Subunit H of the Vacuolar (H\(^+\)) ATPase Inhibits ATP Hydrolysis by the Free V\(_1\) Domain by Interaction with the Rotary Subunit F* 

Received for publication, August 27, 2007, and in revised form, December 6, 2007. Published, JBC Papers in Press, December 21, 2007, DOI 10.1074/jbc.M707144200

Kevin C. Jefferies and Michael Forgac

From the Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts 02111

The vacuum (H\(^+\)) ATPases (V-ATPases) are large, multimeric proton pumps that, like the related family of F\(_{1}\)F\(_{0}\) ATP synthases, employ a rotary mechanism. ATP hydrolysis by the peripheral V\(_1\) domain drives rotation of a rotary complex (the rotor) relative to the stationary part of the enzyme (the stator), leading to proton translocation through the integral V\(_0\) domain. One mechanism of regulating V-ATPase activity \textit{in vivo} involves reversible dissociation of the V\(_1\) and V\(_0\) domains. Unlike the corresponding domains in F\(_{1}\)F\(_{0}\), the dissociated V\(_1\) domain does not hydrolyze ATP, and the free V\(_0\) domain does not passively conduct protons. These properties are important to avoid generation of an uncoupled ATPase activity or an unregulated proton conductance upon dissociation of the complex \textit{in vivo}. Previous results (Parra, K. J., Keenan, K. L., and Kane, P. M. (2000) \textit{J. Biol. Chem.} 275, 21761–21767) showed that subunit H (part of the stator) inhibits ATP hydrolysis by free V\(_1\). To test the hypothesis that subunit H accomplishes this by bridging rotor and stator in free V\(_1\), cysteine-mediated cross-linking studies were performed. Unique cysteine residues were introduced over the surface of subunit H from yeast by site-directed mutagenesis and used as the site of attachment of the photo-activated cross-linking reagent maleimido benzophenone. After UV-activated cross-linking, cross-linked products were identified by Western blot using subunit-specific antibodies. The results indicate that cross-linking, cross-linked products were identified by Western blot using subunit-specific antibodies. The results indicate that subunit H and subunit F (a rotor subunit) in the free V\(_1\) domain but not in the intact V\(_1\)V\(_0\) complex. These results indicate that subunits H and F are proximal in free V\(_1\), supporting the hypothesis that subunit H inhibits free V\(_1\) by bridging the rotary and stator domains.

The pH within intracellular compartments is a critical and highly regulated parameter involved in an array of cellular processes, such as receptor-mediated endocytosis, intracellular membrane transport, prohormone processing, protein degradation, and the coupled transport of small molecules (1). The primary regulator of pH within intracellular compartments is the vacuolar (H\(^+\)) ATPase (V-ATPase)\(^3\) (1–6). The V-ATPases are ATP-dependent proton pumps present in a variety of intracellular compartments including clathrin-coated vesicles, endosomes, Golgi-derived vesicles, lysosomes, secretory vesicles, and the central vacuoles of yeast, \textit{Neurospora}, and plants. V-ATPases also localize to the plasma membrane of various specialized cells and facilitate processes such as bone resorption by osteoclasts (7), acid secretion by renal intercalated cells (3), pH homeostasis in macrophages and neutrophils (8), and sperm maturation and storage in the vas deferens and epididymis (9). Plasma membrane V-ATPases have also been implicated in the invasiveness of certain tumor cell lines (10).

The V-ATPase is a hetero-oligomeric complex composed of 14 different subunits arranged in two structural domains, V\(_1\) and V\(_0\) (1–6). ATP hydrolysis occurs in the peripheral V\(_1\) domain, a 640-kDa complex containing 8 different subunits (A, B, C, D, E, F, G, H), whereas proton translocation occurs through the 250-kDa integral domain, V\(_0\), which is composed of six different subunits (a, d, e, c, c\(^\prime\), c\(^\prime\')). These two domains are connected by both a central stalk containing subunits D, F, and d and peripheral stalks containing subunits C, E, G, H, and the hydrophilic N-terminal domain of subunit a (11–14). The V-ATPase shares considerable structural similarity with the ATP synthase, for which a partial high resolution crystal structure has been obtained (15).

Like the F\(_{1}\)F\(_{0}\) ATP synthase, the V-ATPase employs a rotary mechanism (16, 17). The energy from ATP hydrolysis in the hexameric A\(_3\)B\(_3\) head drives rotation of the central stalk and connected ring of proteolipid subunits (c, c\(^\prime\), and c\(^\prime\')). Rotation of the proteolipid ring relative to subunit a of the V\(_0\) domain is thought to drive transport of protons across the membrane (1). Subunit a is held fixed relative to the hexameric head of V\(_1\) by the peripheral stalk, which functions as a stator in preventing unproductive rotation. Although sequence homology exists between the V and F\(_{1}\)F\(_{0}\) ATP synthases in both the nucleotide binding and the proteolipid subunits, there is virtually no sequence similarity for the remaining subunits.

An important mechanism of regulating V-ATPase activity \textit{in vivo} involves dissociation into its component V\(_1\) and V\(_0\) domains and the subsequent silencing of their respective functions (1, 2). In yeast, dissociation occurs rapidly and reversibly.
in response to glucose withdrawal (18). This mechanism has been shown to regulate V-ATPase activity in cells of higher eukaryotes as well, including mammalian renal and dendritic cells and insect midgut cells (19–21). Activation of antigen processing in dendritic cells causes assembly of the V-ATPase (20), which enhances proteolytic cleavage of antigen, whereas in renal cells the assembly status of the V-ATPase is also modulated by glucose (19). V-ATPase dissociation occurs in goblet cells of the insect midgut during molting as a means to preserve energy stores and is reversed after completion of the molting process (21). A general property of this mechanism is that upon dissociation of the two domains, the ATPase activity of the V$_1$ domain and the passive proton conductance of the V$_0$ domain are blocked (22, 23). This is critical as rapid dissipation of intracellular proton gradients would occur if V$_0$ passively conducted protons, and cellular energy stores would be significantly depleted upon the release of an uncoupled ATPase into the cytosol.

Subunit H of the V-ATPase in yeast has been shown to play an essential role in silencing ATP hydrolysis by V$_1$. Thus, isolated V$_1$ complexes depleted of subunit H show significant levels of ATPase activity compared with wild-type V$_1$ complexes containing subunit H (22). The importance of suppressing the activity of the free V$_1$ domain is highlighted by the observation that in mutant yeast strains in which all of the V$_1$ domains are free in the cytosol, the absence of subunit H is lethal (24). In addition, yeast strains bearing half the normal amount of subunit H show a conditional lethal phenotype and possess free V$_1$ domains displaying elevated Mg-ATPase activity (25). Cross-linking and electron microscopy studies performed on the V-ATPase from yeast and clathrin-coated vesicles suggest that subunit H is localized to the peripheral stator near the interface between the V$_1$ and V$_0$ domains (13). Consistent with this localization of subunit H are co-immunoprecipitation and co-localization studies demonstrating the interaction between subunit H and other peripheral stator subunits, including subunit E and the N-terminal domain of subunit a (26–28). Based upon these observations, we hypothesize that subunit H inhibits ATP hydrolysis by the free V$_1$ domain by bridging the peripheral and central stalks, serving as a mechanical brake to prevent ATP-driven rotation.

In this study we have employed cysteine-mediated cross-linking using the photo-activated cross-linking reagent maleimido benzophenone (MBP) (11) to probe the arrangement of subunits relative to subunit H in both free V$_1$ and fully assembled V$_1$V$_0$. Unique cysteine residues were introduced over the surface of subunit H by site-directed mutagenesis using the available high resolution structure of the yeast protein (29). These cysteine residues were then used as the site of attachment of MBP. The results indicate that subunit H and subunit F (one of the rotor subunits) are proximal in the free V$_1$ domain but not in the intact V$_1$V$_0$, supporting the hypothesis that subunit H inhibits ATP hydrolysis in V$_1$ by bridging the rotor and stator stalks.

**EXPERIMENTAL PROCEDURES**

**Materials and Strains**—Concanamycin A and 9-amino-6-chloro-2-methoxyacridine were purchased from Fluka Chemical, respectively. Pre-cast polyacrylamide Ready-Gels, Tween 20, SDS, nitrocellulose membranes (0.45 μm pore size), and horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG were from Bio-Rad. Zymolyase 100T was purchased from Seikagaku America, Inc. 4-(N-Maleimido)benzophenone (MBP) and most common chemicals were purchased from Sigma. PEG-maleimide (Sunn-Bio Co.) was a gift from Dr. Carol Deutsch. The chemiluminescence substrate for horseradish peroxidase was from KPL Laboratories. Kodak BioMax MR film was used for Western blotting. Amicon Ultra concentration tubes (100K) were used for buffer exchange and concentration of the V$_1$ subcomplex. Polyvinylidene difluoride syringe filters (0.45 μm) were purchased from Millipore.

The VMA13 gene in yeast encodes the H subunit. A yeast strain lacking subunit H was constructed from YPH500 by replacing the VMA13 gene with the TRP1 gene (vma13Δ::TRP1). The strain was first selected on plates lacking tryptophan, and the growth phenotype was then assessed on YEPD plates buffered with 50 mM KH$_2$PO$_4$ and 50 mM succinic acid to either pH 7.5 or 5.5.

**Antibodies**—Monoclonal antibodies against subunits a, A, and B were purchased from Molecular Probes. The polyclonal antibody against subunit E (Vma4p) was a gift from Dr. Daniel Klionsky (University of Michigan). The polyclonal antibodies against subunits H, D, and F were a gift from Dr. Tom Stevens (University of Oregon).

**Cloning of the VMA13 Gene Encoding Subunit H**—Genomic DNA was isolated from an adenine-deficient yeast strain of YPH500 and used to amplify the VMA13 gene. The following primers with unique restriction enzymes sites for SacI and KpnI (underlined) were used: forward, 5’-GCGGAGCTCTGTCGCCAGGGAGGTTACC-3’; reverse, 5’-GGCGGTACCGCGCCCCATATTACCCGACT-3’. The resulting PCR product was then digested with SacI and KpnI and ligated into pRS316.

**Construction of Mutants**—Mutations of Vma13p were performed using Promega Altered Sites II in vitro site-directed mutagenesis system. The VMA13 gene was cloned into the pALTER-1 vector using the SacI and KpnI restriction sites. To construct a cysteine-less (Cys-less) mutant of VMA13, the seven endogenous cysteine residues at positions 92, 183, 186, 290, 297, 298, and 369 were replaced with serine residues using the oligonucleotides listed in Table 1. Single cysteine residues were introduced into the Cys-less form of Vma13p also using Altered Sites II. The oligonucleotides for the single cysteine mutants constructed are listed in Table 1. After mutagenesis, each plasmid was digested with SacI and KpnI, and the resulting fragment was purified by agarose gel electrophoresis and ligated into pRS316. The final sequence of all mutants was confirmed by DNA sequencing.

**Transformation and Selection**—YPH500 yeast cells lacking functional endogenous Vma13p (vma13Δ::TRP1) were transformed by electroporation. The transformants were selected on uracil–minus plates, and the growth phenotypes were evaluated on yeast extract–peptone–dextrose plates buffered with KH$_2$PO$_4$ and 50 mM succinic acid to either pH 7.5 or 5.5.

**Preparation of the V$_1$ Subcomplex**—125 ml of yeast cells were grown overnight at 30 °C to an absorbance (600 nm) of ~1.7 in
Subunit H of the V-ATPase Bridges Rotor and Stator in V1

TABLE 1
Sequences of oligonucleotides used to generate VMA13 mutants

| Mutant | Oligonucleotide (5′→3′) |
|--------|------------------------|
| C92S  | CACGGAACGCAAATAATC     |
| C183S and C186S | GAGCAGATTGCTACTAGTGAAGCATCAGCTA |
| C290S, C297S, and C298S | AATGATCCACCGCCAATA |
| C369S | CAGCTGCTACTGCTGCTC    |
| S24C  | TCCCTCCGCTTGCTGCGGAC   |
| S24C  | GAGCTGCTACTGCTGAGCAA   |
| S78C  | ATCCGAGCAATACTGCTATTTAATTTCTACCTTA |
| P121C | TTTCCAAGAACCGCCAACTGGA |
| S140C | AAAAAACTGACGCTGCACTAGGACCTGAT |
| L189C | TACTGAGCACTGTAGCTGCAAGACTGCTCC |
| P197C | GAACTGCGTCTGATAATTGAATTCTACGATGAA |
| Q265C | GACCAAGACCGCTGCTGAAACTTTGTTAGAT |
| T300C | CTTGCAAAATATTCCTGCTGAGCC |
| S331C | AGAAAGTATTGCGACGAAGAA |
| T354C | TACCAAGAATGAGACCTCTCCCTGCTGATAG |
| S369C | CAGCTGCTACTGCTGCTGCTC |
| S381C | GTTGTTGGCTGCAAAATCCTT |
| D390C | GAGCTGCAAGAAAGGGCAGT |
| E400C | TTGTAAGCAATGCTTGCTGCTGCTGAG |
| G409C | GTGCCGTCAGGCTGCAAGCTCA |
| S457C | TCTGGAACATCCGCTTCTGAGT |

selective medium and washed twice with water. After resuspension in 5 ml of alkaline buffer (100 mM Tris-Cl, 10 mM DTT, pH 9.4), cells were incubated with gentle agitation at 30 °C for 20 min. Cells were pelleted again, washed with YEPD medium (0.7 M sorbitol, 2 mM dithiothreitol, 100 mM MES-Tris, pH 7.5), and resuspended with 2.5 ml of YEPD medium and 166 μg of zymolase. Cells were incubated for 1 h at 30 °C with gentle agitation. The resulting spheroplasts were washed with ice-cold 1.2 M sorbitol, resuspended in 2 ml of osmotic lysis buffer (50 mM Tris-Cl, 200 mM sorbitol, 1 mM EDTA, pH 7.5), and incubated on ice for 45 min. After incubation the spheroplasts were centrifuged at 100,000 × g to pellet all membranes, and the supernatant was collected and forced through a Millex-HV polyvinylidene difluoride 0.45 μm syringe filter.

Isolation of Vacuolar Membrane Vesicles—Vacuolar membrane vesicles were isolated as described in our previous study (14). Briefly, 1 liter of yeast cells were grown overnight at 30 °C to an absorbance at 600 nm of ~1.7 in selective medium, pelleted, washed twice with water, and re-suspended in 50 ml of alkaline buffer (described above). After incubation at 30 °C for 20 min with gentle agitation, cells were pelleted again, washed once in 25 ml of YEPD medium, and resuspended in the same YEPD medium including 2 mg of zymolase 100T. Cells were incubated for 1 h at 30 °C with gentle agitation. The resulting spheroplasts were osmotically lysed, and the vacuolar membranes were isolated by centrifugation on two consecutive Ficoll gradients (12 and 8%) and diluted in transport buffer (15 mM MES-Tris, 4.8% glycerol, pH 7).

Photochemical Cross-linking of the Intact V1V0 ATPase or V1 Subcomplex with MBP—MBP-mediated cross-linking was performed on both the yeast cytosolic fraction, which contain the dissociated V1 subcomplex, and the vacuolar membranes, which contain the intact V1V0 complex. MBP dissolved in dimethylformamide was added to the purified vacuolar membranes or the V1 subcomplex-containing fraction suspended in 100 μl to a final concentration of 1 mM followed by incubation in the dark for 30 min at 23 °C. Unreacted MBP was quenched by the addition of 10 mM DTT. The vacuolar membranes were pelleted and washed twice with PBS-EDTA with protease inhibitors resuspended in 100 μl of PBS-EDTA. The V1-containing fractions were washed twice with PBS-EDTA using a 100K Amicon Ultra centrifugal filter and spun to a final volume of 100 μl of PBS-EDTA. Samples were then irradiated with a long wavelength (366 nm) ultraviolet lamp (Mineralight® model UVGL-25) at 4 °C for 5 min.

Analysis of Cross-linked Products—After cross-linking with MBP, vacuolar membranes were solubilized with 2% polyoxyethylene 9-lauryl ether, and the intact V1V0 complexes were immunoprecipitated using an antibody against subunit A. The immunoprecipitated intact complexes or the cytosolic fraction containing the V1 complex were separated by SDS-PAGE on 7.5% acrylamide gels and then transferred to nitrocellulose. Western blotting using antibodies specific for subunit H or other V-ATPase subunits was used to detect MBP-dependent cross-linked products as previously described (11, 14).

Sulfhydryl Modification with PEG-maleimide—To determine the accessibility of the sulfhydryl groups of various mutants, both isolated vacuoles and crude V1 preparations were incubated with 1 mM polyethylene glycol maleimide (PEG-Mal, 5 kDa) in PBS for 30 min at room temperature and quenched with 10 mM DTT. After quenching, the samples were mixed with sample buffer and separated by SDS-PAGE on 7.5% acrylamide gels. Samples were transferred to nitrocellulose and blotted with antibodies against subunit H. Although the PEG-maleimide has a molecular mass of 5 kDa, it causes a shift in apparent mobility of 10–15 kDa, depending upon the exact site of modification.

Other Procedures—Protein concentrations were determined by the Bradford and Lowry methods. ATPase activity was measured using a coupled spectrophotometric assay in the presence or absence of 1 μM concanamycin A as described previously (30). ATP-dependent proton transport was measured using the fluorescence probe 9-amino-6-chloro-2-methoxyacridine in the presence or absence of 1 μM concanamycin A as described previously (30).

RESULTS

Construction of Single Cysteine-containing Mutants of Vma13p and the Effect of Mutations on Growth Phenotype, ATPase Activity, and Proton Transport Activity—To determine the proximity of other V-ATPase subunits to specific sites within subunit H in both the V1 domain and the assembled V1V0 complex, we employed a cysteine mediated cross-linking strategy using the photoactivable cross-linker, MBP (11). These studies allowed us to test the hypothesis that subunit H is able to interact with subunits of the central rotary stalk in V1 but not V1V0. The available crystal structure of subunit H (29) facilitated the introduction of single cysteine residues into structurally defined sites on the surface of subunit H.

Before the construction of single cysteine-bearing mutants of subunit H, it was first necessary to prepare a construct of Vma13p (yeast subunit H) in which the endogenous cysteines were removed and replaced with serine residues. Serine resi-
Subunit H of the V-ATPase Bridges Rotor and Stator in V₁

![Diagram of subunit H](image)

**Figure 1. Location of unique cysteine residues introduced into the yeast V-ATPase subunit H (Vma13p).** The high resolution structure of the yeast Vma13p (29) is shown, with coordinates obtained from the Protein Data Bank (accession code 1H08). The model was constructed using the Swiss-pdb viewer. The view in the right panel is rotated 90° along the vertical axis relative to the image in the left panel. Residues mutated to cysteine are shown in space-filling mode, with residues in black facing out of the page, and those shown in gray facing in.

dues were chosen as a substitute for cysteine because they most closely resemble cysteine in charge and size. The Cys-less mutant of subunit H was constructed by replacing each of the seven endogenous cysteine residues (which occur at positions 92, 183, 186, 290, 297, 298, and 369) with serine residues using site-directed mutagenesis, as described under “Experimental Procedures.” Using the available high-resolution crystal structure of subunit H as a guide (29), single cysteine residues were then introduced by site-directed mutagenesis into 17 sites over the surface of the Cys-less form of Vma13p (Fig. 1). All constructs were cloned into the pRS316 expression vector, which contains the URA3 selectable marker. Additionally, the wild-type VMA13 gene was cloned from YPH500 genomic DNA and inserted into pRS316 as well. All subsequent references to wild-type VMA13 refer to this construct in the vma13Δ yeast strain.

All constructs were then transformed by electroporation into a vma13Δ strain of YPH500 yeast bearing the TRP1 marker (vma13Δ::TRP1) that had been prepared by homologous recombination. Wild-type VMA13 was included as a positive control for growth phenotype and activity assays (as described below), whereas the vma13Δ strain transformed with the pRS316 plasmid alone was used as a negative control.

All mutants were selected from uracil-minus plates, and the growth phenotypes were assessed on YEPD plates buffered to either pH 7.5 or 5.5. The wild-type, Cys-less, and single cysteine mutants of Vma13p were all able to grow at both acidic and neutral pH, indicating that each mutant can complement the vma− phenotype of the vma13Δ strain. Previous studies have shown that V-ATPase mutants that can grow at pH 7.5 have at least 20% of wild-type levels of V-ATPase activity (31, 32).

Concanamycin-sensitive proton transport and ATPase activity were then determined for vacuolar membranes isolated from each yeast strain (Fig. 2). Vacuolar membranes from all mutants that gave identifiable cross-linked products (see below) possessed concanamycin-sensitive ATPase and proton pumping activities that were between 40 and 100% that of wild-type activity, suggesting that all of these single cysteine mutants of subunit H are able to assemble into a V-ATPase complex possessing significant function. Interestingly, the T354C mutant had higher than wild-type levels of ATPase activity but was not further pursued, as no cross-linked products were observed for this mutant.

**Photochemical Cross-linking of V-ATPase Complexes Containing Mutant Forms of Vma13p Using MBP—** The structural arrangement of V-ATPase subunits with respect to the single cysteine mutations of subunit H was determined using the photoreactive reagent maleimido MBP. This reagent possesses a linker arm of 10 Å and forms a covalent bond with sulphydryl groups via the maleimide moiety (11). Upon exposure to UV light, a reactive species is generated that is capable of covalently reacting with proximal residues, thus forming a cross-link between the sulphydryl-bearing subunit H and proteins within 10 Å.

Because many of the other V-ATPase subunits possess endogenous cysteine residues, even in complexes containing a Cys-less form of subunit H, it is critical to determine whether MBP could react with these sulphydryl groups and cross-link to subunit H upon UV exposure. To test this, both vacuolar membranes and cytosolic V1 complexes were isolated from the yeast strain expressing the Cys-less form of Vma13p and reacted with 1 mM MBP for 30 min in the dark followed by quenching of the reagent with 10 mM DTT. After washing to remove unreacted reagent and irradiation with a long-wavelength UV lamp, the products were separated by SDS-PAGE and analyzed by Western blot using antibodies against subunit H. As shown in Figs. 3A and 4A, no reactive bands of molecular weight greater than that of subunit H (54 kDa) appear in the presence of MBP. This demonstrates that any cross-linked products involving the single cysteine mutants of subunit H are not a result of MBP reacting with the sulphydryl groups of other subunits.
Subunit H of the V-ATPase Bridges Rotor and Stator in \( V_1 \)

To identify subunits within close proximity to subunit H in free \( V_1 \) complexes, the cytosol from each mutant strain and wild type was collected, concentrated, incubated with MBP, quenched with DTT, exposed to UV light, and analyzed by Western blot. Of the 18 strains tested (which includes 17 cysteine mutants and the cysteine-less mutant as a negative control), five produced MBP-dependent products that react with an antibody directed against subunit H. These include mutants P197C, Q265C, T300C, S381C, and D390C. Of these mutants only S381C and D390C gave cross-linked products that could be conclusively identified with antibodies against other subunits. In each case the cross-linked product had an apparent molecular weight larger than subunit H. Mutant D390C produced an MBP-dependent product recognized by the anti-H subunit of molecular mass about 105 kDa when cross-linking is performed from P197C could be identified with antibodies against another subunit. Mutant D390C did not show any cross-linked products in \( V_1 V_0 \) (data not shown). Mutant P197C produced an MBP-dependent band at about 105 kDa that reacts with both anti-H and anti-E subunit antibodies (Fig. 4B). Although the molecular mass of this cross-linked product is greater than that expected of an H/E heterodimer (81 kDa), it is likely that, due to cross-linking near the middle of either subunit, this species migrates aberrantly on SDS-PAGE. We have previously observed that cross-linked products between the same two subunits migrated differently depending upon where within the primary sequences the cross-linking occurred (11, 12). These results suggest that subunits H and E are in close proximity in the fully assembled complex near the middle of subunit H.

Interestingly, mutant P197C also shows an MBP-dependent product of molecular mass about 105 kDa when cross-linking is performed in free \( V_1 \), as noted above. Although this product did not react with antibodies against subunit E, it is possible that the site within subunit E that undergoes cross-linking is different in free \( V_1 \) and intact \( V_1 V_0 \) and that, as a result, the epitope recognized by the anti-E subunit antibody becomes masked in \( V_1 \). Although the antisera against subunit E is polyclonal and it is unlikely that all of the epitopes become masked by a single cross-linking event, cross-linking may have masked the primary epitope in subunit E. It is also possible that the cross-link involves a subunit not tested for (such as A or C) or involves a novel protein.

Significantly, mutant S381C, which resulted in H-F cross-linking in the free \( V_1 \) domain, did not show cross-linking in \( V_1 V_0 \) (Fig. 4A). One explanation of this negative result is that...
the S381C residue is inaccessible to sulfhydryl modifying reagents in the intact V-ATPase complex. To test this possibility, both V₁ and vacuolar membrane preparations containing V₁V₀ from the S381C mutant and four other mutants were reacted with the sulfhydryl reagent PEG-maleimide followed by SDS-PAGE and blotted with the anti-H antibody. Because reaction with PEG-maleimide shifts the mobility of the protein by 10–15 kDa, this experiment identifies mutants possessing cysteine residues that are solvent-exposed (33). As can be seen in Fig. 5, the S381C mutant shows reactivity with PEG-maleimide in both free V₁ and the intact V₁V₀ complex that is comparable with that observed for the other cysteine residues tested. These results suggest that the absence of an S381C cross-link in the intact complex is not due to the inaccessibility of the cysteine residue at this position. It should be noted that the variable mobility of the PEG-maleimide-modified cysteine mutants, depending upon the location of the cysteine residue within the primary sequence, is consistent with the proposed explanation for the aberrant mobility of some of the observed cross-linked products noted above.

**DISCUSSION**

Eukaryotic cells are able to modulate the pH of their intracellular compartments independently and in response to a variety of stimuli. In yeast, removal of glucose from the media causes reversible dissociation of V₁V₀ complexes, thus preserving cellular ATP stores (18). Because reversible dissociation is widely used as a mechanism of regulating V-ATPase activity in vivo, it is important that the ATPase activity of free V₁ and the passive proton conductance of free V₀ be suppressed as a means to avoid physiologically catastrophic consequences.

Based upon the inhibitory effect of subunit H on the ATPase activity of the free V₁ domain and on its localization to the peripheral stator, we hypothesize that subunit H inhibits catalysis by bridging the rotor and stator domains and, thus, physically blocking rotation. The results presented in this study suggest that subunit H comes within 10 Å of subunit F (a rotor subunit) in the free V₁ domain but not in the intact V-ATPase complex and are, thus, consistent with the proposed mechanism by which subunit H inhibits rotary catalysis. Moreover, they identify the C-terminal domain of subunit H as the critical region for inhibition of activity.

The crystal structure of subunit H reveals that it is an elongated protein consisting of an N-terminal domain (residues 2–352) and a C-terminal domain (residues 353–478), which are separated by a four-residue loop, suggesting structural flexibility between the two domains (29). The N-terminal domain, composed largely of stacked α-helices, contains a hydrophobic groove, similar to that observed in importins, which may function as a protein-protein binding domain. In fact, subunit H has been shown to function as the binding site for a number of other proteins that associate with the V-ATPase, including the HIV-NEF protein, the adaptor protein complex 2, and the Golgi

**FIGURE 3. Subunit H mutant cross-links in free V₁ complexes using MBP.** Cytosolic protein (2 mg) isolated from the vma1Δ strain expressing the Cys-less VMA13 gene (panel A), the D390C mutation (panel B), the S381C mutation (panel C), and the P197C mutation (panel D) was incubated in the presence (+) or absence (−) of 1 mM MBP for 30 min at room temperature. Excess MBP was quenched by the addition of 10 mM dithiothreitol and removed by washing with a 100K concentration tube. The samples were then irradiated with a long-wavelength ultraviolet lamp (366 nm) for 5 min at 4 °C, mixed with sample buffer, and separated on either 7.5% (Cys-less, P197C, D390C) or 10% polyacrylamide gels (S381C) and transferred to nitrocellulose membranes. Membranes were then probed with the antibodies indicated in each panel. 

The numbers shown on the right of each figure correspond to the molecular mass of each band, whereas the letters on the left-hand side indicate the monomers and any cross-linked products. Images shown were adjusted for contrast.
Subunit H of the V-ATPase Bridges Rotor and Stator in V₁

ectoapyrase (34, 35). Interestingly, the N-terminal domain of subunit H has been shown to partially support an uncoupled ATPase activity by the V-ATPase complex but requires the C-terminal domain to recover coupling of proton transport and ATPase activity (36).

Subunit F is a 14-kDa protein that has been shown along with subunit D to form part of the rotor by direct rotational studies performed on the V-ATPase from Thermus thermophilus (16). Therefore, any direct interaction between subunit H and subunit F would be expected to prevent rotary catalysis. It should be noted, however, that these results do not rule out other possible mechanisms by which subunit H may inhibit activity. The inhibitory protein of the mitochondrial ATP synthase, for example, has been shown to inhibit ATP hydrolysis by intercalating into an interface of the H9251/H9252 subunits and preventing a critical conformational change from occurring that is required for catalysis (37). It is possible that subunit H may inhibit ATP hydrolysis in V₁ by a similar mechanism. Nevertheless, the results do point toward a reversible interaction of the C-terminal domain of subunit H and the rotor stalk upon dissociation of V₁ and V₀ domains and are, thus, supportive of a bridging mechanism.

Significantly, the lack of cross-linking between subunits H and F in the assembled complex is not due to an inaccessibility of the cysteine residue at position 381 to the cross-linking reagent, since this residue is still capable of reacting with PEG-maleimide in the intact complex. Rather, this result suggests that a conformational change moves the C-terminal domain of subunit H toward the rotor stalk upon dissociation of V₁ and V₀ domains and are, thus, supportive of a bridging mechanism.

The recent high resolution crystal structure of subunit F from T. thermophilus reveals that the N- and C-terminal domains are connected by a flexible loop, allowing the protein to adopt either a “retracted” or an “extended” conformation, both of which were detected by fluorescence resonance energy
transfer analysis (38). This is similar to the situation observed for the F_{1}F_{0} ATP synthase \epsilon subunit, which also forms part of the rotary stalk in the ATP synthase (39), although the two proteins are not otherwise structurally related. It is possible that either a change in conformation of subunit F or a movement of the C-terminal domain of subunit H or both allow these two proteins to become proximal upon dissociation of V_{1}. Interestingly, because it is the retracted form of subunit F that is observed in the absence of ATP, it is predicted that it would be this form of the protein that would interact with subunit H upon dissociation of V_{1}. Although the assembly status of the V1VO complex, previous studies have shown that the C-terminal domain of H participates in this interaction in the V_{1}-domain. Because the sites of cross-linking to subunit F and subunit B in V_{1} are in quite close proximity to each other on the surface of subunit H, it is possible that it is only the C-terminal domain of H that is required to bridge the rotor and stator. By contrast, the N-terminal domain of subunit H interacts with subunit E, which in turn interacts with subunit B via a region in its N terminus (27). Subunit E is a 26-kDa protein present in multiple copies per complex (41) that appears to stretch a considerable distance perpendicular to the membrane (11, 12) and has been shown to interact with many of the peripheral stalk subunits including subunits B, C, and G (11, 14, 41, 42).

In summary, we have demonstrated that the N-terminal domain of subunit H is in proximity to subunit B in the free V_{1} domain, whereas the C-terminal domain is in proximity to subunit B in the intact V_{1} domain. Moreover, the C-terminal domain of subunit H becomes proximal to the rotary subunit F only in the free V_{1} domain, consistent with the proposed mechanism of inhibition by subunit H of catalysis by the V_{1} domain via bridging of the rotor and stator portions of the complex.

Acknowledgments—We thank Drs. Takao Inoue, Ayana Hinton, Dan Cipriano, and Yanru Wang as well as Jie Qi and Sarah Bond for helpful discussions. We also thank Dr. Tom Stevens and Daniel Klionsky for generously providing anti-Vma13p and anti-Vma4p antibodies. Additionally, we thank Dr. Carol Deutsch for providing helpful discussions. We also thank Drs. Tom Stevens and Daniel Cipriano, and Yanru Wang as well as Jie Qi and Sarah Bond for helpful discussions. We also thank Dr. Carol Deutsch for providing anti-Vma13p and anti-Vma4p antibodies. Additionally, we thank Dr. Carol Deutsch for providing helpful discussions.