Structural Analysis of the N-terminal Domain of Subunit a of the Yeast Vacuolar ATPase (V-ATPase) Using Accessibility of Single Cysteine Substitutions to Chemical Modification

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Background: The N terminus of the V-ATPase subunit a functions in intracellular targeting and regulation of assembly.

Results: A homology model of this domain was tested by determining aqueous accessibility of introduced cysteines.

Conclusion: The results identify residues located at the peripheral/integral domain interface.

Significance: The identified sites may participate in interactions that regulate V-ATPase assembly.

The vacuolar ATPase (V-ATPase) is a multisubunit complex that carries out ATP-driven proton transport. It is composed of a peripheral V1 domain that hydrolyzes ATP and an integral V0 domain that translocates protons. Subunit a is a 100-kDa integral membrane protein (part of V0) that possesses an N-terminal cytoplasmic domain and a C-terminal hydrophobic domain. Although the C-terminal domain functions in proton transport, the N-terminal domain is critical for intracellular targeting and regulation of V-ATPase assembly. Despite its importance, there is currently no high resolution structure for subunit a of the V-ATPase. Recently, the crystal structure of the N-terminal domain of the related subunit I from the archaeabacterium *Meiothermus ruber* was reported. We have used homology modeling to construct a model of the N-terminal domain of Vph1p, one of two isoforms of subunit a expressed in yeast. To test this model, unique cysteine residues were introduced into a Cys-less form of Vph1p and their accessibility to modification by the sulphydryl reagent 3-((N-maleimido-propionyl) biocytin (MPB) was determined. In addition, accessibility of introduced cysteine residues to MPB modification was compared in the V1,V0 complex and the free V0 domain to identify residues protected from modification by the presence of V1. The results provide an experimental test of the proposed model and have identified regions of the N-terminal domain of subunit a that likely serve as interfacial contact sites with the peripheral V1 domain. The possible significance of these results for in vivo regulation of V-ATPase assembly is discussed.

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The vacuum H+-ATPases (V-ATPases) are a family of ATP-driven proton pumps that are ubiquitously expressed in eukaryotes and are present in both intracellular compartments and the plasma membrane (1–3). Acidification of intracellular compartments by V-ATPases is necessary for many pH-dependent cellular processes, including receptor-mediated endocytosis, intracellular trafficking, protease activation, and proton coupled transport of small molecules, such as neurotransmitters (1). Plasma membrane V-ATPases also serve diverse functions, including urinary acidification, bone resorption, sperm maturation, and cancer cell invasion (3–6). Altered functioning of V-ATPases has therefore been implicated in many human disease states, including osteoporosis, renal disease, and cancer. A greater understanding of the structure of the V-ATPases would likely aid in the development of therapeutics for diseases in which they participate. Although the structures of several individual subunits and subcomplexes have been solved by x-ray analysis (7–9), there is currently no high resolution structure for either the intact pump or several key components. It is therefore necessary to employ alternative strategies to elucidate the structure of this important enzyme.

The V-ATPases are structurally and mechanistically related to both the F-type ATPases (or ATP synthases) from mitochondria, chloroplasts, and bacteria and the A-type ATPases from archaeabacteria (10). All three classes are composed of a peripheral domain (V1, F1, or A1) that hydrolyzes ATP and an integral domain (V0, F0, or A0) that translocates protons. Moreover, all three classes operate by a rotary mechanism in which ATP hydrolysis in the peripheral domain drives rotation of a central rotor domain (composed of both peripheral and integral subunits) relative to the remainder of the complex (termed the stator domain) (11, 12). This rotation in turn drives proton translocation through the integral domain. However, unlike the F- and A-type ATPases, which function to synthesize ATP, the physiological function of the V-ATPases is to translocate protons.

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Structural Analysis of the N Terminus of V-ATPase Subunit a

For the V-ATPases, the V$_1$ domain is a 670-kDa complex composed of eight subunits (A, B, C, D, E, F, G, and H) present in the stoichiometry $A_3B_3CDEFGFH$ (1, 13). The A and B subunits are arranged alternately in a hexameric ring, with the catalytic sites where ATP is bound and hydrolyzed located at three A/B subunit interfaces (14). ATP hydrolysis at these sites drives rotation of a central stalk, which is composed of the D and F subunits and subunits of V$_0$ (described below). The remaining subunits of V$_1$ form peripheral stalks, which serve to bridge the V$_1$ and V$_0$ domains and to keep the complex assembled despite the torque generated during rotary catalysis. The V$_0$ domain is composed of 6 subunits (a, c, c$'$, c''', d, and e) in the stoichiometry $a_3d_1e_1c_6c''c'''_1$ (1, 13). The proteolipid subunits (c, c$'$, and c''') form a 10-membered ring (as first suggested by the crystal structure of the Na$^{+}$-translocating A-ATPase from Enterococcus hirae (15)), which is attached via subunit d to subunits D and F in V$_1$ to form a central rotor complex (13). The a subunit is a 100-kDa integral membrane protein composed of a 50-kDa cytoplasmic N-terminal domain and a 50-kDa membrane-embedded C-terminal domain containing eight transmembrane helices (16). The C-terminal domain is postulated to form two proton-conducting hemichannels, one in contact with the cytoplasmic compartment and the other in contact with the luminal (or extracellular) compartment (17), as originally proposed for subunit a of the F-ATPase (18–20). During proton translocation, protons enter via the cytoplasmic hemichannel and protonate a conserved glutamic acid residue on one of the proteolipid subunits. Rotation of the central rotary complex driven by ATP hydrolysis moves this protonated carboxyl group into contact with a buried arginine residue on subunit a (21, 22). Interaction with this positively charged side chain stabilizes the carboxyl group in its deprotonated form, displacing the proton into the luminal hemichannel and completing proton transport.

The N-terminal domain of subunit a serves several important functions. First, it contains information necessary to target V-ATPases to specific cellular destinations (23). Yeast express two isoforms of subunit a: Vph1p, which targets complexes to the vacuole, and Stv1p, which targets complexes to the Golgi (23, 24). Mammalian cells express four isoforms of subunit a (a1–a4), with a1 and a2 localized predominantly to intracellular sites and a3 and a4 able to target V-ATPases to the plasma membrane of osteoclasts and renal intercalated cells, respectively (1, 3). In addition, the N-terminal domain of subunit a functions in regulated assembly of the V-ATPase complex. Regulated assembly of the V$_1$ and V$_0$ domains represents an important mechanism of controlling V-ATPase activity in both yeast and higher eukaryotes (1, 2). In yeast, in vivo dissociation occurs rapidly in response to glucose depletion, is reversible upon glucose readdition, and does not require new protein synthesis (25, 26). Interactions between the N terminus of subunit a and subunits in V$_1$ must be broken during in vivo dissociation and thus may be sensitive to signaling events resulting from changes in glucose concentration.

A high resolution structure has not been solved for the holoenzyme of any of the rotary ATPases. Although x-ray structures have been obtained for both individual subunits and partial complexes of the V-ATPase (7–9), subunit a has been the most difficult to characterize structurally. The C terminus of subunit a is composed of eight transmembrane helices which contain a number of buried polar and charged residues that are thought to contribute to the proton-conducting hemichannels described above (17). Despite its importance, no high resolution structure of subunit a or either domain has been obtained. Recently, the structure of N-terminal domain of subunit I (homologous to subunit a of the V-ATPases) was solved for the A-ATPase from M. ruber (27). Although the sequence homology between subunit a of the V-ATPase and subunit I of the A-ATPase is relatively low (33% similarity), the predicted secondary structure is well conserved between these domains (28).

Here, we have used the coordinates of the subunit I structure to construct a model of the N terminus of Vph1p from yeast. We have tested this model by examining the accessibility of unique cysteine residues introduced throughout the N terminus of Vph1p to the sulfhydryl-labeling reagent 3-(N-maleimido-propionyl) biocytin (MPB). Additionally, we have examined how the accessibility of these residues changes depending upon the assembly state of the V-ATPase, offering insight into residues of subunit a present at the interface of V$_1$ and V$_0$.

EXPERIMENTAL PROCEDURES

Materials—Zymolase 20T was obtained from Seikagaku American, Inc. Protease inhibitors (aprotinin, pepstatin, leupeptin) were purchased from Roche Applied Science. MPB was purchased from Molecular Probes. The monoclonal antibody 10D7 against the yeast V-ATPase a subunit was purchased from Abcam, and the monoclonal antibody 13D11 against the yeast V-ATPase B subunit was purchased from Invitrogen (29). Neutra-avidin was purchased from Pierce. Immunoblotting reagents were purchased from GE Healthcare. The QuiChase lightning mutagenesis kit was obtained from Stratagene. ATP, phenylmethylsulfonyl fluoride, concanamycin A, and other chemicals were purchased from Sigma.

Strains and Culture Conditions—Yeast strain MM112 (MATa\alpha vph1::LEU2\ast sv1::LYS2his3\ast Δ200leu2lys2ura3\ast 52) lacking the endogenous Vph1p and Stv1p subunit a isoforms was used to generate all Vph1p mutants (24). Yeast cells were grown in unbuffered SD-Ura minimal medium.

Modeling—The coordinates of the structure of the N terminus M. ruber subunit I (Protein Data Bank code 3RRK) were used as a structural template for the structural model of the N terminus of Vph1p (27). The sequences of the N terminus of M. ruber subunit I and Vph1p were aligned using the ClustalW2 program and entered into the online program I-TASSER (30) for homology modeling, as described previously (28).

Selection of Residues to be Mutagenized—Each residue in the N terminus of Vph1p was assigned a predicted accessibility score ranging from 0 (buried) to 7 (accessible). We selected for mutagenesis all residues with scores of 6 or 7 and one residue from each dispersed stretch of residues with a score of 0. We selected several residues with an accessibility score of 2 or 3 to determine the degree of solvent accessibility that would permit labeling.

Mutagenesis and Transformation—The Cys-less form of Vph1p, in which the seven endogenous cysteines were substi-
tuted with serine, was used as a background for single cysteine mutagenesis (31). The QuikChange Lightning site-directed mutagenesis kit was used to introduce single cysteine mutations into the Cys-less form of Vph1p in the pRS316 plasmid. Sequences of the Cys-less 

\( VPH1 \) and the single cysteine mutants were confirmed by DNA sequencing. The yeast strain MM112 was then transformed using the lithium acetate method with the Cys-less form of 

\( VPH1 \) or each of the single cysteine mutants of 

\( VPH1 \) (32). Transformants were selected on uracil-deficient plates as described previously (17). The vma growth phenotype of the mutants was examined on YEPD plates buffered with 50 mM phosphate/succinate to either pH 7.5 or 5.5 (16).

**Isolation of Vacuolar Membrane Vesicles**—Vacuolar membranes vesicles were isolated as described previously (16). Yeast cells were grown overnight to an 

\( A_{600} \) of 1.0–1.5 in 2 liters of selective medium. Cells were pelleted, washed once with water, and resuspended in 100 ml of 100 mM Tris-HCl, pH 9.4, containing 10 mM dithiothreitol. After incubation at 30 °C for 20 min, cells were pelleted again, washed once with 50 ml of YEPD medium containing 0.7 M sorbitol, 2 mM dithiothreitol, and 100 mM MES-Tris, pH 7.5, resuspended in 50 ml YEPD containing 0.7 M sorbitol, 2 mM dithiothreitol, 100 mM MES-Tris, pH 7.5, and 20 mg Zymolyase 20T, and incubated at 30 °C for 1 h with gentle shaking. The resulting spheroplasts were osmotically lysed, and the vacuoles were isolated on two consecutive Ficoll gradients and diluted in transport buffer (15 mM MES-Tris, pH 7.0, 4.8% glycerol).

**Proton Transport and ATPase Activity**—ATP-dependent proton transport was measured by fluorescence quenching using the fluorescence probe 9-amino-6-chloro-2-methoxy-acridine in transport buffer (25 mM MES-Tris, pH 7.2, 5 mM MgCl₂) in the presence or absence of 1 mM ATPase inhibitor concanamycin A, as described previously (16, 33). ATP hydrolysis was measured using a coupled spectrophotometric assay as described previously (17).

**MPB Labeling and Detection**—150 μg of vacuolar membrane protein was washed in labeling buffer (PBS-EDTA containing 137 mM NaCl, 1.2 mM KH₂PO₄, 15.3 mM Na₂HPO₄, 2.7 mM KCl, 2 mM EDTA (pH 7.2), 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 1 mM PMSF) twice at 16,000 × g for 5 min. Membrane vesicles were then resuspended in 100 μl of labeling buffer. MPB was added to a final concentration of 50 μM (or 2, 10, 50, or 100 μM as specified in the concentration dependence experiments), and the membrane vesicles were incubated on ice for 15 min (or 5 or 10 min as specified in the time course experiments). The reaction was quenched for 20 min on ice by adding DTT to a final concentration of 10 mM. Vesicles were washed twice with 1 ml of labeling buffer and resuspended in 100 μl of labeling buffer. Membranes were solubilized for 1 h at 4 °C with 2% C₁₂E₉. Solubilized vesicles were split into equal aliquots. One portion was immunoprecipitated with the mouse monoclonal antibody 10D7 directed against Vph1p, which immunoprecipitates only the free \( V_0 \) domain (29). The other portion was immunoprecipitated with the mouse monoclonal antibody 13D11 directed against subunit B present in the \( V_1 \) domain, which precipitates both intact \( V_0V_1 \) and free \( V_1 \) (17). Following precipitation of antibody-antigen complexes with protein A-Sepharose, samples were separated by SDS-PAGE on 10% acrylamide gels (34) and transferred to nitrocellulose membranes for detection of MPB. For MPB detection, blots were probed with horseradish peroxidase-conjugated NeutraAvidin and, in parallel, the monoclonal antibody 10D7 against the 100-kDa a subunit to ensure the presence of equal amounts of subunit a. For immunoblot analysis, blots were probed with a horseradish peroxidase-conjugated secondary antibody (BioRad) as described previously (16). The blots were developed using a chemiluminescent detection method obtained from GE Healthcare. All labeling experiments were performed a minimum of two times per mutant, employing separate vacuolar preparations.

**RESULTS**

**Construction of a Model of the N-terminal Domain of Vph1p Based on the Structure of Subunit I of M. ruber**—Because of its importance in intracellular targeting, complex assembly, and other aspects of V-ATPase function (1, 23), we first wished to construct a molecular model of the N-terminal domain of subunit a. This was made possible by the recently published crystal structure of the N-terminal domain of the homologous subunit I of the A-ATPase from 

\( M. \) ruber (27). We chose for our modeling studies the N-terminal domain of Vph1p, one of two isoforms of subunit a expressed in yeast, because of the wealth of structural data about the V-ATPase complex from yeast (1, 2) and because of the ease of introducing point mutations into the yeast protein. Fig. 1 shows the sequence alignment of the N-terminal domain of Vph1p, Stv1p, and subunit I using the ClustalW2 program. Vph1 and subunit I display 13% identity and 33% similarity. The web-based program I-TASSER (30) was then used to create a structural model of Vph1p based upon the coordinates of the 

\( M. \) ruber protein (Fig. 2) (27). A model of Stv1p has previously been published using this approach, and the derived models are similar in overall shape (28). Only residues of Vph1p corresponding to residues 1–344 of subunit I were employed in the analysis as the published structure lacks the C-terminal most 43 residues of the N-terminal domain of subunit I.

To test this model, we determined the accessibility of unique cysteine residues introduced into the N-terminal domain of Vph1p to modification by the sulphydryl-labeling reagent MPB (31). residues exposed on the surface of the protein should be accessible to modification by MPB, whereas those buried within the protein should not. Solvent accessibility scores for each residue were determined using the I-TASSER program and varied from 0 (most buried) to 7 (least buried). Twenty-one residues were selected for mutagenesis to cysteine (Table 1 and Fig. 2), including four predicted to be solvent-inaccessible (Ser-26, Gln-140, Gly-189, and Ala-235), two predicted to be minimally accessible (Gln-40 and Gly-181), and 15 predicted to be highly accessible (Lys-47, Lys-82, Asp-87, Asp-167, Asn-172, Asp-194, Glu-219, Lys-304, Asp-333, Ala-347, Ala-358, and Ile-362).

**Construction of Single Cysteine-containing Mutants of Vph1p and the Effect of Mutations on Growth Phenotype and V-ATPase Activity**—Vph1p contains seven endogenous cysteine residues. A construct encoding Vph1p that lacks the seven
endogenous cysteine residues was previously constructed (31). Vacuoles isolated from yeast expressing this Cys-less form of Vph1 display 78% of wild-type V-ATPase activity (31). Site-directed mutagenesis was used to introduce unique cysteine residues into the Cys-less form of Vph1p at positions described above. These mutant constructs were transformed into the yeast strain MM112, which lacks both Vph1p and Stv1p (the two isoforms of subunit a expressed in yeast), and thus has no functional V-ATPase activity (24). All mutant strains were tested for growth on YEPD buffered to pH 5.5 or 7.5. Yeast that contain <20% wild-type V-ATPase activity are unable to grow at neutral pH (35). This conditional lethality is referred to as the vma" phenotype (36). As shown in Table 1, each of the 21 mutants was able to grow at both pH 5.5 and pH 7.5, indicating that mutant complexes possessing at least 20% wild-type V-ATPase activity were formed in all strains. By contrast, the MM112 strain lacking both Vph1p and Stv1p is unable to grow at neutral pH.

FIGURE 1. Alignment of primary sequences of Vph1p, Stv1p, and subunit I. Primary sequences were aligned using ClustalW2 as described previously (28). Identical residues in the sequences of Vph1p, Stv1p, and subunit I are highlighted in red. Identical residues in the sequences of only Vph1p and subunit I are highlighted in green. The sequences of Vph1p and subunit I display 13% identity and 33% similarity.

FIGURE 2. Homology model of the N terminus of subunit a. The I-TASSER online threading program (30) was used to construct a structural model of the N terminus of Vph1p using the coordinates of the M. ruber subunit I crystal structure (Protein Data Bank code 3RRK) (27). Positions at which unique cysteine residues were introduced into a Cys-less form of Vph1p are indicated by the black circles and amino acid residue numbers.
TABLE 1

| Mutant     | Exposure score* | Vma* phenotype* |
|------------|-----------------|-----------------|
| MM112      | -1              | -amen             |
| Cys-less   | 0               | +amen             |
| Ser-26     | 0               | +amen             |
| Glu-40     | 2               | +amen             |
| Lys-47     | 7               | +amen             |
| Lys-82     | 6               | +amen             |
| Asp-87     | 6               | +amen             |
| Glu-140    | 0               | +amen             |
| Asp-167    | 6               | +amen             |
| Asn-172    | 6               | +amen             |
| Gly-181    | 3               | +amen             |
| Gly-189    | 0               | +amen             |
| Asp-194    | 7               | +amen             |
| Glu-219    | 7               | +amen             |
| Lys-226    | 7               | +amen             |
| Ala-235    | 0               | +amen             |
| Asp-257    | 6               | +amen             |
| Ser-265    | 5               | +amen             |
| Lys-304    | 6               | +amen             |
| Asp-344    | 6               | +amen             |
| Ala-347    | 7               | +amen             |
| Ala-358    | 6               | +amen             |
| Ile-362    | 7               | +amen             |

* Predicted solvent accessibility scores for residues in the N-terminal domain of Vph1p were calculated using the I-TASSER program. Scores ranged from 0 (least accessible) to 7 (most accessible).

** A positive sign indicates wild-type growth; a minus sign indicates no growth.

To further test the function of V-ATPase complexes containing the mutant forms of Vph1p, vacuolar membranes were isolated from each strain and concanamycin-sensitive, ATP-dependent proton transport was measured by 9-amin-6-chloro-2-methoxyacridine fluorescence quenching as described previously (37). Activity is expressed relative to the activity of an antibody against subunit B (Vma2p, which is part of the V0 domain). Each mutant showed a significant increase in ATPase activity that was at least 60% of the control strain. Furthermore, the assembly competence of each of the mutants was tested by the ability of an antibody against subunit B (Vma2p, which is part of the V0 domain) to co-immunoprecipitate subunit a (Vph1p, which is part of the V0 domain). Each mutant showed normal levels of V-ATPase assembly by this assay (data not shown).

Unexpectedly, the I362C mutation resulted in nearly a 3-fold increase in proton transport activity compared with the Cys-less background strain. To further probe this finding, we tested the concanamycin-sensitive ATPase activity of the I362C mutant to determine whether the increase in proton transport activity was accompanied by an increase in its ATPase activity. As shown in Fig. 3A, the concanamycin-sensitive ATPase activity of I362C shows a similar 3-fold increase relative to that of the Cys-less background strain. Point mutations in V-ATPase subunits that increase both proton transport and ATPase activity have previously been identified. It is theorized that these mutations partially “loosen” the V-ATPase complex, giving a conformation with increased catalytic activity (38). By contrast, other mutations have been identified, which lead to changes in the amount of ATP hydrolysis relative to proton transport, and thus lead to a change in the coupling efficiency of the V-ATPase (39, 40).

Modification of Unique Cysteine Residues in Mutant Forms of Vph1p by the Sulfhydryl Reagent MPB in Both V1V0 and Free V0 Complexes—The sulfhydryl-labeling reagent MPB was used to test the aqueous accessibility of the unique cysteine residues introduced into the N terminus of Vph1p (41). We have previously used this reagent to test cysteine accessibility in V-ATPase subunits (16, 17, 31, 42). We expect that cysteine residues exposed on the surface of the protein will react with MPB, whereas cysteine residues buried within the protein or at the interface of two subunits will be protected from reaction. Labeling was performed as described below such that modification by MPB of subunit a in both intact V1V0 and free V0 domains was determined. In this way, residues present at the interface of V1 and V0 could be identified by their modification in free V0 but not intact V1V0 complexes.

Vacuolar membranes were isolated from the strains expressing the Cys-less and mutant forms of Vph1p and reacted with 500 μM MPB for 15 min on ice followed by quenching of the reagent with 10 mM DTT and washing by sedimentation. The membranes were solubilized with C12E8 and assembled and disassembled pumps were separately isolated as follows. To isolate intact V1V0 complexes, immunoprecipitation was performed using an antibody directed against subunit B, such that both V1 and V0 complexes were precipitated. By contrast, to isolate free V0 complexes, immunoprecipitation was performed using the antibody 1D7 against Vph1p. This antibody recognizes its epitope only in the absence of the V1 domain such that immunoprecipitation with 1D7 brings down only free V0 domains (29). Labeling by MPB was detected by separation of the proteins by SDS-PAGE followed by Western blot analysis using horseradish peroxidase-conjugated avidin as described previously (42). Equal loading of subunit a was ensured by Western blotting of a parallel sample with the antibody against Vph1p. Fig. 4 shows representative Western blots from multiple labeling experiments using vacuoles isolated from separate vacuole preparations.

As shown in Fig. 4A, four of the Vph1p mutants tested (S26C, Q140C, G189C, and A235C) showed only faint background labeling comparable with that observed for the Cys-less mutant, consistent with the predicted buried nature of all four residues. By contrast, 17 of the Vph1p mutants (Q40C, K47C, K82C, D87C, D167C, N172C, G181C, D194C, E219C, K226C, D257C, S265C, K304C, D344C, A347C, A358C, and I362C) showed significant labeling by MPB in the free V0 as indicated by the band observed in the lane labeled “a” for each mutant (Fig. 4B and C). These results are again consistent with the predicted surface accessibility of these residues in the model shown in Fig. 2. It should be noted that even those residues having a relatively low accessibility score (such as Q40C and G181C) showed significant modification by MPB under the labeling conditions as described in the “Experimental Procedures.” To determine whether differences in MPB labeling could be detected for residues with different accessibility scores, we compared the time course of MPB labeling of A347C (which has an accessibility score of 7), with two residues which are predicted to have reduced accessibility (Q40C and G181C,
which have scores of 2 and 3, respectively) by varying the incubation time from 5 to 15 min. As shown in Fig. 5A, Q40C does in fact show reduced MPB labeling at shorter times relative to the other two mutants. By contrast, the G181C mutant displays a time course of labeling similar to A347C. To further investigate whether G181C is less accessible than A347C, we compared the concentration dependence of modification by MPB after 5 min for these two mutants over the concentration range of 2–100 μM MPB. As shown in Fig. 5B, G181C shows reduced labeling at lower MPB concentrations as compared with A347C, indicating that it is in fact less accessible.

Next, to identify potential subunit a residues at the interface of the V1 and V0 domains, labeling by MPB in both the assembled and disassembled states was compared. As shown in Fig. 4C, five mutants (K47C, K82C, D167C, D194C, and D344C) showed reduced labeling in the V1V0 complex relative to that observed in the free V0 complex. This reduced accessibility in the intact V-ATPase complex suggests that these residues are likely located at the interface of the V1 and V0 domains.

DISCUSSION

In the current report, we have constructed a molecular model of the N-terminal domain of subunit a of the yeast V-ATPase and tested this model by determining the accessibility of unique cysteine residues introduced into the sequence of Vph1p to chemical modification by the sulfhydryl reagent...
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MPB. The V-ATPases are large, multisubunit, membrane-embedded complexes that have thus far eluded crystallization efforts. Although a number of individual V-ATPase subunits and subcomplexes have been crystallized (7–9), a crystal structure of subunit a of the V₀ domain has not yet been solved. Therefore, structural information regarding subunit a has been derived by more indirect and less high resolution methods, including chemical modification, covalent cross-linking, and electron microscopy (13, 16, 17, 22, 43, 44). These approaches, while not providing the atomic detail of x-ray crystallography, do afford valuable complementary information on interactions between subunits and on structural changes associated with processes such as in vivo dissociation of the V-ATPase complex, which represents an important mechanism of regulating activity.

Recently, the high resolution structure of the N-terminal domain of the homologous subunit (subunit I) from the A-ATPase present in the archaeabacterium M. riber has been solved (27). We have employed this structure to construct a model of the N-terminal domain of Vph1p, one of two isoforms of subunit a expressed in yeast. A similar approach was recently taken in modeling the N-terminal domain of Stv1p, the Golgi-localized isoform of subunit a (28). Small angle x-ray scattering data demonstrate that the N termini of subunit I and Vph1p have a similar overall shape (45, 46). As shown in Fig. 2, the generated model has a hairpin structure with globular proximal

![Figure 4](https://example.com/figure4.png)

**FIGURE 4.** MPB labeling of single cysteine-containing mutants of subunit a in free V₀ and V₁V₀ complexes. Vacuolar membrane vesicles (150 μg of protein) isolated from cells expressing the Cys-less mutant and single cysteine-containing mutants of Vph1p were labeled with 500 μM MPB for 15 min on ice, except where indicated below. The membranes were then solubilized with C₅E₅, and the solubilized membranes were split into equal aliquots for immunoprecipitation. One aliquot was immunoprecipitated with a mouse monoclonal antibody against subunit a (10D7) to immunoprecipitate the free V₀ domain (labeled IP: a). The other aliquot was immunoprecipitated with a mouse monoclonal antibody against subunit B (13D11) to immunoprecipitate the intact V₁V₀ complex (labeled IP: B). Samples were separated by SDS-PAGE followed by transfer to nitrocellulose. Western blotting (WB) was performed using horse radish peroxidase-conjugated NeutrAvidin to detect MPB modification (labeled WB: Avidin) or the monoclonal antibody 10D7 to detect subunit a (labeled WB: a), as described under “Experimental Procedures.” A, 4 residues (marked Buried Residues) showed virtually no MPB labeling for either free V₀ or intact V₁V₀; B, 12 cysteine mutants (marked Exposed Residues) showed equal MPB labeling of subunit a in both free V₀ and intact V₁V₀; C, 5 residues (marked Interfacial Residues) showed reduced MPB labeling in V₁V₀ complexes relative to that observed in free V₀. The above Western blots are representative images of multiple experiments.
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and distal lobes connected by a helical linker region, similar to that observed for subunit C of the V-ATPase (8). The proximal lobe contains, approximately, residues 1–100 and 320–362, whereas the distal lobe contains residues 145–272. Because of the relatively low sequence homology between Vph1p and the M. ruber subunit I, and because the reported structure was of the isolated N-terminal domain of subunit I in the absence of the remainder of the complex, it was important to provide an experimental test of the proposed model. Here, we have tested the accessibility of inserted cysteine residues to the sulfhydryl labeling reagent MPB and compared their accessibility between the assembled and disassembled states of the V-ATPase. Although this approach does not provide a definitive test of the proposed model, it does provide an experimental test of that model within the context of the intact V-ATPase complex. Hence, the information derived will be valuable even after a high resolution structure of the N-terminal domain of Vph1p is solved.

We have found that the four cysteine residues predicted to be buried within the structure of the N-terminal domain of Vph1p were inaccessible to modification by MPB. Similarly, all 17 cysteine residues with solvent accessibility scores ranging from 2 to 7 were found to be accessible to MPB modification in the free V0 domain. To determine whether cysteine residues with non-zero accessibility scores showed variations in MPB labeling, time courses for MPB modification of a mutant with high predicted accessibility were compared with those of mutants with low predicted accessibility. The results demonstrate that the Q40C mutant does in fact have reduced accessibility relative to the other two mutants. By contrast, for the G181C mutant, as with the highly accessible residue, maximal labeling was already observed at 5 min and was unchanged for labeling times up to 15 min. To further examine whether G181C is less accessible than A347C, we determined the concentration dependence of MPB modification for these two mutants. The results demonstrate that G181C exhibits less reactivity toward MPB at lower concentrations than does A347C, which is consistent with the lower predicted accessibility of G181C. Overall, these results support the prediction that G181C is more accessible than Q40C but less accessible than A347C.

The results presented both provide support for our structural model and support its validity for the solution structure of the N terminus of Vph1p in the context of the assembled V0 domain. In addition, they suggest that none of the surface accessible residues of Vph1p tested here are completely shielded from solvent by interaction with other V0 subunits. This latter point is important as electron microscopic studies have suggested that the N terminus of subunit a associates closely with other V0 subunits (47). Our model predicts the accessibility of residues in the isolated N-terminal domain. The fact that none of the predicted surface accessible residues are protected from labeling by other subunits in the free V0 domain suggests that residues at these interfaces may only be partially shielded or that thermal fluctuations in the structure may allow their modification.

To identify surfaces of the N-terminal domain of subunit a that are involved in interaction with subunits in the V1 domain, we have compared the accessibility of introduced cysteine residues to MPB modification in the free V0 and intact V1V0 complex. We have identified five residues (Lys-47, Lys-82, Asp-194, Asp-167, and Asp-344) that when mutated to cysteine were accessible to MPB in the free V0 domain but were partly shielded from labeling in the holoenzyme. These results (summarized together with the earlier results in Fig. 6), suggest that these residues lie at the interface of the V0 and V1 domains. Interactions between the V1 domain and the N-terminal domain of subunit a are important for several reasons. First, these interactions must be sufficient to withstand the torque generated during rotary catalysis because subunit a represents the sole membrane integral subunit of the V-ATPase stator complex. Second, because reversible dissociation of the V1 and V0 domains represents an important mechanism of regulating V-ATPase activity \textit{in vivo}, interactions between V1 and the N-terminal domain must be broken to facilitate this dissociation. Thus, these interactions cannot be so stable that they prevent \textit{in vivo} dissociation of the complex.

A number of previous studies have identified interactions between V1 subunits and the N-terminal domain of subunit a that represent candidates for the shielding interactions identified in the present study. Yeast two-hybrid experiments together with co-immunoprecipitation studies have demonstrated that the N-terminal domain of subunit a interacts with both the catalytic A subunit and subunit H of V1 (48). More recent studies using co-immunoprecipitation indicate that it is
the C terminus of subunit H that binds to the N-terminal domain of subunit a (49). Cross-linking studies using the photolabile maleimide reagent maleimidobenzophenone demonstrated proximity between both subunit E and subunit G of V₁ and subunit a in Vₒ (43, 50). Importantly, cross-linking to subunits E and G occurs in both the proximal and distal lobes of the N-terminal domain of subunit a (51). Because maleimidobenzophenone has a cross-linking arm of ~10 Å, these earlier studies could only provide information about proximity rather than proof of actual subunit contact. The current data demonstrating shielding from solvent accessibility of residues in close proximity to those observed to cross-link to subunits E and G suggest that shielding of residues in both the proximal and distal lobes of the a subunit N terminus is likely due to binding of an EG heterodimer to each site. Additional support for interaction between the N-terminal domain of subunit a and an EG heterodimer comes from recent studies using isothermal calorimetry (45), peptide mapping analysis (52), and a cryoelectron microscopy study of the yeast V-ATPase (44). This latter study also suggests that subunit H makes contact with the N-terminal domain of subunit a in the flexible region between the distal and proximal lobes. We would thus suggest that it is interaction with subunit H that restricts accessibility of residue Lys-82, whereas interactions with separate EG heterodimers sequester the other residues shown to be protected in the proximal and distal lobes of the N-terminal domain.

Interaction between the N-terminal domain of subunit a and subunit H is of particular interest due to the regulatory role of subunit H in preventing ATP hydrolysis by the free V₁ domain (53). Subunit H is present in eukaryotic V-ATPases but not in F-ATPases or A-type ATPases, consistent with regulated assembly generating free peripheral domains in the former but not the latter complexes. In the intact complex, subunit H binds to the N-terminal domain of subunit a, most likely through the

FIGURE 6. Homology model of the N-terminal domain of subunit a indicating the accessibility of unique cysteine residues to modification by MPB. The homology model prepared as described in the legend to Fig. 2 is shown with cysteine residues that were accessible to modification by MPB in both free Vₒ and V₁Vₒ complexes highlighted in green, those that were inaccessible to modification in both free Vₒ and V₁Vₒ complexes highlighted in red, and those that showed reduced accessibility to modification by MPB in V₁Vₒ complexes relative to free Vₒ highlighted in orange. Orange residues that show differential labeling in free Vₒ and V₁Vₒ complexes are postulated to be located at the interface of the V₁ and Vₒ domains. Top and bottom images are related by a 90° rotation about a horizontal axis.
C terminus of subunit H (49). Under these conditions, the rotary complex of the V-ATPase (including subunits D, F, and the ring of proteolipid subunits) is free to rotate, allowing the intact complex to hydrolyze ATP. Upon dissociation of the V₁ and V₀ domains, interaction between subunit H and the N terminus of subunit a is broken, freeing subunit H to interact with the rotary subunit F, thus preventing ATP hydrolysis by free V₁ by physically preventing ATP-driven rotation (54). Maximal inhibition of ATP hydrolysis by subunit H requires the intact subunit (49). This mechanism of silencing ATP hydrolysis by the free V₁ domain allows reversible disassembly of the V-ATPase complex to function as a means of regulating acidification in vivo without excessive hydrolysis of ATP by an uncoupled V₁ domain. Ongoing experiments in our laboratory are aimed at testing whether subunit H and the N-terminal domain of subunit a directly interact via the identified site and whether blocking this interaction could alter pump activity or assembly.

In addition to making contact with other V-ATPase subunits, the N-terminal domain of subunit a has been shown to interact with a number of signaling molecules, which may play a role in regulating V-ATPase assembly in vivo. For example, aldolase has been shown to bind the N-terminal domain of subunit a as well as the V₁ subunits E and B (55). Interaction between V-ATPase subunits and aldolase stabilizes the enzyme in an assembled state. Disruption of this interaction through mutation of specific aldolase residues that do not affect catalytic activity leads to dissociation of the V-ATPase complex (56). In addition to glucose, pH has been implicated as a regulatory cue for the pump assembly state (57). The N terminus of subunit a has recently been shown to interact with the Sec7 domain of cytohesin-2, a GDP/GTP exchange factor involved in pH sensing (58). Moreover, it has been suggested that the V-ATPase regulates cytohesin-2 activity through this interaction. We hypothesize that the exposed regions of the subunit a N terminus identified in the present study are candidate sites for these interactions and may prove useful in the design of regulatory small molecules.

In conclusion, we have mapped the surface of the N-terminal domain of subunit a of the V-ATPase and have obtained support for a model of this domain based upon homology to subunit I of the A-ATPase from M. ruber. These data suggest that very little of the N-terminal domain of subunit a is sequestered through interaction with other V₀ subunits and that the proposed model appears accurate in the context of the remainder of the V-ATPase complex. In addition, we have identified residues that are solvent-inaccessible in the intact V-ATPase and thus likely lie at the interface of the V₁ and V₀ domains. Further studies will be required to confirm the identity of V₁ subunits responsible for sequestering these regions of subunit a and to explore the possibility of modulating these interactions through small molecule inhibitors.

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