Site-directed Mutagenesis of the 100-kDa Subunit (Vph1p) of the Yeast Vacuolar (H\textsuperscript{+})-ATPase*

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Vacuolar (H\textsuperscript{+})-ATPases (V-ATPases) are multisubunit complexes responsible for acidification of intracellular compartments in eukaryotic cells. V-ATPases possess a subunit of approximate molecular mass 100 kDa of unknown function that is composed of an amino-terminal hydrophilic domain and a carboxyl-terminal hydrophobic domain. To test whether the 100-kDa subunit plays a role in proton transport, site-directed mutagenesis of the VPH1 gene, which is one of two genes that encodes this subunit in yeast, has been carried out in a strain lacking both endogenous genes. Ten charged and twelve polar residues located in the seven putative transmembrane helices in the COOH-terminal domain of the molecule were individually changed, and the effects on proton transport, ATPase activity, and assembly of the yeast V-ATPase were measured. Two mutations (R735L and Q634L) in transmembrane helix 6 and at the border of transmembrane helix 5, respectively, showed greatly reduced levels of the 100-kDa subunit in the vacuolar membrane, suggesting that these mutations affected stability of the 100-kDa subunit. Two mutations, D425N and K358A, in transmembrane helix 1 and at the border of transmembrane helix 3, respectively, showed reduced assembly of the V-ATPase, with the D425N mutation also reducing the activity of V-ATPase complexes that did assemble. Two mutations, H743A and K583A, in transmembrane helix 6 and at the border of transmembrane helix 4, respectively, have significantly greater effects on activity than on assembly, with proton transport and ATPase activity inhibited 40–60%. One mutation, E789Q, in transmembrane helix 7, virtually completely abolished proton transport and ATPase activity while having no effect on assembly. These results suggest that the 100-kDa subunit may be required for activity as well as assembly of the V-ATPase complex and that several charged residues in the last four putative transmembrane helices of this subunit may play a role in proton transport.

The vacuolar (H\textsuperscript{+})-ATPases (V-ATPases)

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are a family of proton pumps responsible for acidification of intracellular compartments in eukaryotic cells (for reviews see Refs. 1–9). Among the compartments acidified by V-ATPases are clathrin-coated vesicles, endosomes, lysosomes, secretory vesicles, such as synaptic vesicles and chromaffin granules, and the central vacuoles of plants, Neurospora and yeast. Acidification of these compartments, in turn, plays an important role in such processes as receptor-mediated endocytosis, intracellular membrane traffic, protein processing and degradation, and coupled transport of small molecules. In yeast, acidification of the central vacuole is important both to maintain the activity of degradative enzymes and to drive uptake of solutes such as Ca\textsuperscript{2+} and amino acids (5).

The V-ATPases are multisubunit complexes composed of two structural domains. The peripheral V\textsubscript{1} domain is a 500-kDa complex responsible for ATP hydrolysis, whereas the integral V\textsubscript{0} domain is a 250-kDa complex responsible for proton translocation (10). In Saccharomyces cerevisiae, the V\textsubscript{1} domain is composed of seven different subunits of molecular masses approximately 100 kDa (subunit A encoded by VMA1 (11, 12)), 60 kDa (subunit B/VMA2 (13)), 54 kDa (VMA3 (14)), 42 (subunit C/VMA5 (15, 16)), 32 (subunit D/VMA8 (17)), 27 (subunit E/VMA4 (15, 18)), and 14 kDa (subunit F/VMA7 (19, 20)). The V\textsubscript{0} domain is composed of at least four subunits of molecular masses approximately 100 kDa (encoded by VP\textsubscript{H1} and STV1 (21, 22)), 36 kDa (VMA6 (23)), 13 kDa (VMA10 (24)), and 17 kDa (subunit C/VMA3 and VMA11 (25, 26)). By analogy with the bovine coated vesicle V-ATPase (27), the V\textsubscript{1} domain has the structure A\textsubscript{3}B\textsubscript{3}C\textsubscript{1}D\textsubscript{1}E\textsubscript{1}F\textsubscript{1}, whereas the V\textsubscript{0} domain has the structure 100\textsubscript{A}36\textsubscript{B}c\textsubscript{5}. No mammalian homolog to Vma10p has yet been identified, and the yeast counterpart to the bovine 19-kDa V\textsubscript{0} subunit is also uncertain.

Although the V-ATPases are homologous to the F-ATPases (28–31), both in overall structure (10, 27) and in sequence homology of several of the subunits (11–13, 25, 32–38), no obvious structural homolog exists for the 100-kDa subunit in the F-ATPases. The 100-kDa subunit of the V-ATPase in yeast is encoded by two homologous genes, VP\textsubscript{H1} (21) and STV1 (22). VP\textsubscript{H1} encodes a 95-kDa protein, which possesses a hydrophilic amino-terminal domain of approximately 45 kDa and a carboxyl-terminal hydrophobic domain of approximately 50 kDa containing 6–7 putative transmembrane helices (21). STV1 encodes a 102-kDa protein that shares the same domain arrangement and is 54% identical in amino acid sequence with the product of the VP\textsubscript{H1} gene (Vph1p) (22). Disruption of the VP\textsubscript{H1} gene leads to somewhat reduced growth at neutral pH relative to acidic pH (21) and disruption of STV1 has no obvious phenotypic consequences (22). By contrast, disruption of both VP\textsubscript{H1} and STV1 leads to the typical Vma\textsuperscript{--} phenotype, including the inability to grow at neutral pH and hypersensitivity to Ca\textsuperscript{2+} (22). These results suggest that the VP\textsubscript{H1} and STV1 gene products are at least partially able to substitute for each other. The reason that yeast possess two genes encoding the 100-kDa subunit is uncertain, but the results available thus far suggest that V-ATPases possessing Vph1p and Stv1p may be targeted to different intracellular membranes (22). In order to determine whether the 100-kDa subunit plays a direct role in proton transport.

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1 The abbreviations used are: V-ATPase, vacuolar proton-translocating adenosine triphosphatase; Mes, 4-morpholineethanesulfonic acid.
transport by the V-ATPase complex, site-directed mutagenesis of the VPH1 gene product has been carried out in a strain lacking endogenous Vph1p and Stv1p.

**EXPERIMENTAL PROCEDURES**

**Materials and Strains—**Zymolyase 100T was obtained from Seikagaku America, Inc. [35S]Trans-label was purchased from ICN. Bafilomycin A1 was a kind gift from Dr. K. Kane (University of Osnabruck). Leupeptin was from Boehringer Mannheim. 9-Amino-6-chloro-2-methoxyacridine was from Molecular Probes, Inc. ATP, phenylmethylsulfonylfluoride, and most other chemicals were purchased from Sigma.

**VPH1 Mutagenesis—**Mutagenesis was performed on SacI-EcoRI or EcoRI-BamHI fragments of the wild type vph1 gene in the vector pALTER-1 (Promega) following the manufacturer’s protocol. The mutagenesis oligonucleotides were as follows with substitution sites underlined:

- T414A, 5′-GTGACAATTGCGGGTAAACCAG-3′; T414A, 5′-GTGACAATTGCGGGTAAACCAG-3′
- Y585F, 5′-GAAACCTGCGTACATGG-3′; Y585F, 5′-GAAACCTGCGTACATGG-3′
- H547A, 5′-ATAAGAATAGGTCA-3′; H547A, 5′-ATAAGAATAGGTCA-3′
- K538A, 5′-GAAACCTGCGTACATGG-3′; K538A, 5′-GAAACCTGCGTACATGG-3′
- T475A, 5′-GTGGT-3′; T475A, 5′-GTGGT-3′
- S472A, 5′-GTCATTAAGAACCCGGCACCCATATCACC-3′; S472A, 5′-GTCATTAAGAACCCGGCACCCATATCACC-3′
- T781V, 5′-GTGGT-3′; T781V, 5′-GTGGT-3′
- Y463F, 5′-AACAAAATAATGTATACACCAGTGAAGGCCA-3′; Y463F, 5′-AACAAAATAATGTATACACCAGTGAAGGCCA-3′

Fragments containing the indicated mutations were sequenced (22) to generate and study VPH1 mutants. Yeast cells were grown in YPD medium (yeast extract-peptone-dextrose) to a coupled spectrophotometric assay (45) with the modification of using 0.5 mM NADH and 0.5 mM bacto Na₂HPO₄ for 35 min at 4°C. The pelleted protein was measured in transport buffer (25 mM Mes/Tris, pH 7.2, 5 mM MgCl₂, and 25 mM KCl) using the fluorescence probe aminophenolic-6-chloro-2-methoxyacridine as described previously (46) in the presence or the absence of 10 mM bafilomycin A₁. Protein concentration was measured using the Lowry method (47). SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (48). Silver staining was performed using the method of Oakley et al. (49).

Western blots were probed with mouse monoclonal 8B1-F3 against the 69-kDa subunit, (from Molecular Probes, Inc.) or 10D7 against the 100-kDa subunit, (a generous gift from Dr. P. Kane), followed by horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Blots were developed using a chemiluminescent detection method obtained from KPL. Quantitations were done using an IS-1000 Digital imaging system (Alpha Innotech Corporation).

Immunoprecipitations were carried out as described (50), with the following modifications. Cells were grown overnight in supplemented minimal medium lacking methionine and then converted to spheroplasts by incubation for 20 min with 0.5 unit of zymolase/10⁷ cells in SD-Met, 1.2 m sorbitol, 50 mM Tris-Mes, pH 7.5. Aliquots containing 5 × 10⁷ spheroplasts were then incubated with [35S]Trans-label (50 μCi) for 60 min at 30°C. Spheroplasts were then pelleted, lysed in phosphate-buffered saline with C₆E₉, and immunoprecipitated (50) using 7.5 μg of purified 8B1-F3 antibody and protein A-Sepharose followed by SDS-polyacrylamide gel electrophoresis on 12% acrylamide gels and autoradiography as described (50).

**RESULTS**

It has previously been shown that deletion of genes encoding subunits of the yeast V-ATPase leads to a conditional lethal phenotype such that strains carrying such deletions are unable to grow at neutral pH but are able to grow at acidic pH (51). We have employed a strain in which both the VPH1 and STV1 genes encoding the 100-kDa subunit of the V-ATPase have been disrupted, leading to the typical Vma⁻ phenotype (22). Expression of the VPH1 gene on a CEN plasmid in this strain leads to growth at pH 7.5. Twenty-two individual site-directed mutations were introduced into the VPH1 gene and the mutant proteins expressed in the double knock out strain. The residues selected for mutation all correspond to polar or charged residues located within the seven putative transmembrane helices in the COOH-terminal half of the 100-kDa subunit. These residues were selected in an effort to identify buried polar or charged amino acids that might directly contribute to the proton conduction pathway in the V-ATPase complex. Polar residues were changed to nonpolar residues of similar size (for example substituting alanine for serine or phenylalanine for tyrosine), whereas charged residues were replaced either by similar polar amino acids (i.e. glutamine for glutamic acid) or by nonpolar amino acids (usually alanine). It is anticipated that replacement of a polar or charged amino acid that directly participates in proton movement across the membrane will disrupt ATP-dependent proton transport. Of these 22 mutations, only three showed greatly reduced growth at neutral pH (E789Q, D425N, and R735I) (data not shown), with the latter two having an identical growth phenotype to the double knock out strain. Because as little as 20% of the wild type V-ATPase activity is sufficient to rescue the growth phenotype of a Vma⁻ strain, it was necessary to measure the vacuolar proton pumping and V-ATPase activity in each mutant strain to assess the effect of the mutation on V-ATPase activity. Fig. 1 shows the ATP-dependent proton transport and the bafilomycin-sensitive ATPase activity for isolated vacuoles purified from a Δvph1Δstv1 strain expressing the pRS316 plasmid alone, pRS316 containing the wild type VPH1 gene, or the VPH1 gene bearing the indicated point mutations. Bafilomycin A1 has been shown to be a specific inhibitor of the V-ATPases

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*P. Kane, personal communication.*
As expected on the basis of the growth phenotype, three mutations (D425N, R735L, and E789Q) showed 20% or less of the wild type proton transport and V-ATPase activity, with both D425N and R735L having virtually no detectable activity. Four additional mutations (K538A, K593A, Q634L, and H743A) showed between 20 and 70% of wild type activity, with Q634L having the lowest activity of this group. The remaining mutants all displayed 70% or greater of wild type activity. None of the mutations resulted in significant uncoupling of proton transport from ATP hydrolysis (i.e., a greater loss of proton transport than ATPase activity), although one mutation (Q634L) did decrease ATPase activity to a somewhat greater extent than proton transport, suggesting a possible increase in coupling efficiency.

The most obvious ways in which mutations in the 100-kDa subunit might lead to a decrease in V-ATPase activity is if these mutations resulted in a 100-kDa subunit that was unstable and rapidly degraded or that was unable to correctly assemble with the remaining subunits of the V-ATPase complex. To assess these possibilities, Western blot analysis was performed on isolated vacuoles using antibodies directed against Vph1p and the A subunit of the V-ATPase. It has previously been shown that disruption of the VPH1 gene leads to the inability of the V1 domain (including the A subunit) to assemble onto the vacuolar membrane (21). Moreover, the absence of any of the V1 subunits (with the exception of the 54-kDa subunit (14)) leads to loss of assembly of the entire V1 domain onto the vacuolar membrane (53, 54). Thus the presence of the 100-kDa and A subunits on the vacuolar membrane provides a reasonable measure of V-ATPase assembly.

Fig. 2 shows a Western blot carried out on vacuolar membranes from the wild type, deletion, and mutant strains using antibodies directed against either Vph1p or the A subunit, whereas Fig. 3 shows the results of quantitative analysis of the Western blot as well as the activity data on each mutant for comparison. As can be seen, two mutants (R735L and Q634L) showed greatly reduced levels of the 100-kDa subunit in the vacuolar membrane as well as greatly reduced levels of A subunit. These mutations thus appear to affect stability of the 100-kDa subunit. Two other mutations (D425N and K538A) show significantly reduced levels of A subunit associated with the vacuolar membrane with only slightly reduced levels of the 100-kDa subunit. These mutations thus appear to have an effect on assembly of the V-ATPase complex. It should be noted from Fig. 3 that the D425N mutant, while still having detectable levels of assembly as assessed by the presence of the A subunit, is completely devoid of proton transport and ATPase activity, suggesting that any V-ATPase that does assemble is inactive. This result suggests that Asp425 may play a role in both assembly and activity of the V-ATPase.

The remaining mutants have near normal levels of the 100-kDa and A subunits. In particular, the E789Q mutant shows wild type levels of both subunits while possessing less than 20% of the wild type levels of proton transport and ATPase activity. Similarly, examination of Fig. 3 reveals that both the K593A and H743A mutants have approximately half as much...
the V-ATPase complex. It should be noted, however, that the basis of the data shown in Fig. 3. Thus, all three of these only the V1 subunits are immunoprecipitated in the vector asphereoplasts and metabolically labeled with [35S]Trans-label the following experiment was performed. Cells were converted to spheroplasts and metabolically labeled with [35S]Trans-label for 60 min at 30 °C followed by cell lysis, detergent solubilization, and immunoprecipitation using the anti-A subunit antibody 8B1-F3 and protein A-Sepharose. As can be seen in Fig. 4, the complete complement of V1 and V0 subunits are immunoprecipitated from cells expressing wild type Vph1p, whereas only the V1 subunits are immunoprecipitated in the vector control. Of the three mutants listed above, H743A and E789Q showed wild type patterns of immunoprecipitation, whereas K593A showed the normal pattern of subunits but with some-what reduced levels of the V0 subunits, as predicted on the basis of the data shown in Fig. 3. Thus, all three of these mutations appear to impair activity rather than assembly of the V-ATPase complex. It should be noted, however, that the absence of one of the smaller V-ATPase subunits (i.e. the VMA7 or VMA10 gene products) might not be detectable by this method and that no test for “correct” assembly of the V-ATPase complex has been performed.

To further characterize these mutants, we have determined the K_m for ATP and V_{max} values as well as the pH optima for the wild type and for each of the mutants affecting activity as well as for Q634L. As can be seen from the data in Table I, no change greater than 40% was observed in K_m values for any of the mutations tested, although the V_{max} values are in good agreement with the activity data shown in Fig. 1. These data indicate that the observed decrease in activity in these mutants is not due to a greatly diminished affinity for ATP. Interestingly, two of the mutations did result in a very significant shift in the pH optima of the enzyme. Thus, whereas the optimum pH for the wild type V-ATPase was approximately 7.2, that for H743A was 8.2, whereas that for E789Q was 9.7. In addition, the activity at the pH optimum of these mutants was approxi-mately 75% (for H743A) and 30% (for E789Q) of the activity at the optimum pH of the wild type.

FIG. 2. Effect of Vph1p mutations on stability of the 100-kDa subunit and association of the 68-kDa A subunit with the vaculear membrane. Vacuolar membrane vesicles (5 μg) isolated from the Δvph1a9ts1 strain expressing the wild type VPH1 gene, the VPH1 gene bearing the indicated mutations, or the vector alone were subjected to SDS-polyacrylamide gel electrophoresis on a 12% acrylamide gel followed by transfer to nitrocellulose and Western blot analysis using the c subunit (57) that are present in a stoichiometry of 100, 38, 19, and 17 kDa (sub-

**DISCUSSION**

The V-ATPases resemble the F-ATPases of mitochondria, chloroplasts, and bacteria (28–31) both in overall structure (10, 27) and in sequence homology between several of the subunits, including the nucleotide binding A and B subunits (32–37, 11–13) and the dicyclohexylcarbodiimide-reactive c subunit (25, 38). As with the F-ATPases, the V-ATPases possess a peripheral domain (V1) responsible for ATP hydrolysis and an integral domain (V0) responsible for proton translocation. Unlike the corresponding F-ATPase domains, however, the V1 and V0 domains do not retain their respective activities when separated from each other (55–57).

An important question with regard to the V-ATPases is the mechanism by which they translocate protons. For the bovine coated vesicle V-ATPase, the V0 domain, which is responsible for proton translocation (58), is composed of four subunits of approximate molecular masses 100, 38, 19, and 17 kDa (subunit c) (57) that are present in a stoichiometry of 100, 38, 19, 17 (60). Dicyclohexylcarbodiimides reacts with a buried carboxyl group located near the middle of the last of four putative transmembrane helices. Although a low level of proton translocation has been reported for the isolated, reconstituted c subunit (61), optimal levels of dicyclohexylcarbodiimide-inhibitable proton transport are only observed when the complete complement of V0 subunits are re-assembled prior to reconstitution (58). The basis for difference in proton conduc-tion properties of the native and reassembled V0 domains remains uncertain.

In the case of the F-ATPases, the F_{0} domain of Escherichia coli is composed of three subunits of molecular masses 30 (a), 17 (b), and 8 kDa (c) that are present in a stoichiometry of a_{2}b_{2}c_{10–12} (62). Reassembly studies indicate that for F_{0} all three subunits are required to form a functional proton channel (63, 64). Mutational analysis has demonstrated that the buried carboxyl group located in the second of two transmembrane helices of the c subunit is critical for proton translocation (60). Moreover, genetic analysis of the a subunit indicates the presence of several buried polar and charged residues, particularly in the last two transmembrane helices, which also play a critical role in proton translocation (65, 66). Thus, substitutions at Ser{sup 229}, Arg{sup 210}, and Glu{sup 236} in the fifth putative transmembrane helix or His{sup 245} in the sixth putative transmembrane helix of the a subunit significantly impair proton translocation through F_{0}. These studies have led to a model in which the proton conduction pathway is composed of several residues of the a subunit, possibly arranged as an amphipathic helix, with the buried carboxylate of the c subunit providing a gate in the conduction pathway (60, 65).

By analogy with the F-ATPases, we would predict that the V-ATPases should have some homolog to the a subunit, which would serve a comparably important role in proton translocation. Of the V_{0} subunits besides the c subunit (which contains no more buried polar or charged residues than the F_{0} c subunit (38, 25)), the 36-kDa subunit (Vma6p) contains no putative transmembrane helices (25, 27). The sequence of the highly hydrophobic mammalian 19-kDa subunit has not been ob-tained; however, no yeast counterpart to this subunit has yet been identified. The remaining yeast V_{0} subunit, the 13-kDa product of the VMA10 gene (24), resembles the product of the VMA6 gene in possessing no putative transmembrane helices. Thus the only yeast V_{0} subunit that has been identified that might serve the role of the a subunit in the V-ATPase complex is the 100-kDa subunit.
The 100-kDa subunit is composed of an NH₂-terminal hydrophilic domain of 45 kDa and a COOH-terminal hydrophobic domain of 55 kDa containing 6–7 putative transmembrane helices (21, 22, 68). In addition to the two yeast 100-kDa subunit genes (\textit{VPH1} and \textit{STV1}), three additional cDNAs from mouse, rat, and bovine have been cloned (68–70), with 40–95% amino acid identity observed between pairs of these sequences (22). Based upon hydropathy analysis (21), a tentative model for the folding of the 100-kDa subunit in the membrane is shown in Fig. 5. The amino-terminal hydrophilic domain has been placed on the lumenal side of the membrane based upon two observations. First, labeling of the coated vesicle 100-kDa subunit by membrane impermeant reagents is only observed after detergent permeabilization of the membrane (27), suggesting that the large hydrophilic domain is sequestered within the lumen of the coated vesicle. Second, proteolysis of the 100-kDa subunit in the membrane is shown in Fig. 5. The amino-terminal hydrophilic domain has been placed on the luminal side of the membrane based upon two observations. First, labeling of the coated vesicle 100-kDa subunit by membrane impermeant reagents is only observed after detergent permeabilization of the membrane (27), suggesting that the large hydrophilic domain is sequestered within the lumen of the coated vesicle. Second, proteolysis of the 100-kDa subunit by trypsin in intact coated vesicles results in cleavage at a site between transmembrane helices five and six.⁵

Because coated vesicles are oriented with the cytoplasmic surface exposed, this places the loop between H5 and H6 on the cytoplasmic side of the membrane. Tracing of the polypeptide back to the amino terminus places the amino-terminal domain on the luminal side of the membrane. There are other data, however, that make this assignment tentative. Thus protease treatment of intact yeast vacuoles leads to disappearance on Western blots of any band recognized by a polyclonal antiserum raised against a peptide located in the amino-terminal domain of \textit{Vph1p}, suggesting that this epitope is exposed on the cytoplasmic side of the vacuole.⁴ Further work is thus necessary to resolve the actual orientation of the 100-kDa subunit in the membrane.

Like the F₀ a subunit, the 100-kDa subunit possesses multiple polar and charged residues located within the putative transmembrane helices in the hydrophobic COOH-terminal domain. To test whether the 100-kDa subunit might be playing an analogous role to the a subunit in proton translocation, we carried out site-directed mutagenesis of 22 such polar and charged residues in this domain. The residues selected for mutagenesis (shown circled in Fig. 5) are all conserved between the available 100-kDa sequences from yeast, mouse, rat, and bovine.

Interestingly, most of the mutations tested did not show any impairment of proton translocation. These residues are presumably not individually critical for proton translocation by the V-ATPase, although it is possible that replacement of several of these residues together might impair proton transport. Mutations at Arg⁷³⁵ and Gln⁶³⁴ in H6 and the border of H5, respectively, led to the nearly complete absence of the 100-kDa subunit in the vacuolar membrane, suggesting that the proteins containing these mutations were either unstable and rapidly degraded or else mistargeted to some other intracellular membrane. Given the results that suggest that the 100-kDa subunit possesses targeting information in yeast (22), the latter possibility should not be ruled out. However, the fact that for most integral membrane proteins in yeast targeting to the vacuole represents the default pathway (71) makes it more

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³ I. Adachi and M. Forgac, unpublished data.

⁴ M. Manolson, unpublished observations.
likely that these mutations have affected stability of the 100-kDa subunit. Mutations at Asp\(^{425}\) and Lys\(^{538}\) in H1 and at the border of H3, on the other hand, did not prevent folding and targeting of the 100-kDa subunit to the vacuolar membrane but did interfere with proper assembly of the V-ATPase complex as demonstrated by the reduced level of the peripheral A subunit on the vacuolar membrane. Because these four mutations prevented the appearance of a V-ATPase complex in the vacuolar membrane, it is not possible to determine the role of the corresponding residues in proton transport.

The mutations of greatest interest are those that inhibited proton transport and ATPase activity but did not have obvious effects on assembly or stability of the V-ATPase complex. The three residues that fell into this last category are Lys\(^{593}\) at the border of H4, His\(^{743}\) in H6, and Glu\(^{789}\) in H7. For both K593A and H743A, proton transport and ATPase activity were only 50% of that predicted on the basis of the amount of assembly observed, whereas for E789Q, the V-ATPase complex was vir-

![FIG. 4. Effect of Vph1p mutations on V-ATPase assembly.](http://www.jbc.org/content/274/13/22492/F4)

Fig. 4. Effect of Vph1p mutations on V-ATPase assembly. Yeast cells (the Δvph1Δstv1 strain) expressing the wild type VPH1 gene, the indicated mutations or the vector alone were grown overnight in methionine-free medium followed by conversion to spheroplasts and incubation with \[^{35}S\]Trans-label (50 \(μ\)Ci/5 \(× 10^6\) spheroplasts) for 60 min at 30 °C. Spheroplasts were then pelleted and lysed in phosphate-buffered saline with C\(_s\)E\(_a\), and the V-ATPase immunoprecipitated using the monoclonal antibody 8B1-F3 directed against the 69-kDa A subunit and protein A-Sepharose followed by SDS-polyacrylamide gel electrophoresis on a 12% acrylamide gel and autoradiography as described under “Experimental Procedures.” The positions of the V-ATPase subunits are indicated and were confirmed by comparison with the migration of \(^{14}C\)-labeled molecular mass standards. WT, wild type.

![FIG. 5. Model for folding of the 100-kDa subunit (Vph1p) of the yeast V-ATPase and the location and effects of point mutations in Vph1p.](http://www.jbc.org/content/274/13/22492/F5)

Fig. 5. Model for folding of the 100-kDa subunit (Vph1p) of the yeast V-ATPase and the location and effects of point mutations in Vph1p. The orientation of the 100-kDa subunit in the membrane is based upon labeling and proteolysis data obtained for the 100-kDa subunit of the bovine coated vesicle V-ATPase (see text). In particular, the position at which trypsin cleaves the bovine 100-kDa subunit from the cytoplasmic side of the membrane is indicated. Residues that were mutated in this study are circled. Two mutations (R735L and Q634L) affected stability of the 100-kDa subunit, two mutations (D425N and K538A) affected assembly of the V-ATPase complex, whereas three mutations (H743A, K593A, and E789Q) affected proton transport and ATPase activity of the V-ATPase complex.

| Mutation | pH optimum | \(K_m\) for ATP (mM) | \(V_{max}\) rel |
|----------|------------|---------------------|-----------------|
| Wild type | 7.2        | 0.70 ± 0.08         | 1.00 ± 0.07     |
| K593A    | 7.0        | 0.61 ± 0.17         | 0.33 ± 0.03     |
| Q634L    | 7.0        | 0.43 ± 0.11         | 0.18 ± 0.02     |
| H743A    | 8.2        | 0.79 ± 0.35         | 0.50 ± 0.02     |
| E789Q    | 9.7        | 0.41 ± 0.04         | 0.11 ± 0.02     |
tually completely devoid of activity. In addition, both the H743A and E789Q mutations resulted in a significant change in the pH optimum of the enzyme. While it is difficult to assign a precise interpretation to these findings, they may reflect an alteration in the environment of residues whose protonation state is important for transport to occur. Interestingly, as with the F_2 subunit, positively and negatively charged residues in the last two putative transmembrane helices appear to be important for activity, possibly serving to line a polar channel necessary to allow protons to gain access to the buried carboxyl group of the c subunit. These results suggest that the 100-kDa subunit may serve an analogous role to the a subunit in the V-ATPase complex, such as coupling of proton transport to ATP hydrolysis.

We have previously presented data that suggest the 100-kDa subunit may also possess the binding site for the specific V-ATPase inhibitor bafilomycin A1 (58). Thus, reconstituting the V_0 complex, such as coupling of proton transport to ATP hydrolysis, whether they serve some other function in the V-ATPase complex, remains a possibility. One could imagine a series of events that might lead to conversion of the 100-kDa subunit into the functional V_0 subunit. For example, the 100-kDa subunit may be selectively stabilized by the a subunit, and this stabilization might be accompanied by the loss of necessary residues from the a subunit and the exposure of new residues to the cytosolic surface.

In summary, mutation of the residues in the last two putative transmembrane helices greatly enriched the field of the vacuolar ATPases. Further studies will be necessary to resolve this question.

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