Mutational Analysis of the Subunit C (Vma5p) of the Yeast Vacuolar H\textsuperscript+-ATPase*

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Subunit C is a V\textsubscript{1} sector subunit found in all vacuolar H\textsuperscript{+}-ATPases (V-ATPases) that may be part of the peripheral stalk connecting the peripheral V\textsubscript{1} sector with the membrane-bound V\textsubscript{0} sector of the enzyme (Wilkins, S., Vasilyeva, E., and Forgac, M. (1999) J. Biol. Chem. 274, 31804–31810). To elucidate subunit C function, we performed random and site-directed mutagenesis of the yeast VMA5 gene. Site-directed mutations in the most highly conserved region of Vma5p, residues 305–325, decreased catalytic activity of the V-ATPase by up to 48% without affecting assembly. A truncation mutant (K360stop) identified by random mutagenesis suggested a small region near the C terminus of the protein (amino acids 382–388) might be important for subunit stability. Site-directed mutagenesis revealed that three aromatic acids 382–388) might be important for subunit stability.

This paper is available on line at http://www.jbc.org

Received for publication, December 7, 2001, and in revised form, January 2, 2002
Published, JBC Papers in Press, January 3, 2002, DOI 10.1074/jbc.M111708200
the vacuolar membrane (16). These data indicate that subunit C is an essential subunit of the V-ATPase.

Certain features of the C subunit distinguish it from most other V1 subunits, however. Deletion of the A, B, D, E, F, or G subunits disrupts assembly of the remain V1 subunits to a much greater extent than deletion of the C or H subunits (17–19). This suggests that the C and H subunits could play a role in bridging the V1 and V0 sectors rather than acting as "core" subunits or either sector. Consistent with such a role for Vma5p, Puopolo et al. (20) report that partial proton pumping activity could be restored to clahtin-coated vesicles stripped of V1 by the addition of a V1 fraction lacking the C subunit, but the reconstituted complexes were unstable. In addition, all subunits of the V0 and V1 sectors except subunit C remain assembled with their respective sectors when reversible disassembly of the V-ATPase is induced by glucose deprivation (13, 21). The C subunit is released from cytosolic V1 sectors upon disassembly and reattached to the V1 and V0 sectors upon reassembly (13). These studies suggest that subunit C is involved in the unique requirements for regulation of the enzyme and may be important for achieving the balance of stability and instability described above. To better understand the structural and functional roles of the C subunit, we have begun a mutagenesis study of the yeast VMA5 gene. Our studies reveal several distinct mutant classes that implicate subunit C in the V-ATPase structure but also suggest that this subunit may not be essential for ATP hydrolysis and proton pumping by the V-ATPase.

**EXPERIMENTAL PROCEDURES**

**Materials and Strains—**Zymolyase 100T and Tran™S-label were purchased from ICN. Dithiothreitol(succinimidyldipropionate) was purchased from Pierce. Concaminacin A was purchased from Wako Biochemicals. Trypsin phosphatase-conjugated secondary antibodies were from Promega. Restriction enzymes were purchased from New England Biolabs. All other reagents were purchased from Sigma.

The vma5Δ strain, SF383–1Da vma5Δ (MATa ade6 leu2-3,112 ura3-52 pep4-3, his4-519 vma5Δ::LEU2) (16), was obtained from Tom Stevens. The wild type strain referred to throughout the following study is the vma5Δ strain that has been transformed with a CEN plasmid carrying wild-type VMA5, pMH14, pMH14 fully complements the growth phenotypes of a vma5Δ strain (16).

Cells were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) buffered to pH 5 or fully supplemented minimal media (SD) lacking components used for selection (22). Mutants were tested for growth on YEPD buffered to pH 7.5 containing 60 mM CaCl2 prepared as described (23). For comparison of growth rates by serial dilution, cells were grown overnight to log phase in supplemented minimal medium lacking uracil (SD-uracil). Cells were diluted to the indicated density in water and then plated onto the media indicated.

**Random Mutagenesis—**VMA5 was randomly mutagenized using a PCR-based protocol. The VMA5 gene was cloned from pMH10, which consisted of VMA5 cloned into pBluescript KS+, using the SpeI and HindIII restriction sites into a vector of the yeast shuttle vector pRS216 (24) in which the ClaI and XbaI sites had been destroyed to form plasmid pCX5. VMA5 was amplified from pMH10 using Taq polymerase and T3 and T7 oligonucleotides following the low frequency mutagenesis protocol described by Liu and Kane (25). pCX5 was digested with EcoRI and ClaI, removing most of the VMA5-coding sequence. The vma5Δ strain was co-transformed with the gapped pCX5 vector and the mutagenized PCR product (23). Transformants were selected on SD-uracil plates, then were patched onto YEPD pH 5.0 plates and incubated for 30°C. These plates were then replica-plated to YEPD pH 7.5, SD-uracil, and YEPD pH 5.0. Colonies able to grow on SD-uracil and YEPD pH 5.0 but not YEPD pH 7.5 were selected as potential mutants and further characterized.

**Site-directed Mutagenesis—**Mutant constructs were produced using the Altered Sites II in vitro mutagenesis system from Promega. Full-length VMA5 was subcloned from pMH10 into pALTER using the HindIII and SacI restriction sites. Mutations were made with the use of different mutagenic oligonucleotides listed in Table I.

The double mutant Y382A,Y388A was constructed using the Y388A mutation as the template and the Y382A mutagenic oligonucleotide. All mutations were confirmed by sequencing at the BioResource Center DNA Sequencing Facility, Cornell University. All mutations were confirmed by sequencing at the BioResource Center DNA Sequencing Facility, Cornell University. All mutations were confirmed by sequencing at the BioResource Center DNA Sequencing Facility, Cornell University. All mutations were confirmed by sequencing at the BioResource Center DNA Sequencing Facility, Cornell University.

**Biochemical Characterization of Mutants—**Whole cell lysates were prepared and subjected to SDS-PAGE followed by Western blot analysis as previously described (27).

Vacular vesicles were isolated as described by Roberts et al. (28) with the following modifications. Cells were grown overnight in YEPD pH 5.0 or SD-uracil and converted to spheroplasts, but spheroplasts were washed two times in 0.25 M Suc and incubated for 30°C. During the last wash cells were incubated at 30°C for 10 min to ensure V-ATPase assembly, then cells were lysed and homogenized in 50 ml of lysis buffer (10 mM Mes-Tris, pH 6.9, 0.1 mM MgCl2, 12% Ficoll). ATPase activity was determined by the coupled enzyme assay described by Lotersche et al. (29) in the presence and absence of 100 nM concanamycin A, a specific V-ATPase inhibitor (30). The concanamycin A-sensitive ATPase activity is shown. Protein pumping was monitored by quinacrine quenching as described (31).

**Western Blot Analysis of Vacular Vesicles—**Protein concentrations of isolated vacuolar vesicles were determined as described by Lowry et al. (32). Vacuolar vesicles were solubilized using cracking buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% SDS, 5% β-mercaptoethanol) and heating for 15 min at 75°C. Equal amounts were analyzed by SDS-PAGE followed by transfer to nitrocellulose. The presence of V-ATPase sub-
units was determined by Western blotting with the subunit-specific antibodies 10D7, 8B1, 13D11, and 7A2, mouse monoclonal antibodies raised against the α, β, γ, and δ subunits (27), followed by an alkaline phosphatase-conjugated goat anti-mouse secondary antibody or a rabbit polyclonal antibody raised against the E subunit (a generous gift from Tom Stevens) followed by an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody.

Immunoprecipitations—V-ATPase complexes were isolated as described by Kane (13). Spheroplasts were labeled with Tras 35S-label for 60 min while shaking at 30 °C. Labeled spheroplasts were then solubilized in the presence of 0.67 mM dithiobis(succinimidyl propionate), and V-ATPase complexes were immunoprecipitated with the indicated monoclonal antibody followed by protein A-Sepharose. Immunoprecipitated complexes were visualized by SDS-PAGE followed by analysis on a Molecular Dynamics Storm PhosphorImager.

RESULTS

Random Mutagenesis—The S. cerevisiae subunit C gene VMA5 was both cloned based on protein sequence (33) and isolated in a genetic screen for V-ATPase mutants. The gene was demonstrated to encode a 42-kDa V1 sector subunit (16). There is no homologue of VMA5 in F-ATPases, and relatively little is known subunit C function in any species. In an attempt to better understand the structure and function of this subunit we initiated a mutagenesis study.

First, a random mutagenesis approach was taken. The VMA5 gene was mutagenized using a PCR-based protocol that relies on the reduced fidelity of Taq polymerase (see “Experimental Procedures”). A vma5Δ strain was transformed with constructs containing mutagenized VMA5 PCR products in combination with a gapped plasmid, and mutant plasmids were generated in vivo by gap repair (34). Alternatively, a VMA5-containing plasmid was mutagenized by passage through an Escherichia coli mutator strain (23). Approximately 5000 transformants containing mutant plasmids generated by the two methods were analyzed for a Vma/q/H11002 growth phenotype, characterized by inability to grow at elevated pH and calcium levels (35, 36). Although we and others have successfully isolated random mutations in several other V-ATPase subunit genes by this method (23, 25, 37), we recovered very few vma5 mutants, and most of those that were recovered did not appear to express any Vma5p based on immunoblots of whole cell lysates. Two vma5 mutants were identified that did express the subunit protein; both proved to encode truncations. One mutation resulted in a frameshift at amino acid 353 that introduced a stop codon 6 amino acids later as well as a missense mutation, R310K. The second was a truncation mutan,
K360stop, which exhibited a tight Vma\(^-\) phenotype (Fig. 2A). Western blot analysis of whole cell lysates revealed that the K360stop mutant produced a small amount of Vma5p with a molecular mass lower than the wild-type subunit (Fig. 2B). Isolated vacuolar vesicles from this mutant displayed no V-ATPase activity (Fig. 2C) and almost completely lacked V1 subunits (subunits A, B, and E) associated with the vacuolar membrane but contained wild-type levels of the V0 subunit \(\alpha\) (Fig. 2D). Both the vma5\(^{\Delta}\) mutant and the K360stop mutant vesicles do contain a small amount of B subunit at the vacuolar membrane, as described previously (16).

Genes for subunit C have now been identified and cloned from a variety of different species. Fig. 1 is a multisequence alignment of subunit C genes cloned from five evolutionarily distant organisms, yeast, Arabidopsis, C. elegans, Drosophila, and Bos taurus (bovine). The K360stop mutation deleted the last 33 amino acids of Vma5p. The region after Lys-K360 is quite poorly conserved among the organisms shown in Fig. 1, except for a small region rich in aromatic amino acids between 380 and 389. We targeted this region for site-directed mutagenesis to examine the role of these amino acids in Vma5p stability and function.

Investigating the C terminus of VMA5—Two truncation mutations, which changed Thr-380 and Ile-389 to stop codons, were designed to fall just before and just after this conserved C-terminal region. If this region were in fact needed for wild-type Vma5p function, then the T380stop mutant might display a classical Vma\(^-\) phenotype, whereas I389stop mutant would not. Plasmids carrying the different truncation mutants were transformed into a vma5\(^{\Delta}\) strain, and transformants were screened for a Vma\(^-\) growth phenotype (Fig. 2A). The T380stop mutant exhibited a strong Vma\(^-\) phenotype; the I389stop mutant grew similarly to wild type. Immunoblot analysis of whole cell lysates showed decreased levels of Vma5p in cells containing the T380stop mutation and wild type levels in cells containing the I389stop mutation (Fig. 2B). These results implicated residues 381 through 388 in subunit stability.

In previous mutagenesis studies, the presence of the Vma\(^-\) phenotype typically suggested that there was less than 25% wild-type V-ATPase activity present in the cell (25). We iso-

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**Fig. 2.** Phenotypes of truncation mutants near the C terminus of VMA5. A, Vma\(^-\) growth phenotypes of mutants. Strains indicated were streaked onto YEPD pH 7.5 + 60 mM CaCl\(_2\) or YEPD pH 5.0 plates and incubated for 48 h at 30 °C. B, levels of Vma5p in whole cell lysates of strains harboring different Vma5p mutants. Lysates prepared from equal cell numbers of the different strains were subjected to SDS-PAGE and then transferred to nitrocellulose. Subunit C was detected using the 7A2 monoclonal antibody, as described under “Experimental Procedures.” C, concanamycin-sensitive ATPase activities in isolated vacuolar vesicles. Vacular vesicles were isolated as described under “Experimental Procedures” and assayed for V-ATPase activity. Activities shown are sensitive to 100 nm concanamycin A. The data shown represent the mean of \(n\) separate vacuole preparations from each strain; the bars indicate S.E. D, levels of V-ATPase subunits at the vacuolar membrane. Equal concentrations of vacuolar vesicles were solubilized and subjected to SDS-PAGE followed by transfer to nitrocellulose. 10 \(\mu\)g of total vesicle protein was loaded to identify the \(\alpha\), A, and E subunits, 12 \(\mu\)g was loaded to visualize the B subunit, and 8 \(\mu\)g was used to detect the C subunit. Immunoblots were probed with the 10D7, 8B1, 13D11, and 7A2 monoclonal antibodies against the \(\alpha\), A, B, and C subunits, respectively, or polyclonal antibody against the E subunit.
lated vacuolar vesicles to quantitate the amount of V-ATPase activity in the presence of the T380stop and I389stop mutations. Consistent with the growth phenotype, Fig. 2 demonstrates that compared with a wild-type strain, only 7.7% as much ATPase activity is found in vacuolar vesicles from the T380stop mutant, and 90% as much activity is found in vesicles from the I389stop mutant. Immunoblots of vesicle protein indicated decreased levels of V1 subunits assembled at the vacuolar membrane of the T380stop mutant (Fig. 2D), whereas the I389stop mutation allowed assembly of wild-type levels. These results focused our mutagenesis efforts on the amino acids between Thr-380 and Ile-389.

We performed alanine-scanning mutagenesis in this region, focusing on the charged and aromatic residues. Glu-381, Tyr-382, Phe-385, and Tyr-388 were all individually mutated to alanine. Mutant constructs were transformed into vma5Δ cells. We did not observe a Vma− phenotype with any of the point mutations, even in the more sensitive serial dilution assay for growth (Fig. 3A). Levels of Vma5 protein were determined for all strains; all produced wild-type levels of Vma5p (Fig. 3B). Some point mutants displayed a reproducible effect on the mobility of Vma5p.

We determined that the mobility shifts were due to secondary structure differences, not covalent modifications, because they disappeared in gels run in the presence of high concentrations of urea (data not shown). Vacuolar vesicles were isolated from all strains, and V-ATPase activity was evaluated. Mutants E381A, Y382A, F385A, and Y388A supported an average of 106, 35, 90, and 41% of the wild-type V-ATPase activity, respectively, in vacuolar vesicles (Fig. 3C). To ascertain if the reduced activity of mutants Y382A and Y388A was the result of a catalytic or an assembly defect, vacuolar vesicles were analyzed for V-ATPase subunit levels. Fig. 3D demonstrates that vacuolar vesicles isolated from all of the strains contained comparable levels of the V0 subunit a, but strains carrying the Y382A or Y388A mutations contained reduced levels of V1 sector subunits A, C, and E (once again the effect on subunit B is less pronounced). This suggests that the reduction in V-ATPase activity in these two mutants is due to decreased assembly of V-ATPase subunits at the vacuolar membrane. Surprisingly, there are reduced levels of all of the V1 subunits in vacuolar vesicles isolated from cells containing the F385A mutant as well despite the near wild-type ATPase activity detected in the isolated vacuolar vesicles. This suggests that in

Fig. 3. Phenotypes resulting from site-directed mutagenesis in the Glu-381–Tyr-388 region of VMA5. A, Vma− growth phenotypes. The indicated strains were grown overnight and diluted to the same density. A series of 10-fold serial dilutions were made for each strain, and the diluted and undiluted suspensions were plated onto YEPD pH 7.5 + 60 mM CaCl2 or YEPD pH 5.0 and incubated at 30 °C for 48 h. B, levels of the C subunit in whole cell lysates. Whole cell lysates were analyzed for the level of C subunit protein by immunoblot as described in Fig. 2. C, concanamycin A-sensitive ATPase activities in wild-type and mutant vacuolar vesicles. Vacuolar vesicles were isolated, and ATP hydrolysis activity was measured; all activities shown are the average n different experiments and are sensitive to 100 nM concanamycin A. The bars represent the S.E. of the measurements. D, levels of V-ATPase subunits in isolated vacuolar vesicles. Equal amounts of vacuolar vesicle protein were solubilized and separated by SDS-PAGE followed by immunoblotting. 6 μg of total vacuolar vesicle protein was analyzed for a, A, and E subunits, 12 μg was used to detect the B subunit, and 15 μg was loaded to detect the C subunit. The immunoblots were analyzed as described in Fig. 2 and under “Experimental Procedures.” Wt, wild type.
the presence of the F385A mutant, there is a reduction in the amount of assembled V-ATPase, but the enzyme that is assembled has a higher $k_{cat}$ than wild-type. This point is addressed below.

To establish whether the decreased levels of subunits found at the vacuolar membrane were due to a defect in enzyme assembly or stability, we examined the assembly of the V-ATPase in the Y382A, Y388A, and F385A mutants by immunoprecipitation. Strains harboring the different vma5 mutants were biosynthetically labeled, and V-ATPase complexes were immunoprecipitated under nondenaturing conditions in the presence of cross-linker. Fig. 4 indicates no differences between the immunoprecipitated complexes from wild-type and mutant strains. This implies that there are no fundamental defects in V-ATPase biosynthesis in strains containing the Y382A, F385A, or Y388A mutations, even though there is an effect on V-ATPase subunit levels in isolated vacuolar vesicles. We hypothesize that the enzyme is stable enough to exhibit function in vivo and remain assembled through immunoprecipitation in the presence of the cross-linker but is not stable enough to survive vacuole isolation. This phenomenon has previously been observed in mutagenesis studies of the G subunit (38).

Because tyrosine can be post-translationally modified, we mutated the Tyr-382 to phenylalanine, which cannot be phosphorylated. Fig. 3 shows that Y382F supports full wild-type function in vivo and remain assembled through immunoprecipitation in the presence of the cross-linker. However, it is not stable enough to survive vacuole isolation. This phenomenon has previously been observed in mutagenesis studies of the G subunit (38).

Because tyrosine can be post-translationally modified, we mutated the Tyr-382 to phenylalanine, which cannot be phosphorylated. Fig. 3 shows that Y382F supports full wild-type function, suggesting that an aromatic residue at 382 may be sufficient for Vma5p function. Both Y382A and Y388A had effects on the V-ATPase activity, so we determined whether there was an additive effect of simultaneously mutating both residues to alanine by constructing a double point mutant, Y382A, Y388A. Cells containing the double point mutant exhibit at most a very weak Vma- phenotype, with slightly slower growth on pH 7.5 plates containing 60 mM CaCl$_2$. There was no effect on Vma5p stability in whole cell lysates (Fig. 3B). Vacular vesicles isolated from the double point mutant strain exhibited only 15% wild-type V-ATPase activity, however. Even lower levels of the C and E subunits were at the vacuolar membrane than in either of the single mutants, suggesting there is a cumulative effect of the mutations. Non-denaturing immunoprecipitation
showed wild-type V-ATPase assembly in the Y382A,Y388A strain (Fig. 4). This appears to be another example of a V-ATPase structure that is capable of assembly and some activity in vivo but is too unstable to survive preparation of vacuolar vesicles.

Site-directed Mutagenesis of Conserved Regions of Vma5p—Although Vma5p homologues from different organisms show less conservation than some other V-ATPase subunits, there is evidence that they are structurally and functionally interchangeable. Beltran et al. (33) replaced more than half of the yeast VMA5 gene with the bovine subunit C gene and showed that the chimera could complement the growth defects of a vma5Δ mutant. Oka et al. (39) complemented the defects of a vma5Δ strain with the C. elegans C subunit. In Arabidopsis, the C subunit is encoded by the det3 gene; Schumacher et al. (40) found that the yeast VMA5 gene could complement the defects of a det3 mutant. These results suggest that the structure of subunit C is retained in evolutionarily distant organisms and that the amino acids conserved between the different homologues could be involved in structural and functional interactions with conserved regions of other V-ATPase subunits. Based on these observations, we performed alanine-scanning mutagenesis of a number of the most conserved charged and aromatic amino acids in VMA5.

The region between amino acids 305 and 326 of the yeast VMA5 sequence is the most conserved among all species, so we decided to focus on mutagenesis of this part of the protein. Many of the conserved aromatic and charged residues surrounding this region, including Phe-255, Phe-260, Tyr-262, Tyr-296, Phe-300, Lys-307, Tyr-312, Ser-315, Arg-318, Tyr-318, and Phe-325, were individually mutated to alanine (some of these amino acids proved not to be conserved in Arabidopsis, but the mutagenesis was initiated before the Arabidopsis sequence was available). Fig. 5A shows that only one of the point mutations, F255A, resulted in a VmaΔ phenotype. Whole cell lysates were prepared from all strains to evaluate Vma5p stability. F255A was the only mutant that exhibited an unstable subunit C; all other mutants produced wild-type levels of the C subunit (Fig. 5B). Although it is interesting that only the F255A point mutation renders subunit C unstable, we did not characterize this mutant further because it is essentially a null mutant. Vacuolar vesicles were isolated, and V-ATPase activity was measured in all of the other strains. Compared with wild type, the mean ATPase activities varied from 120 to 52% (Fig. 5C). Two mutants, F260A and Y262A, showed average ATPase activities comparable with or slightly higher than wild type, and the Y312A mutant behaved like wild type. The rest of the mutations resulted in reduced V-ATPase activities. To further investigate we analyzed vacuolar vesicles for subunit levels. Strains carrying the mutations Y296A through F325A had wild-type levels of subunits at the vacuolar membrane (Fig. 5D). Although the Y319A mutant appears to have higher levels of V1 subunits at the membrane than wild-type in this blot, this effect was not observed consistently. This suggests that, with the exception of Y312A, mutations in the region from Y296A through F325A had modest effects on V-ATPase activity without affecting enzyme assembly.

Western blot analysis of vacuolar vesicles isolated from F260A and Y262A revealed greatly reduced levels of V1 sector subunits (Fig. 5D). In fact, the C subunit was undetectable in vacuolar vesicles from these mutants unless the amount of protein loaded was increased relative to that shown for the other mutants in Fig. 5D. This is surprising because vacuolar vesicles isolated from these two mutants contained V-ATPase activities at least as high as the wild-type on average (Fig. 5C). To determine whether the V-ATPase was ever capable of normal assembly in the F260A and Y262A mutants, we immunoprecipitated V-ATPase complexes from whole cells solubilized in the presence of cross-linker as described above. Fig. 6 reveals that V-ATPase complexes formed in the F260A and Y262A mutants are indistinguishable from wild-type complexes. These two mutants are therefore similar to the F385A mutant in that they appear to generate unstable V-ATPase complexes with a higher $k_{cat}$ than the wild-type enzyme.

Because this phenotype is quite novel, we probed the characteristics of the F260A, Y262A, and F385A mutants more closely. We first determined whether the ATPase activity observed in vesicles from these mutants was coupled to proton pumping. We monitored quinacrine quenching as a measure of ATP-driven proton pumping at high (2.5 m) ATP concentrations. Under these conditions, vacuolar vesicles from the 3 mutants showed 88–100% the extent of quinacrine quenching seen in the wild-type vesicles; concanamycin-sensitive ATPase activities in this experiment were 84–89% of wild-type for the three mutants, as indicated (Fig. 7A). These results indicate that the V-ATPase complexes in these mutants are functionally coupled. To more accurately estimate the $k_{cat}$ of the mutant vesicles, we performed a titration of the wild-type vesicles from the same preparation assayed in Fig. 7A and directly compared the level of V1 subunits in the wild-type and mutant vesicles (Fig. 7B). Vesicle samples used for ATP hydrolysis and proton pumping assays were not centrifuged and are comparable with the (~ lanes) in Fig. 7B. Identical concentrations of the wild-type and mutant vesicles were loaded, and based on comparison to the titration, we conclude that the F260A, Y262A, and F385A mutant membranes contain only approximately one-third as much A subunit as the wild-type vesicles. The levels of the C subunit were even lower, particularly in the F260A mutant (less than 16% wild-type levels). Therefore, the mutant ATPases support coupled proton transport with a $k_{cat}$ at least three times that of the wild-type enzyme, and they are active in...
the presence of very little C subunit. We next asked whether structural coupling of the V₁ and V₀ sectors was affected in the mutants. In general, V₁ sectors remain tightly bound to the membrane V₀ sectors in vitro unless the interaction is disrupted by chaotropes such as nitrate or iodide (41–43). Even chaotrope treatment is relatively inefficient at removing V₁ from V₀ unless MgATP is present (41) and the enzyme is able to bind and hydrolyze ATP (25). However, we found that the A and B subunits (Fig. 7B) from the F260A, Y262A, and F385A mutants were very poorly pelleted with V₀ in vacuolar membranes by centrifugation, even in the absence of chaotrope and catalytic substrate. The C subunit is removed almost completely from the Y262A and F385A mutant vesicles by centrifugation.

**DISCUSSION**

**Classification of VMA5 Point Mutants**—Table II summarizes the phenotypes of the mutants described here. Based on their phenotypes, the mutants fall into several distinct classes. Three are essentially null mutants that destabilize Vma5p and, therefore, exhibit a full set of Vma⁻ phenotype. This class includes the C-terminal truncation mutants K360stop and T380stop and the missense mutation F255A. A second class of point mutants affect ATPase activity of the V-ATPase without affecting assembly of the complex. Mutations in the most highly conserved region of VMA5 fell quite consistently in this class. Certain mutations in stalk subunits D and G result in catalytic defects without any obvious assembly defect (37, 38), and the mutations in subunit D also had relatively modest effects on activity and did not result in a Vma⁻ phenotype. Like subunit D, subunit C may play a role in the long range conformational interactions necessary for efficient ATP hydrolysis.

A number of the VMA5 point mutations destabilize V-ATPase complexes in vitro, resulting in lower levels of V-ATPase activity in isolated vacuoles, but allow sufficient function in vivo to permit growth at elevated pH and calcium concentrations. In these mutants, the mutant subunit C is able to assemble into the V-ATPase and to support assembly of V₁ with V₀ as determined by non-denaturing immunoprecipitations in the presence of cross-linker. However, when vacuolar vesicles are isolated, activities are low, and assembly of V₁ subunits at the vacuolar membrane is severely reduced. Mutagenesis studies of the yeast VMA10 gene have produced this class of mutants as well (38), and it is particularly interesting that one of those mutants, vma10-E14A, appeared to have specific effects on Vma5p. Our interpretation of this phenotype is that the V-ATPase in these mutants is functional but fragile. Although it can be isolated by immunoprecipitation in the presence of cross-linker, it cannot survive the multiple steps involved in vacuole purification. Mutations in the conserved aromatic amino acids near the C terminus of subunit C gener-
ated this phenotype; these amino acids may be involved in stabilizing Vma5p association with the V-ATPase or in stabilizing the whole V-ATPase complex.

The F260A, Y262A, and F385A mutants exhibit the most novel phenotype. The direct effect of these mutations may be to partially destabilize the interaction of the C subunit with the V-ATPase complex. The V-ATPase complexes that result are somewhat similar to those obtained biochemically by Puopolo et al. (20) through in vitro reconstitution of a bovine clathrin-coated vesicle V-ATPase lacking the C subunit. However, Fig. 7 suggests that the yeast V-ATPase complexes carry out A TP-driven proton transport at an even higher rate than wild-type complexes. Because a low level of C subunit does remain, even in the F260A mutant vesicles (Fig. 5 D), we cannot eliminate the possibility that the population of V-ATPase complexes containing the mutant C subunits is responsible for the activity we observe. However, if this is true, these complexes must have an extremely high turnover rate, because in Fig. 7, less than 16% of the wild-type level of the C subunit is present in the F260A vesicles. Regardless of whether the ATPase complexes are conducting ATP-driven proton pumping in the presence or absence of subunit C, the apparent increase in $k_{cat}$ in these mutants is intriguing. No previous V-ATPase mutant has exhibited this phenotype. Future studies will examine the structural basis of this change in more detail.

At first it seems counterintuitive that mutations that structurally destabilize the V-ATPase also enhance its ATPase activity in vitro. However, there is substantial evidence associating catalytic activity with destabilization of $V_1^{-}V_0$ interactions in the wild-type ATPase, and these mutations may simply enhance this effect. Chaotrope-induced dissociation of the yeast V-ATPase in vitro is enhanced in the presence of MgATP, and point mutations in the catalytic subunit that inhibit ATP hydrolysis also inhibit in vitro dissociation (25, 41, 44). In vivo dissociation of the V-ATPase in response to glucose deprivation is also inhibited by concanamycin A or by mutations that affect catalytic activity (44, 45). Taken together, these results suggest that during its catalytic cycle, the V-ATPase assumes a conformation that makes the $V_1$ sector susceptible to detachment from $V_0$. The conformation susceptible to detachment may involve changing interactions with the C subunit in the stator and may be mimicked in these three vma5 mutants even in the absence of catalysis.

What Is the Essential Function of Vma5p in the V-ATPase?—If the C subunit is not required for ATP-driven proton pumping, why do vma5 mutant strains exhibit growth defects as severe as mutants lacking any other structural subunit of the V-ATPase? The F260A, Y262A, and F385A mutations do not result in a Vma$^-$ phenotype, so the mutant C subunits must support a critical aspect of V-ATPase function that is lacking in the vma5 mutant, even though they are present at low levels in vacuoles. Isolated vma5 vesicles have no V-ATPase activity and even lower levels of the other $V_1$ subunits than these three point mutants. It is possible that Vma5p is critical at some stage in assembly or biosynthesis of the complex or that the mutant C subunits impart a minimal level of stability to the enzyme in vivo, even if they are ultimately dispensable for its catalytic function in vitro. In this study VMA5 proved to be very resistant to mutational inactivation (only mutations that dramatically destabilized the protein itself resulted in a Vma$^-$ phenotype); this suggests that the essential contribution of Vma5p to V-ATPase function is not very sensitive to single amino acid changes.

Although the mutations described here provide no direct evidence for structural placement of subunit C in the V-ATPase, their phenotypes are consistent with the C subunit playing an role in the stator of the enzyme. Although the stator of the F-type ATPases is envisioned as imparting a structural stability to the enzyme critical for productive rotation of the central stalk (12), V-ATPase$\alpha$es strike a delicate balance between productive coupling of ATP hydrolysis and proton transport and the dynamic instability that is an important feature of their regulation. The characteristics of the vma5 mutants described here, including both subtle and dramatic effects on enzyme stability, some of which are coupled to effects on catalytic activity, would be consistent with a critical role for Vma5p in reversible stabilization of the V-ATPase stator. Future experiments will address this role in more detail.

Acknowledgments—We thank Tom Stevens for the vma5 strain and VMA5-containing plasmids and Tom Duncan and Moshe Reuveni for helpful discussions.

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Table II
Summary of phenotypes for VMA5 mutants

| Mutation | Growth on pH 7.5 + CaCl$_2$ | C subunit stability | By IP | In vacuoles | V-ATPase activity |
|----------|-----------------------------|---------------------|-------|-------------|------------------|
| None     | +++                         | +                   | ND    | +++         | +++              |
| E381A    | +++                         | +                   | ND    | +++         | +++              |
| Y312A    | +++                         | +                   | ND    | +++         | +++              |
| K360stop | −                           | −                   | −     | −           | −                |
| T380stop | −                           | −                   | −     | −           | −                |
| F255A    | −                           | −                   | −     | ND          | ND               |
| Y296A    | +++                         | +                   | ND    | +++         | +++              |
| F300A    | +++                         | +                   | ND    | +++         | +++              |
| K307A    | +++                         | +                   | ND    | +++         | +++              |
| S315A    | +++                         | +                   | ND    | +++         | +++              |
| R318A    | +++                         | +                   | ND    | +++         | +++              |
| Y319A    | +++                         | +                   | ND    | +++         | +++              |
| F325A    | +++                         | +                   | ND    | +++         | +++              |
| Y382A    | +++                         | +                   | ND    | +++         | +++              |
| Y382F    | +++                         | +                   | ND    | +++         | +++              |
| F385A    | +++                         | +                   | ND    | +++         | +++              |
| Y388A    | +++                         | +                   | ND    | +++         | +++              |
| Y382A,Y388A | +                   | +                   | ND    | +++         | +++              |
| F260A    | +++                         | +                   | ND    | +++         | +++              |
| Y262A    | +++                         | +                   | ND    | +++         | +++              |

IP, immunoprecipitation; ND, not determined.

What Is the Essential Function of Vma5p in the V-ATPase?—If the C subunit is not required for ATP-driven proton pumping, why do vma5 mutant strains exhibit growth defects as severe as mutants lacking any other structural subunit of the V-ATPase? The F260A, Y262A, and F385A mutations do not result in a Vma$^-$ phenotype, so the mutant C subunits must support a critical aspect of V-ATPase function that is lacking in the vma5 mutant, even though they are present at low levels in vacuoles. Isolated vma5 vesicles have no V-ATPase activity and even lower levels of the other $V_1$ subunits than these three point mutants. It is possible that Vma5p is critical at some stage in assembly or biosynthesis of the complex or that the mutant C subunits impart a minimal level of stability to the enzyme in vivo, even if they are ultimately dispensable for its catalytic function in vitro. In this study VMA5 proved to be very resistant to mutational inactivation (only mutations that dramatically destabilized the protein itself resulted in a Vma$^-$ phenotype); this suggests that the essential contribution of Vma5p to V-ATPase function is not very sensitive to single amino acid changes.

Although the mutations described here provide no direct evidence for structural placement of subunit C in the V-ATPase, their phenotypes are consistent with the C subunit playing an role in the stator of the enzyme. Although the stator of the F-type ATPases is envisioned as imparting a structural stability to the enzyme critical for productive rotation of the central stalk (12), V-ATPase$\alpha$es strike a delicate balance between productive coupling of ATP hydrolysis and proton transport and the dynamic instability that is an important feature of their regulation. The characteristics of the vma5 mutants described here, including both subtle and dramatic effects on enzyme stability, some of which are coupled to effects on catalytic activity, would be consistent with a critical role for Vma5p in reversible stabilization of the V-ATPase stator. Future experiments will address this role in more detail.

Acknowledgments—We thank Tom Stevens for the vma5 strain and VMA5-containing plasmids and Tom Duncan and Moshe Reuveni for helpful discussions.

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J. Biol. Chem. 2002, 277:8979-8988.
doi: 10.1074/jbc.M111708200 originally published online January 3, 2002

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