Transmembrane Topography of the 100-kDa a Subunit (Vph1p) of the Yeast Vacuolar Proton-translocating ATPase*

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The membrane topography of the yeast vacuolar proton-translocating ATPase a subunit (Vph1p) has been investigated using cysteine-scanning mutagenesis. A Cys-less form of Vph1p lacking the seven endogenous cysteines was constructed and shown to have 80% of wild type activity. Single cysteine residues were introduced at 13 sites within the Cys-less mutant, with 12 mutants showing greater than 70% of wild type activity. To evaluate their disposition with respect to the membrane, vacuoles were treated in the presence or absence of the impermeant sulfhydryl reagent 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) followed by the membrane permeable sulfhydryl reagent 3-(N-maleimidylpropionyl) biocytin (MPB). Three of the 12 active cysteine mutants were not labeled by MPB. The mutants E3C, D89C, T161C, S266C, N447C, K450C, and S703C were labeled by MPB in an AMS-protectable manner, suggesting a cytoplasmic orientation, whereas G602C and S840C showed minimal protection by AMS, suggesting a luminal orientation. Factor Xa cleavage sites were introduced at His-499, Leu-560, and Pro-606. Cleavage at 560 was observed in the absence of detergent, suggesting a cytoplasmic orientation for this site. Based on these results, we propose a model of the a subunit containing nine transmembrane segments, with the amino terminus facing the cytoplasm and the carboxyl terminus facing the lumen.

The vacuolar proton-translocating ATPases (or V-ATPases) are multisubunit complexes found in a variety of intracellular compartments, such as clathrin-coated vesicles, endosomes, lysosomes, Golgi-derived vesicles, chromaffin granules, synaptic vesicles, and the central vacuoles of yeast, Neurospora, and plants (1–9). Acidification of these intracellular compartments is in turn essential for a variety of cellular processes, including receptor-mediated endocytosis, intracellular targeting, protein translocation (27–30), although more recent studies have suggested that some of these residues can be replaced without complete loss of function (31).

Although considerable information has been obtained concerning the membrane topography of the F-ATPase a subunit (32, 33), essentially no information has yet been reported concerning the folding of the V-ATPase a subunit. In the present study, we have employed a combination of cysteine-scanning mutagenesis and chemical modification together with introduction of factor Xa cleavage sites in putative loops to investigate the topographical arrangement of the V-ATPase a subunit.

EXPERIMENTAL PROCEDURES

Materials and Strains—Zymolyase 100T was obtained from Seikagaku America, Inc. Bafilomycin A1 was a kind gift from Dr. Karlheinz Altendorf (University of Osnabruck). Factor Xa protease and protease inhibitors were from Roche Molecular Biochemicals. 9-Amino-6-chloro-2-methoxyacridine (ACMA), 3-(N-maleimidylpropionyl) biocytin (MPB), and 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) were purchased from Molecular Probes. The monoclonal antibody 10D7
against the yeast V-ATPase a subunit (34) was also from Molecular Probes. NeutrAvidin and immunoblotting reagents were obtained from Pierce. *Escherichia coli* and yeast culture media were purchased from Difeo Laboratories. Restriction endonucleases, T4 DNA ligase and other molecular biology reagents were from Life Technologies, Inc., Promega, and New England Biolabs. ATP, phenylmethylsulfonyl fluoride, and most other chemicals were purchased from Sigma.

Yeast strain MM112 (MATa Δvph1::LEU2 attvl:1;LYS2 his3-(2000 leu2 lys2 ura3-52) and plasmid MM322 (VPH1 in pRS316) were used to generate and study VPH1 mutations (24). Yeast cells were grown in yeast extract-peptone-dextrose or synthetic dropout medium (35).

**Isolation of Vacuolar Membrane Vesicles—**Vacuolar membrane vesicles were isolated as described previously (34). For the labeling experiment, yeast cells were grown in medium without glucose for 20 min at 30 °C. The vesicles were isolated by centrifugation at 100,000 g for 15 min and washed using labeling buffer (10 mM Tris-Mes (pH 7.0), 0.25 mM MgCl2 and 20 mM Tris-Mes (pH 7.5), even in the presence of elevated Ca2+). This allowed immunoprecipitation of the V0-V1 complex and reduced variability due to dissociation of the V-ATPase complex.

**Results**

**Construction of the Cys-less Form of Vph1p—**To probe the membrane topology of the 100-kDa subunit of the V-ATPase using a cysteine modification approach, we first needed to construct a Cys-less form of Vph1p in which all seven of the endogenous cysteine residues were replaced. Site-directed mutagenesis of *VPH1* was performed to replace each of the endogenous cysteine residues with serine. The final construct was confirmed by DNA sequencing. Transformation of a yeast strain in which both VPH1 and STV1 were disrupted with the Cys-less form of Vph1p gave rise to a strain showing a wild type growth phenotype. That is, cells were able to grow at both pH 5.5 and 7.5, even in the presence of elevated Ca2+. This is in contrast to the deletion strain that displays a vma- phenotype (i.e., unable to grow at pH 7.5 or in the presence of elevated Ca2+)

**Isolation of Vacuolar Membrane Vesicles—**Vacuolar membrane vesicles were isolated as described previously (25). For the labeling experiment, the membrane vesicles were washed using labeling buffer followed immediately by addition of 50 μM MPB and incubation for 15 min at 25 °C. Dilution was employed rather than washing to stop reaction with AMS to avoid variability due to losses associated with an additional pelleting step. The labeling reaction was then stopped by addition of 15 mM 2-mercaptoethanol.

**Detergent Solubilization, Immunoprecipitation, and Detection of MBP Labeling of the a Subunit—**After MBP labeling, vesicles were pelleted by centrifugation at 100,000 × g for 15 min and solubilized in ice-cold phosphate-buffered saline containing 1% C12E9, and the V0 domain was immunoprecipitated using the mouse monoclonal antibody 10D7 specific for Vph1p plus protein A-Sepharose. In cases where gluconeic acid was not employed after solubilizing, immunoprecipitation was carried out using the monoclonal antibody 8B1-F3 (Molecular Probes) specific for the 70-kDa A subunit, which precipitated both V0-V1 and the V-ATPase complex (25). Vesicle samples were then subjected to SDS-PAGE on 10% acrylamide gels and transferred to nitrocellulose membranes for MBP detection as described below.

For MBP detection, the blots were probed with horseradish peroxidase-conjugated NeutrAvidin and developed using the Supersignal ULTRA chemiluminescent system (Pierce). After MBP detection, blots were stripped and reprobed with the mouse monoclonal antibody 10D7 against the 100-kDa subunit to ensure the presence of equal amounts of the a subunit. For immunoblot analysis, original blots or stripped blots were probed with 10D7, followed by horseradish peroxidase-conjugated secondary antibody (Bio-Rad) as described previously (25). Blots were developed using a chemiluminescent detection method obtained from Kirkegaard and Perry Laboratories.

**Factor Xa Cleavage of Wild Type and Vph1p Mutants Bearing Factor Xa Labeling Sites—**Vacuolar membrane vesicles (50 μg protein) prepared from cells transformed with wild type VPH1 or VPH1 mutants bearing the factor Xa sites as described above were pelleted and resuspended in buffer containing 400 mM potassium iodide, 1.6 mM MgATP, 50 mM Tris-Mes (pH 7.0) and incubated on ice for 60 min. The purpose of the potassium iodide treatment was to remove the V1 domain, thus exposing any factor Xa sites that may be shielded by the presence of V1. Membrane vesicles were then sedimented at 100,000 × g for 45 min and resuspended in factor Xa buffer (100 mM NaCl, 1 mM CaCl2, 1 mM glycerol, 50 mM Tris-HCl (pH 7.4), 2 mM dithiothreitol). The resuspended vesicles were divided into three aliquots. One received no factor Xa, whereas two received 2 μg of factor Xa protease. One of the samples receiving the factor Xa also received 1% C12E9. All samples were incubated overnight at 4 °C followed by addition of Laemmli sample buffer and separation of half of each sample on a 7.5% polyacrylamide gel. The proteins were then blotted to nitrocellulose and probed with the monoclonal antibody 10D7 against the 100-kDa subunit followed by secondary antibody (Bio-Rad). We have observed that the antibody 10D7 recognizes a site in the amino-terminal half of the protein, so that the size of the fragments generated by factor Xa cleavage that would be recognized by 10D7 could be predicted. Blots were developed using a chemiluminescent detection method obtained from Kirkegaard and Perry Laboratories.

**Other Procedures—**Protein concentrations were determined by the method of Lowry et al. (39). ATP-dependent proton transport was measured in transport buffer (25 mM MES/Tris, pH 7.2, 5 mM MgCl2) using the fluorescence probe ACMA in the presence or absence of 10 nM bafilomycin A1 as described previously (40). SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (41).

**Results**

**Construction of the Cys-less Form of Vph1p—**To probe the membrane topology of the 100-kDa a subunit of the V-ATPase using a cysteine modification approach, we first needed to construct a Cys-less form of Vph1p in which all seven of the endogenous cysteine residues were replaced. Site-directed mutagenesis of *VPH1* was performed to replace each of the endogenous cysteine residues with serine. The final construct was confirmed by DNA sequencing. Transformation of a yeast strain in which both VPH1 and STV1 were disrupted with the Cys-less form of Vph1p gave rise to a strain showing a wild type growth phenotype. That is, cells were able to grow at both pH 5.5 and 7.5, even in the presence of elevated Ca2+. This is in contrast to the deletion strain that displays a vma- phenotype (i.e., unable to grow at pH 7.5 or in the presence of elevated Ca2+). Isolation of vacuoles from the wild type and Cys-less strains and measurement of bafilomycin-sensitive ATP-dependent proton transport revealed that the Cys-less form of Vph1p gave rise to a V-ATPase complex that possessed approximately 78 ± 16% of wild type levels of transport activity (Table I). Thus, the Cys-less Vph1p represents an appropriate genetic background in which to construct mutants bearing unique cysteine residues.

**Vph1p Mutants Bearing Single, Unique Cysteine Residues—**Unique cysteine residues were introduced into the Cys-less form of Vph1p at positions 3, 89, 161, 266, 447, 450, 561, 564, 602, 703, 761, 763, and 840 by site-directed mutagenesis, and the mutations were confirmed by DNA sequencing. The mutant forms of Vph1p were then expressed in the deletion strain, and the growth phenotype was analyzed. All mutants displayed a wild type growth phenotype. Isolation of vacuoles and measurement of bafilomycin-sensitive ATP-dependent proton transport (Table I) revealed that all of the single cysteine mutants

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2 X.-H. Leng, T. Nishi, and M. Furgac, unpublished observations.
**Table I**

| Form of Vph1p | Relative bafilomycin-sensitive ATP-dependent ACMA quenching* |
|---------------|---------------------------------------------------------------|
| Wild type     | 1.00                                                          |
| Vector alone  | 0.0                                                           |
| Cys-less      | 0.78                                                          |
| Mutants in the Cys-less background |                     |
| E3C           | 0.79                                                          |
| D89C          | 0.82                                                          |
| T161C         | 0.91                                                          |
| S266C         | 0.92                                                          |
| N447C         | 0.84                                                          |
| K450C         | 0.80                                                          |
| Y561C         | 0.81                                                          |
| S564C         | 0.91                                                          |
| G602C         | 0.96                                                          |
| S703C         | 0.77                                                          |
| F761C         | 0.77                                                          |
| G763C         | 0.49                                                          |
| S840C         | 0.72                                                          |
| Factor Xa mutants in wild type background |                     |
| fXa-499       | 1.03                                                          |
| 2fXa-560      | 0.78                                                          |
| 5fXa-606      | 0.45                                                          |

* ATP-dependent proton transport (as assessed by ACMA quenching) was measured on aliquots of purified vacuolar membrane vesicles containing 2 μg of protein as described under “Experimental Procedures.” Activities are expressed relative to the ΔVph1 Δstv1 strain expressing the pRS316 plasmid containing the wild-type VPH1 gene (defined as 100%), which was completely inhibited by 10 μM bafilomycin. No ATP-dependent ACMA quenching was observed in vacuolar membrane vesicles isolated from cells transformed with the vector alone. Representative values for each mutant are shown.

 except G763C displayed greater than 70% of wild type levels of activity, with G763C having approximately 50% of wild type activity. Western blot analysis of isolated vacuoles using a monoclonal antibody specific for Vph1p (10D7) showed that all the mutants expressed nearly wild type levels of Vph1p (data not shown). These results suggest that, except for G763C, none of the introduced cysteine residues significantly perturb the structure or function of Vph1p.

**Labeling and Protection of Cysteine Mutants of Vph1p**—To assess the orientation of the cysteine residues introduced into Vph1p with respect to the membrane, modification by two sulfhydryl reagents was employed. The first reagent, MPB, is a membrane permeable reagent that can react with sulfhydryl groups exposed on both sides of the vacuolar membrane (32, 33). The second reagent, AMS, is a membrane impermeant reagent that should react only with sulfhydryl groups on the exposed cystoplasmic surface of the vacuolar membrane (32, 33).

Previous studies indicate that intact vacuoles isolated from yeast are both uniformly sided with the cystoplasmic side exposed and sealed with respect to small molecules (45). The specific activity of the V-ATPase in the vacuolar membrane vesicles employed in the current study (3–4 μmol of ATP/min/mg of protein) is very similar to the values we have measured in intact vacuoles (46), suggesting that very little of the V-ATPase is oriented with the cystoplasmic face sequestered inside the vesicles. Moreover, the vacuolar membrane vesicles used here display a ratio of proton transport (as measured by ACMA uptake) to bafilomycin-sensitive ATPase activity that is nearly the same as that we measure for either intact vacuoles or purified, bovine brain clathrin-coated vesicles. We have previously shown that coated vesicles are both tightly sealed and uniformly oriented with the cystoplasmic surface exposed on the outside (47). These results suggest that the vacuolar membrane vesicles employed here have the same properties.

Labeling of cysteine residues exposed on the cytoplasmic surface of Vph1p by MPB should be blocked by prior treatment with the impermeant reagent AMS. By contrast, cysteine residues exposed on the luminal surface of the protein should show labeling by MPB that is unchanged by pretreatment with AMS. This strategy has been successfully employed to determine the orientation of cysteine residues in several membrane proteins, including MDR (44) and subunit a of the F-ATPase (32, 33).

Labeling of Vph1p by MPB is measured by detergent solubilization, immunoprecipitation, SDS-PAGE, and Western blotting using horseradish peroxidase-conjugated avidin. To avoid possible protection of cysteine residues by the V1 domain, immunoprecipitation of the V0 domain was carried out using the monoclonal antibody 10D7 specific for Vph1p. Because 10D7 recognizes the a subunit only in the free V0 domain (not the intact V1V0 complex (34)), it is necessary to optimize the fraction of V0 in the free state. This was accomplished by including a brief glucose starvation step after spheroplasting of the yeast, a procedure that Kane (38) has shown causes an increase in the fraction of free V0 domain in the vacuolar membrane from approximately 25% to 75%.

Isolated vacuoles were first incubated in the presence or absence of 100 μM AMS for 5 min at 10 °C. These conditions were selected to allow reaction of AMS with cytoplasmically oriented cysteine residues but to minimize its permeation across the membrane and are similar to the conditions previously employed in related studies (32, 33). Vacuoles were then diluted 5-fold and reacted with 250 μM MPB for 15 min at 25 °C. Dilution rather than washing was employed to stop reaction with AMS to eliminate additional variability in yield associated with pelleting and resuspending the membranes. The reaction was then stopped by addition of 15 μl 2-mercaptoethanol and the V-ATPase solubilized and immunoprecipitated as described above.

Preliminary results indicated that four of the introduced cysteine residues at positions 561, 564, 761, and 763 were not labeled by MPB, and these mutants were therefore not further pursued. Fig. 1 shows the labeling of the a subunit by MPB with and without prior treatment with AMS for each of the remaining nine cysteine mutants, as well as the Cys-less control. Fig. 1A shows the results obtained for the cysteine residues introduced into the amino and carboxyl-terminal ends of the a subunit, whereas Fig. 1B shows the results for cysteine residues introduced into the loop regions. Because labeling of G602C by MPB in the absence of AMS was reduced approximately 3-fold (by densitometry) relative to the other loop cysteine residues, a longer exposure was employed for this mutant so that the intensity of the band in the absence of AMS would more closely approximate the other cysteine mutants. This facilitates a more accurate comparison of their relative labeling in the presence and absence of AMS. Western blotting using the antibody 10D7 indicated that the reduced labeling of G602C by MPB is not due to a reduced amount of the a subunit (data not shown).

As can be seen in Fig. 1, seven of the nine cysteine residues (E3C, D89C, T161C, S266C, N447C, K450C, and S703C) showed very effective blocking of MPB labeling by pretreatment with AMS, suggesting that these residues are exposed on the cytoplasmic surface of the protein. By contrast, two of the nine cysteine residues (G602C and S840C) showed very little protection of MPB labeling by AMS, suggesting that these residues are exposed on the luminal side of the membrane. These results were reproducible even when the temperature used in blocking by AMS was raised to 20 °C.
were then solubilized with C12E9, and the a subunit immunoprecipitated with 1% C12E9, and protection by AMS in the absence of detergent. Membrane vesicles (35 μg of protein) isolated from the Cys-less mutant and the single cysteine mutants at the amino and carboxyl termini (E3C, D89C, T161C, S266C, and S840C) were incubated in the presence or absence of 100 μM AMS for 5 min at 10 °C, followed by 5-fold dilution and labeling with 250 μM MPB for 15 min at 25 °C. The membranes were then solubilized with C12E9, and the a subunit immunoprecipitated using the monoclonal antibody 10D7 and protein A-Sepharose. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with horseradish peroxidase-NeutrAvidin. Blots were developed using the Supersignal Ultra system from Pierce. B, vacuolar membrane vesicles (45 μg of protein) isolated from the Cys-less mutant, and each of the a subunit mutants containing single cysteine residues in the loop regions (N447C, K450C, G602C, and S703C) were treated as described in A. Because G602C showed approximately 3-fold lower labeling by MPB (by densitometry) in the absence of AMS relative to the other loop cysteine residues, a longer exposure is shown for this mutant such that the intensity of the band in the absence of AMS is comparable to that observed for the other loop cysteine mutants. This allows a more direct comparison of the effect of AMS pretreatment on MPB labeling.

To test whether G602C and S840C are not protected by AMS due to the inability of the reagent to cross the membrane, reaction with AMS and labeling by MPB was repeated for these two mutants and one of the other cysteine mutants (S703C) in the presence of 0.25% Zwittergent 3-14 in order to allow access of the AMS to the luminal side of the membrane. As can be seen in Fig. 2, the presence of the detergent allowed AMS to effectively block G602C and S840C from reaction with MPB, consistent with a luminal orientation of these residues.

Analysis of Mutants Bearing Factor Xa Cleavage Sites—To further probe the topography of the 100-kDa subunit, factor Xa protease cleavage sites were introduced into three putative loops at positions His-499, Leu-560, and Pro-606. Table I shows the effect of introduction of the four amino acid site (IEGR) preceding His-499 and Pro-606 and the tandem eight amino acid sequence (IEGRIEGR) preceding Leu-560. As can be seen, only the single factor Xa site at position 606 significantly reduced proton transport activity (by approximately 55% relative to wild type), whereas the other two mutants showed near wild type levels of activity. When tested for cleavage by factor Xa in the absence and presence of detergent (1% C12E9), only one of the sites (at position 560) showed significant cleavage. As can be seen in Fig. 3, treatment of the mutant bearing the tandem sites at position 560 with factor Xa resulted in the generation of a 65-kDa fragment in either the absence or presence of detergent, whereas no such fragment was observed for the wild type protein. These results suggest that the tandem sites at position 560 are exposed on the cytoplasmic side of the membrane.

DISCUSSION

We have used cysteine-scanning mutagenesis together with chemical modification by membrane permeable and impermeable reagents, as well as introduction of protease cleavage sites, to analyze the membrane topography of the 100-kDa a subunit of the yeast V-ATPase. The cysteine-scanning method depends upon the successful construction of a functionally active Cys-less form of the protein together with mutants containing unique cysteine residues that do not significantly perturb activity. In the case of the a subunit, this required the simultaneous replacement of seven endogenous cysteine residues. In addition, because the a subunit is part of a multisubunit complex, the mutations introduced must not alter the interactions between the a subunit and the remaining V-ATPase subunits. We observed that both the Cys-less form of the a subunit and all but one of the single cysteine-containing mutants give rise to complexes showing near wild type levels of activity, suggesting that these changes do not significantly alter the structure or function of the a subunit. In the case of G763C, a loss of approximately 50% of proton transport activity was observed.

The labeling strategy employed depends upon the membrane permeability of MPB and the membrane impermeability of AMS. This allows MPB to react with cysteine residues on both sides of the membrane, whereas AMS reacts only with cysteine residues exposed on the surface of the membrane vesicles. In the case of yeast vacuolar membrane vesicles used in the present study, the exposed surface is the cytoplasmic side of the membrane. The orientation and sealed state of the vesicles employed is supported by the similar ratio of proton transport to ATP hydrolysis that we have observed when compared with other sealed, well oriented vesicles, including intact yeast vacuoles (46) and purified clathrin-coated vesicles (47). This strategy has been successfully used to study the membrane topography of several other integral membrane proteins, including in particular subunit a of the E. coli F-ATPase (32, 33). These latter studies have led to the current five membrane spanning model for the F-ATPase a subunit.

In addition to the cysteine modification approach, we have also introduced factor Xa cleavage sites into three putative loop regions of the protein. Although the mutant bearing a factor Xa site at position 606 showed only approximately 50% of wild type levels of activity, the other two mutants showed nearly normal proton transport, suggesting minimal perturbation of structure.

The results obtained in the current study have led to the
model for the folding of the V-ATPase a subunit shown in Fig. 4. In addition, a hydrophathy plot for Vph1p together with the location of each of the putative membrane spanning segments is shown in Fig. 5. The protein is proposed to span the bilayer nine times, with the amino terminus on the cytoplasmic side of the membrane and the carboxyl terminus on the luminal side. All four of the cysteine residues introduced into the amino-terminal soluble domain showed a labeling pattern consistent with a cytoplasmic orientation whereas S840C at the carboxyl terminus showed labeling characteristic of a luminal orientation. This suggests that the amino and carboxyl termini are on opposite sides of the membrane, requiring that the protein span the bilayer an odd number of times.

In our original folding model for the a subunit (25), we had postulated that the amino-terminal soluble domain was exposed on the luminal side of the membrane based upon the increased labeling observed for the bovine a subunit using membrane impermeant reagents when the membrane was disrupted with detergents (47). This result may be explained by a change in conformation of the protein in the presence of detergent such that previously shielded sites on the cytoplasmic side of the membrane become exposed. In addition, because the a subunit is not synthesized with an amino-terminal leader sequence, a cytoplasmic orientation for the amino terminus is more consistent with what is known concerning the requirements for translocation of hydrophilic protein segments across the endoplasmic reticulum membrane during biosynthesis.

A luminal orientation of the carboxyl terminus is also at odds with our previously proposed model, in which we suggested a cytoplasmic orientation for the carboxyl terminus (26). This was based on the identification by random mutagenesis of a cluster of five mutations between residues Leu-800 and Gly-814 that disrupted attachment of the V₁ and V₀ domains, suggesting that this region may be important in assembly of the V-ATPase complex (26). It is nevertheless possible that disruption of assembly in these mutants results from conformational changes in the structure of the 100-kDa subunit or the V₀ domain that prevent attachment of V₁ to V₀. Interestingly, our current model places nearly all of the residues that have been observed to disrupt assembly, including Asp-425, Lys-538, and Arg-735, as well as the five mutations between residues 800 and 814 on the luminal side of the membrane. It is possible that these residues may form a luminal domain that senses the intravesicular pH and conveys this information through conformational changes in the 100-kDa subunit to the cytoplasmic domain of the complex.

With respect to the loop regions, four of the introduced cysteine residues (at positions 561, 564, 761, and 763) are not labeled by MPB, suggesting that they are shielded from reaction by interaction with other V₀ subunits or other regions of the 100-kDa subunit itself. It is also possible that one or more of these cysteine residues may be shielded from reaction with MPB by interaction with the lipid bilayer. Of the remaining introduced cysteine residues, three appear to have a cytoplasmic orientation (N447C, N450C, and S703C) whereas G602C appears to be luminal. The cytoplasmic orientation of residues 447 and 450 requires that the hydrophobic region between residues 405 and 443 span the bilayer twice rather than once (as originally postulated (25)), placing Asp-425 on the luminal side of the membrane. A luminal orientation for Gly-602 and a cytoplasmic orientation for Ser-703 is consistent with a single membrane span between these sites. That Ser-703 is cytoplas-
FIG. 5. Hydropathy plot of Vph1p and location of putative transmembrane segments. The amino acid sequence of Vph1p (24) was analyzed using the method of Kyte and Doolittle (49), and the resultant hydropathy plot shown. The location of the nine putative transmembrane segments are also shown and labeled I–IX. There are four consensus N-linked glycosylation sites (Asn-X-Ser/Thr) in Vph1p at Asn-113, Asn-280, Asn-324, and Asn-374. Because all four sites are located in the amino-terminal soluble domain, which our labeling data clearly shows is cytoplasmic, it is unlikely that any of these four sites are glycosylated in vivo. In fact, it has not clearly been demonstrated that Vph1p is glycosylated in yeast. If Vph1p is glycosylated, it is possible that carbohydrate is attached at O-linked sites, for which there is not a clear consensus sequence.

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**REFERENCES**

1. Stevens, T. H., and Forgac, M. (1997) *Annu. Rev. Cell Dev. Biol.* **13**, 779–808
2. Forgac, M. (1992) *J. Bioenerg. Biomembr.* **24**, 341–350
3. Bowman, B. J., Vazquez-Laslop, N., and Bowman, E. J. (1992) *J. Bioenerg. Biomembr.* **24**, 361–370
4. Kane, P. M., and Stevens, T. H. (1992) *J. Bioenerg. Biomembr.* **24**, 383–394
5. Anraku, Y., Umemoto, N., Hirata, H., and Ohyu, Y. (1992) *J. Bioenerg. Biomembr.* **24**, 395–406
6. Sze, H., Ward, J. M., and Lai, S. (1992) *J. Bioenerg. Biomembr.* **24**, 371–382
7. Gluck, S. L. (1992) *J. Bioenerg. Biomembr.* **24**, 351–360
8. Kibak, H., Taiz, L., Starke, T., Bernasconi, P., and Gogarten, J. P. (1992) *J. Bioenerg. Biomembr.* **24**, 415–424
9. Nelson, N. (1992) *J. Bioenerg. Biomembr.* **24**, 407–414
10. Chatterjee, D., Chakraborthy, M., Leit, M., Neff, L., Jamsa-Kellokumpu, S., Fuchs, R., and Baron, R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 6257–6261
11. Swallow, C. J., Grinstein, S., and Rotstein, O. D. (1990) *J. Biol. Chem.* **265**, 7645–7654
12. Weber, J., and Senior, A. E. (1997) *Biochim. Biophys. Acta* **1319**, 19–58
13. Fillingame, R. H. (1997) *J. Exp. Biol.* **200**, 217–224
14. Cross, R. L., and Duncan, T. M. (1996) J. Bioenerg. Biomembr. 28, 403–408
15. Pedersen, P. L. (1996) J. Bioenerg. Biomembr. 28, 389–395
16. Capaldi, R. A., Agerler, R., Wilkens, S., and Gruber, G. (1996) J. Bioenerg. Biomembr. 28, 407–411
17. Futai, M., and Omote, H. (1996) J. Bioenerg. Biomembr. 28, 409–414
18. Zimniak, L., Dittrich, P., Gogarten, J. P., Kibak, H., and Taiz, L. (1988) J. Biol. Chem. 263, 9102–9112
19. Bowman, E. J., Tenney, K., and Bowman, B. (1988) J. Biol. Chem. 263, 9120–9124
20. Mandel, M., Moriyama, Y., Hulmes, J. D., Pan, Y. C., Nelson, H., and Nelson, N. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5521–5524
21. Hirota, R., Graham, L. A., Takatsuki, A., Stevens, T. H., and Anraku, Y. (1997) J. Biol. Chem. 272, 4785–4803
22. Perin, M. S., Fried, V. A., Stone, D. K., Xie, X. S., and Sudhof, T. C. (1991) J. Biol. Chem. 266, 3877–3881
23. Manolson, M. F., Proteau, D., Preston, R. A., Knoblauch, A., Roberts, B. T., Hoyt, M. Petruzzelli, M., Mulholland, J., Betz, D., and Jones, E. W. (1992) J. Biol. Chem. 267, 14294–14303
24. Manolson, M. F., Wu, B., Proteau, D., Taillon, B. E., Roberts, B. T., Hoyt, M. A., and Jones, E. W. (1994) J. Biol. Chem. 269, 14964–14974
25. Leng, X. H., Manolson, M., Liu, Q., and Forgac, M. (1996) J. Biol. Chem. 271, 22487–22493
26. Leng, X. H., Manolson, M, and Forgac, M. (1998) J. Biol. Chem. 273, 6717–6723
27. Cain, B. D., and Simoni, R. D. (1986) J. Biol. Chem. 261, 10014–10020
28. Cain, B. D., and Simoni, R. D. (1986) J. Biol. Chem. 263, 6606–6612
29. Cain, B. D., and Simoni, R. D. (1989) J. Biol. Chem. 264, 3292–3300
30. Vik, S. B., and Antonio, B. J. (1994) J. Biol. Chem. 269, 30364–30369
31. Valiyaveetil, F. I., and Fillingame, R. H. (1997) J. Biol. Chem. 272, 32635–32641
32. Valiyaveetil, F. I., and Fillingame, R. H. (1998) J. Biol. Chem. 273, 16241–16247
33. Long, J. C., Wang, S., and Vik, S. B. (1998) J. Biol. Chem. 273, 16235–16240
34. Kane, P. M., Kuehn, M. C., Howard-Stevenson, I., and Stevens, T. (1992) J. Biol. Chem. 267, 447–454
35. Guthrie, C., and Fink, G. R. (1991) Methods Enzymol. 194, 13–14
36. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425
37. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds.) (1992) Short Protocols in Molecular Biology, John Wiley & Sons, New York
38. Kane, P. M. (1995) J. Biol. Chem. 270, 17025–17032
39. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
40. Feng, Y., and Forgac, M. (1992) J. Biol. Chem. 267, 5817–5822
41. Laemmli, U. K. (1970) Nature 227, 680–685
42. Yamashiro, C. T., Kane, P. M., Weclzyk, D. F., Preston, R. A., and Stevens, T. H. (1990) Mol. Cell Biol. 10, 3737–3749
43. Nelson, H., and Nelson, N. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3503–3507
44. Loo, T. W., and Clarke, D. M. (1995) J. Biol. Chem. 270, 843–848
45. Kakinuma, Y., Ohsumi, Y., and Anraku, Y. (1981) J. Biol. Chem. 256, 10859–10863
46. Liu, Q., Kane, P. M., Newman, P. R., and Forgac, M. (1996) J. Biol. Chem. 271, 2018–2022
47. Arai, H., Terres, G., Pink, S., and Forgac, M. (1988) J. Biol. Chem. 263, 8796–8802
48. Adachi, I., Arai, H., Pimental, R., and Forgac, M. (1990) J. Biol. Chem. 265, 960–966
49. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132