph1 antibody to isolate complexes as described under "Experimental Procedures." In B, final concentrations in the mixture used for immunoprecipitation were 10.3 μM MBP-Vph1-NT and 47.6 μM MBP-H-CT. In A, final concentrations were 5.9 μM MBP-Vph1-NT and 7.5 μM MBP-H. In C, MBP-tagged H subunit was incubated with protease-cleaved Vph1-NT before immunoprecipitation because the two MBP-tagged proteins have similar molecular masses.

FIGURE 5. Binding of expressed Vph1-NT to expressed H subunit and H-CT fragment. A, amino acids 1–406 of Vph1p were expressed as an MBP fusion protein in E. coli and purified by amylose affinity chromatography. 5, 10, and 20 μg of the purified MBP-Vph1-NT were separated by SDS-PAGE and visualized with Coomassie Blue. B and C, immunoblots probed with anti-MBP antibody. The indicated MBP fusion proteins were loaded singly (MBP-Vph1-NT, MBP-H-CT, or MBP-H) or incubated together for 3 h at 4 °C followed by immunoprecipitation with anti-Vph1p antibody to isolate complexes as described under "Experimental Procedures." In B, final concentrations in the mixture used for immunoprecipitation were 10.3 μM MBP-Vph1-NT and 47.6 μM MBP-H-CT. In A, final concentrations were 5.9 μM MBP-Vph1-NT and 7.5 μM MBP-H. In C, MBP-tagged H subunit was incubated with protease-cleaved Vph1-NT before immunoprecipitation because the two MBP-tagged proteins have similar molecular masses.

Binding to the N-terminal Domain of Vph1p—The two-hybrid assay shown in Fig. 1 indicates that the H-CT domain interacts with the cytosolic N-terminal domain of Vph1p (Vph1-NT). Binding of both intact H and a fragment of the H subunit consisting of amino acids 160–478 to Vph1-NT was documented previously (28). The latter fragment contains both the entire H-CT and approximately half of the H-NT domain and is capable of complementing the growth and biochemical phenotypes of a vma13Δ mutant. To determine whether the H-CT domain alone is able to bind Vph1p-NT in vitro, we first expressed the first 406 amino acids of Vph1p as an N-terminal MBP fusion protein, and purified the protein on amylose resin. Purified MBP-Vph1-NT is shown in Fig. 5A. We then incubated MBP-Vph1-NT with MBP-tagged H-CT, followed by immunoprecipitation with monoclonal antibody (10D7) against Vph1-NT. As shown in Fig. 5B, the antibody was able to co-precipitate H-CT with Vph1-NT. These results support the interaction between the H-CT domain and Vph1-NT suggested by the two-hybrid data in Fig. 1. We also confirmed binding of the intact H subunits to both the expressed MBP-Vph1-NT, which is very close in molecular mass to the MBP-tagged H subunit, and to cleaved Vph1-NT, which is well separated (Fig. 5C).

V₁ sectors isolated from glucose-deprived cells contain the intact H subunit, but have been released from the V₀ sector. Given the evidence that Vph1-NT can bind to the H subunit and H-CT, we tested whether expressed Vph1p-NT can bind to isolated V₁ complexes, because these complexes contain the H subunit. We combined either MBP-tagged Vph1-NT or cleaved Vph1-NT with isolated, FLAG-tagged V₁ containing subunit H (to give final concentrations of 2.2 μM V₁ and 8.6 μM Vph1-NT), then re-purified V₁ complexes on an anti-FLAG column. As shown in Fig. 6 (top panel), no Vph1-NT binding was detected. We confirmed that V₁ had been re-purified by re-probing the same Western blot for V₁ subunits A and B (Fig. 6, data not shown).

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shown here is also consistent with the arrangement of H-CT and the central stalk suggested by the cross-linking experiment, because the D and F subunits are adjacent in the central stalk (38).

However, tethering of the catalytic domain/peripheral stalk to the central stalk as a means of blocking rotation does not necessarily account for all characteristics of V1 MgATPase inhibition. First, both yeast and Manduca sexta V1 complexes retain CaATPase activity even under conditions where MgATPase activity is completely inhibited and the H subunit has presumably assumed its inhibitory conformation (24, 25). The similarity between these V1-ATPases and chloroplast F1, which exhibits CaATP hydrolysis without coupling to proton transport, has been noted previously (25). In an active F1 hybrid ATPase consisting of α and β subunits from Rhodospirillum rubrum and a γ subunit from spinach chloroplast, CaATP was shown to induce rotation of the central stalk with characteristics similar to MgATP-induced rotation (39). It seems unlikely that V1-ATPases could achieve the observed rates of CaATP hydrolysis, which are similar to the rates of MgATP hydrolysis in the absence of subunit H, without rotation (24), although it is possible that CaATP binding perturbs inhibitory interactions between subunit H with the central stalk. In addition, the experiments shown here demonstrate that both the H-NT and H-CT domains individually have some inhibitory activity. Inhibition of MgATPase activity by the expressed H-CT domain could be accommodated by a tethering model for V1 inhibition because Fig. 1 indicates two-hybrid interactions of this domain with multiple V1 subunits, including a subunit of the peripheral stalk (E subunit), the catalytic headgroup (subunit B), and the central stalk (subunit D). However, inhibition of V1-ATPase activity by the H-NT domain alone does not necessarily support tethering to the central stalk as important for inhibition. There is no evidence that this domain of the H subunit approaches the central stalk, yet complexes isolated from cells containing only this domain have only 26% of the NEM-sensitive activity of complexes with no subunit H (Table 1).

It could be argued that the V1 sector does not assemble normally in the presence of H-NT only. However, the ability of the intact H subunit to mediate further inhibition and even partially displace H-NT suggests that the V1(-H) complexes are both capable of activity and susceptible to inhibition. In fact, as summarized in Table 1, if the lower level of MgATPase activity in V1(-H)/H-NT complexes isolated from yeast is considered along with the levels of inhibition achieved by addition of expressed H and H-CT, the V1(-H)/H-NT complexes are inhibited even more completely than V1(-H) complexes. This result is initially surprising. However, one intriguing possibility is that the ATPase activity of the V1(-H)/H-NT complexes shown in Fig. 3C arises largely from a population of V1(-H) complexes without H-NT bound and thus is inhibited to a similar extent as the V1(-H) complexes in Fig. 3B. Consistent with this explanation, H-NT appears to be substoichiometric in V1(-H)/H-NT complexes based on Coomassie staining (Fig. 2C). This interpretation would suggest that there is strong inhibition of ATPase activity in V1(-H)/H-NT complexes. Further experiments will be necessary to confirm this.
In our experiments, binding of H-CT to V₁(-H) complexes is notably weak, unable to survive isolation from yeast cells expressing H-CT or reisolation of purified V₁(-H) complexes incubated with bacterially expressed H-CT (Table 1). It is likely that the H-NT domain provides further binding energy in the intact subunit. Our data do not conclusively demonstrate whether the H-NT and H-CT domains have an additive inhibitory effect. MgATPase activity is further inhibited when H-CT is added to V₁(-H)/H-NT* (Fig. 3), but as described above, occupancy of these complexes with H-NT may not be complete.

Some insights into potential mechanisms for inhibition may be provided by biochemical and structural experiments with the F₁-ATPase. The Walker lab has identified several modes of action that account for mechanisms of multiple inhibitors (40). One class of inhibitor, characterized by the regulatory IF₁ action that account for mechanisms of multiple inhibitors (40).

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Does the H Subunit Contribute to Vph1p Binding to the V₁ Complex?—Previous experiments demonstrated binding of subunit H to the N-terminal cytosolic domain of Vph1p (28). The experiments described here extend those studies by identifying the C-terminal domain of subunit H as the probable site of binding. Binding of H-CT to Vph1p, along with its rather weak affinity for V₁, may help to account for the absence of H-CT in isolated V₁. In addition, improved coupling of ATP hydrolysis and proton pumping in vacuolar vesicles from a strain containing both H-NT and H-CT, relative to the strain with H-NT alone, argued that H-CT provided functionally important interactions with the V₀ sector (19). It is initially surprising, then, that the expressed Vph1-NT did not bind to V₁ containing the intact H subunit. Landolt-Marti cora et al. (28) coprecipitated Vph1p-NT with V₁ subunits, including subunit H, from yeast cells lacking an intact Vph1 subunit. Vph1p-NT has been shown to interact with the E, G, and A subunits, all of which are present in purified V₁, as well as subunit H (28, 44). It is probable that V₁ purified from a cytosolic fraction used in our experiments is in an inhibited conformation that disfavors binding to the membrane sector. The structural basis of this conformation is not understood, but it is tempting to speculate that H-CT binds at a position in free V₁ that does not allow Vph1p binding. Interestingly, the relatively large number of interactions with H-CT suggested by Fig. 1 may reflect binding partners that interact under different circumstances. There must be similar mechanisms for masking Vph1-NT and other V₀ binding sites in V₁ subunits when V₁ is released from the membrane, and it is in fact likely that there are post-translational modifications or additional protein-protein interactions in the cells that modulate binding of V₁ to Vph1NT (33, 45, 46). These cellular factors may impart glucose dependence on interactions between V₁ and Vph1-NT as well.
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