Mutational Analysis of Subunit G (Vma10p) of the Yeast Vacuolar H\textsuperscript{+}-ATPase*

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Vacuolar proton-translocating ATPases (V-ATPases) are highly conserved proton pumps found in all eukaryotic cells (1, 2). V-ATPases acidify multiple compartments of the vacuolar network and, in some cells, also pump protons across the plasma membrane and out of the cytosol (1–3). V-ATPases are composed of two sectors: a peripheral membrane complex containing the sites of ATP binding and hydrolysis (the V\textsubscript{1} complex) and a complex of integral membrane and tightly associated peripheral membrane proteins containing the proton pore (the V\textsubscript{0} complex) (1, 2, 4). Recent low resolution structures of V-ATPases, derived from electron microscopy and image reconstruction, have indicated that the V\textsubscript{0} sector is a large, rather globular complex connected to the membrane domain by at least two narrow stalks (5–7).

Both the A and B (ATP binding catalytic and regulatory) subunits are present primarily in the globular head of the V-ATPases and the proteolipid subunits are embedded in the membrane (5). The other eight subunits of the V-ATPases probably make up most of the stalk structures seen by electron microscopy (5). At the sequence level, these subunits are conserved among eukaryotes from yeast to plants to humans, but show limited, if any, homology with the F-ATPases (11). These subunits are presumed to be involved in structural and functional coupling of the V\textsubscript{1} and V\textsubscript{0} sectors.

Starting from the high resolution structure of the F\textsubscript{1}-ATPase from bovine heart mitochondria (12), major advances have been made in understanding the mechanism by which ATP hydrolysis is coupled to proton transport in F-ATPases. It is now clear that ATP hydrolysis drives rotation of the \gamma subunit of the F\textsubscript{1} complex between the three different catalytic (\beta) subunits (13–15). This rotation is coupled to movement in the F\textsubscript{0} sector that allows proton transport, although the exact mechanism of coupling is still unclear (16–18). This mechanistic picture superimposes elegantly on the electron microscopic and low resolution crystallographic structures of the \textit{F,F,F\textsubscript{0}} complex (8, 9, 19). The “central stalk” in the EM images contains the \gamma subunit and thus comprises the rotor, whereas the second, peripheral stalk is believed to act as a stator, which anchors the catalytic and regulatory subunits to the membrane and allows the relative rotation of the \gamma subunit. In the F-ATPase from \textit{Escherichia coli}, the stator appears to be composed of a dimer of F\textsubscript{0} b subunits and the F\textsubscript{1} \delta subunit bound to a single a/\beta subunit pair (20).

Understanding the structural and functional roles of the V-ATPase stalk subunits is essential, because they may impart to V-ATPases the characteristics that distinguish them from the F-ATPases, including their activity as dedicated proton pumps rather than proton-driven ATP synthases, and their susceptibility to multiple forms of regulation \textit{in vivo}. The yeast \textit{Saccharomyces cerevisiae} has proven to be an excellent model system for studying the V-ATPases of all eukaryotes (1). In this work, we examine the function of the yeast Vma10 protein, subunit G of the V\textsubscript{1} sector. The yeast and bovine VMA10 genes were cloned first (21, 22). The N-terminal halves of the G

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1 The abbreviations used are: V-ATPase, vacuolar proton-translocating ATPase; YEPD, yeast extract-peptone-2% dextrose medium; YEP, yeast extract-peptone medium without dextrose; SD, fully supplemented minimal medium; PAGE, polyacrylamide gel electrophoresis.
subunits were shown to have significant homology to the b subunits of F-ATPases, with the major exception that the G subunits have no transmembrane domain, whereas the b subunits of F-ATPases have at least one transmembrane domain at the N terminus (22). Hunt and Bowman (23) further analyzed the regions of homology between V₁ G and F₀ b subunits and suggested that the N-terminal half of the G subunits may fold into an α helix in which one face is highly conserved both among G subunits and between the b and G subunits. We have addressed the functions of the N-terminal half of the yeast Vma10 protein by site-directed mutagenesis, with mutations designed to perturb and disrupt the conserved face of the predicted helix. Our results support the structural and functional importance of this region of the G subunit but indicate that it can tolerate mutations predicted to disrupt the conserved face of the α helix.

### EXPERIMENTAL PROCEDURES

#### Materials and Strains—Reagents for molecular biology were purchased from New England BioLabs. Concanaamycin A was purchased from Wako Biochemicals. Tran35S-label and zymolyase 100T were purchased from New England BioLabs. Concanamycin A was purchased from Sigma.

The wild-type yeast strain used was SF838-5A (24). The congenic vma10-103a mutant contained a VMA10 allele in which the entire VMA10 open reading frame had been replaced with URA3. The wild-type strain and vma10-103a mutant were grown in yeast extract-peptone-dextrose medium buffered to pH 5.0 with 50 mM sodium phosphate/50 mM sodium succinate (YPEP, pH 5.0) as described (25). After transformation of the vma10-103a mutant with plasmids containing the wild-type or mutant gene, the cells were maintained in fully supplemented minimal medium lacking leucine (SD-leucine) (25). Prior to preparation of vacuoles and serial dilution for comparisons of growth rates, transformants were transferred to YEPD, pH 5.0, for growth overnight. Control experiments indicated that there was minimal plasmid loss under these conditions.

#### Plasmid Constructions and Mutagenesis—The wild-type N-myc VMA10 plasmid contains a single myc epitope introduced immediately after the N-terminal methionine, carried in a pRS315 vector (26). Mutagenesis was performed using the Altered Sites II in vitro mutagenesis system (Promega). A 1.5-kilobase fragment containing the N-myc VMA10 construct was excised from pRS315 with PstI and SacI and subcloned into the same sites of pALTER-1. A single-stranded DNA template was prepared using an R408 helper phage, and mutagenesis was carried out according to the manufacturer’s directions. The sequences of the mutagenic oligonucleotides are listed in Table I. All oligonucleotides were obtained from Genosys. Oligonucleotides VMA10-R25A and VMA10-Y46A also had silent mutations producing restriction enzyme sites AjIII and ClaI, respectively, introduced to facilitate screening of clones. After positive identification of mutants by sequencing, the mutagenized N-myc VMA10 plasmid was excised from pALTER-1 using BamHI and XbaI and subcloned into the same sites in pRS315. VMA10-Y46A and-K55A were cloned into pRS315 with PstI rather than BamHI and XbaI. The plasmids were transformed into SF838-5a vma10-103a yeast cells by the method of Elble et al. (27), and transformants were selected for growth on SD-leucine medium.

#### Biochemical Characterization of Mutants—Vacuoles were prepared from all strains as described (28). Total vacuolar protein was determined by Lowry assay (29). Rates of ATP hydrolysis at 25 °C or 37 °C were determined by coupled enzyme assay of Lotscher et al. (30) in the presence and absence of 100 nM concanamycin A (31) in the assay mixture. V-ATPase-specific hydrolysis was determined by subtracting the rate of hydrolysis in the presence of inhibitor from the rate of hydrolysis in its absence.

Whole cell lysates as described by Hallberg et al. (32). Protein derived from 1 A600 unit of cells was solubilized in 100 μl of cracking buffer (50 mM Tris-HCl, pH 6.8, 8% s urea, 5% SDS, 5% β-mercaptoethanol) and subjected to electrophoresis and immunoblotting as described (33). For immunoblots, the 100–69–60, and 42-kDa subunits were detected using monoclonal antibodies 10D7, 8B1, 13D11, and 7A2, followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies. The 27-kDa subunit was detected with monoclonal antibody 9E10. The 27-kDa subunit was detected with affinity-purified rabbit polyclonal antibodies against the subunit (a generous gift from Tom Stevens) followed by goat anti-rabbit second antibody.

Non-denaturing immunoprecipitations were performed as described (34). Assembly of complexes in the different mutants was assessed by immunoprecipitation after 60 min of labeling with Tran35S-label. Disassembly and reassembly of the complexes were monitored as described previously by Parra and Kane (35). Cells were labeled for 60 min in supplemented minimal medium lacking methionine, excess unlabeled methionine and cysteine were added, and then cells were transferred to YEP, followed by 15-min incubation. Immunoprecipitated proteins were solubilized and separated by SDS-PAGE as described (36), and radioactivity in the 100- and 17-kDa bands was quantitated using a Molecular Dynamics Storm PhosphorImager.

#### RESULTS

Site-directed Mutagenesis of VMA10—Supekova et al. (21, 22) initially cloned the VMA10 genes from yeast and bovine cells and noted the homology of these subunits to the b subunit of the F₀ sector of the E. coli F-ATPase. The absolute identities between the genes from the F- and V-type ATPases were not high, ranging from 17% to 31%, and the most notable difference between the b and G subunits was the absence of any predicted transmembrane domain in the G subunits (22). Hunt and Bowman (23) identified regions of conservation between three V-ATPase G subunits (bovine, Neurospora crassa, and yeast) and the b subunit of the E. coli F₀ complex. By modeling the N-
A. Sequence comparison of amino acids 6 through 55 of the yeast G subunit with corresponding sequences of the G subunit from A. thaliana, tobacco, M. sexta, human, C. elegans, and N. crassa, as well as the b subunit of E. coli F1 F0-ATPase. The position in the subunit sequence of the first amino acid one face of the helix. In Fig. 1, we show a more extensive sequence comparison, including the Arabidopsis thaliana, tobacco, Manduca sexta, Caenorhabditis elegans, and human G subunit sequences along with the yeast and N. crassa, as well as the b subunit of E. coli F1 F0-ATPase. The position in the subunit sequence of the first amino acid included in the sequence alignment is indicated. B. Helical wheel projection for amino acids 6 through 55 of the yeast G subunit. Amino acids that are conserved in six of seven of the G subunit sequences shown in A are circled. Amino acids that were mutagenized in this study are italicized.

terminal half of the b and G subunits as an α-helix, consistent with the demonstrated structure of the b subunit (48), they showed that the conserved residues mapped predominantly to one face of the helix. In Fig. 1A, we show a more extensive sequence comparison, including the Arabidopsis thaliana, tobacco, Caenorhabditis elegans, and human G subunit sequences along with the yeast and N. crassa G subunits and E. coli F1 F0 b subunit. A helical wheel model of amino acids 6 through 55 of the yeast G subunit is shown in Fig. 1B, with the circled amino acids representing those positions that are identical in at least six of the seven V-ATPase G subunits. Once again, it is clear that most conserved residues in this region of the protein map to one face of the helix.

A myc-tagged version of the yeast VMA10 gene was constructed to facilitate detection of the subunit; the tagged subunit fully complemented the growth and activity defects of a vma10Δ strain (see below). To better characterize the structure and function of the yeast G subunit, we constructed several site-directed mutations in the myc-tagged VMA10 gene (10–myc). Five amino acids falling on the conserved face of the helix (Glu-14, Arg-25, Glu-42, Tyr-46, and Lys-50) and one predicted to fall on the opposite face of the helix (Lys-55) were changed to alanine. A second mutation at Arg-25, R25L, was constructed to investigate whether effects seen in the R25A mutant (see below) were attributable to the smaller size of the alanine substitution relative to the native arginine. In addition, a series of mutations designed to disrupt the conserved face of the helix by inserting or deleting 2–4 amino acids were designed. Amino acid pairs Q29D30 and V20S21 were both deleted, to form the ΔQD and ΔVS mutants, and duplicated, to form the 2xQD and 2xVS mutants, in order assess the effects of changing the “register” of the helix at two different places. In addition, a 4-amino acid deletion, removing amino acids Arg-28 to Lys-31 (ΔRQDK), was constructed. This mutation would be predicted to remove approximately one turn of an α-helix, and this would have a significant effect on the helix length but less effect on the register of the helix than the 2-amino acid deletions and insertions.

The growth phenotypes of the different mutant strains are shown in Fig. 2. Mutants lacking V-ATPase activity exhibit a characteristic set of growth phenotypes, including sensitivity to elevated pH and CaCl2 concentrations (1, 37). Wild-type cells and vma10Δ cells carrying the N-myc-tagged wild-type VMA10 gene grow well on YEPD medium buffered to pH 5 or 7.5 as well as on YEPD, pH 7.5, containing 60 mM CaCl2. The VMA10 mutants exhibit a variety of growth phenotypes. All of the mutants grew fairly well on YEPD buffered to pH 5.0. The R25A (Fig. 2), R25L, and E42A (not shown) mutants also grew as well as wild-type (10-myc) cells on medium buffered to pH 7.5, containing 60 mM CaCl2. The E14A, K50A, and ΔQD mutants grew almost as well as did the wild-type (Fig. 2). All of the other mutants showed almost no growth on YEPD, pH 7.5, plates containing CaCl2 and thus were comparable to the untransformed vma10Δ mutant.

Biochemical Characterization of the Vma10 Mutant Strains—Total protein extracts were prepared from all of the mutant strains, and the steady-state level of the myc-tagged Vma10 protein and several other V1 subunits was determined by immunoblotting. As shown in Fig. 3, most of the mutant strains produce near wild-type levels of the myc-tagged Vma10 protein. The steady-state levels of Vma10p appeared to be slightly lower in the 2xQD, ΔRQDK, and 2xVS mutants, but
the difference in protein levels was insufficient to account for the growth defects of these mutants. Tomasek et al. (38) have demonstrated that the V$_1$ subunit E (27-kDa subunit) is unstable in a vma10 strain, suggesting that stability of the E subunit is dependent on the presence of the G subunit. We also observed that the 27-kDa E subunit appeared to be missing in immunoblots of whole cell lysate from a vma10 strain (not shown). Interestingly, the 27-kDa subunit is present at low levels in both the Y46A and K55A mutants, even though the mutant G subunit is present at near-normal levels. The 69-, 60-, and 42-kDa subunits are present at similar levels in all of the mutant strains. The apparent loss of the 42-kDa subunit from the K50A, K55A, and R25A mutants in Fig. 2 was not seen consistently.

Vacuolar vesicles were prepared from all of the mutant strains. The ATPase activity in the isolated vesicles was determined by a coupled enzyme assay, and assays were conducted in the presence and absence of 100 nM concanamycin A, a specific V-ATPase inhibitor, to determine the activity specific to the V-ATPase. The results are shown in Table II. The wild-type strain (SF838-5A) and the congenic vma10 strain carrying the myc-tagged VMA10 gave comparable levels of V-ATPase activity, indicating that introduction of the myc tag did not disrupt subunit function. The E42A mutant showed levels of V-ATPase activity very similar to the myc-VMA10 strain and consistent with its wild-type growth phenotype. The levels of V-ATPase activity in the R25A and R25L mutants were consistently 15–20% higher than the activity of the corresponding wild-type (myc-VMA10) strain. In all of the other mutants, V-ATPase activity was significantly reduced. The QD mutant exhibited 52% of the wild-type activity, and the K50A and K55A mutants exhibited 10.5% and 18.8% of the wild-type activity, respectively. All of the other mutants contained less than 10% of the wild-type V-ATPase activity. This was surprising, given the fact that some of these mutants appeared to be capable of complementing the growth phenotypes of the vma10Δ mutant, but we believe that this may be due to destabilization of the V-ATPase in vitro by these mutations (see below).

To determine whether the changes in V-ATPase activity in the mutants stem from enzyme instability or catalytic defects, the levels of the various subunits in isolated vacuolar vesicles were determined by immunoblotting (Fig. 4). All of the strains contain comparable levels of the 100-kDa V$_0$ subunit. This was expected, because mutants without an intact V$_1$ sector can still assemble a V$_0$ sector and transport it to the vacuole. The E42A mutant contained levels of V$_1$ sector subunits comparable to the wild-type (10-myc) strain, and the R25A and R25L mutant strains appeared to have higher levels of V$_1$ subunits, consistent with their somewhat higher levels of V-ATPase activity and proton pumping. Assembly of the V$_1$ sector with the vacuolar membrane was severely defective in the Y46A, K50A, and K55A mutants, and this assembly defect probably accounts for their observed catalytic defects. The E14A mutant appeared to exhibit specific loss of the Vma10 (16 kDa) and Vma5 (42 kDa) subunits but with near-normal levels of the 69-, 60-, and 27-kDa subunits. Of the deletion and duplication mutants, only the ∆VS mutant showed a severe assembly defect, with almost complete loss of the 27-kDa E and 16-kDa G (Vma10p) V$_1$ subunits. The other deletion and duplication mutants showed a modest decrease in the levels of the E and G subunits. Interestingly, the extent of assembly in all of these mutants, except the ∆VS mutant, was comparable with the QD strain, even though they had less than 10% of the activity of this strain. This suggests that these mutants have a catalytic defect rather than a simple assembly defect.

As an independent test of the ability of the V-ATPase to assemble in the mutant strains, we immunoprecipitated the V-ATPase complex under non-denaturing conditions with an antibody against the 60-kDa subunit (Vma2p) and the anti-myc antibody against the Vma10 protein. All of the strains were converted to spheroplasts, proteins were biosynthetically labeled with Tran35S-label for 60 min, and V-ATPase complexes were immunoprecipitated as described previously (34). The results are shown in Fig. 5. In these autoradiograms it is not possible to clearly distinguish between the myc-tagged Vma10 protein and the Vma3p and Vma11p proteolipid subunits, which run as a diffuse band of the same molecular mass. The immunoprecipitation with the anti-myc antibody (Fig. 5B) clearly distinguishes the complexes formed with the myc-tagged mutant Vma10 proteins, however. Immunoprecipita-

![Fig. 3. Levels of V-ATPase subunits in whole cell lysates of wild-type and mutant cells. SF838-5A cells transformed with a low copy plasmid carrying myc-tagged wild-type VMA10 (10-myc) or myc-tagged VMA10 with the indicated mutation were grown overnight until mid-log phase in YEPD, pH 5.0, medium. A culture volume corresponding to 1 Å$_{600}$ unit of each strain was removed, and cells were rapidly lysed as described (32). The whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. The blots were probed with mouse monoclonal antibodies 8B1, 13D11, 7A2, and 9E10, against the 69-, 60-, 42-kDa, and myc-tagged Vma10 subunits, respectively, followed by alkaline-phosphatase-conjugated goat anti-mouse antibodies, or a rabbit polyclonal antiserum against the 27-kDa anti-rabbit antibody. The bound antibodies were detected using ECF substrate on a Molecular Dynamics Storm PhosphorImager. Lysate corresponding to 0.3 Å$_{600}$ unit of the original culture was loaded for detection of the 42-kDa and myc-tagged Vma10 subunits, and lysate corresponding to 0.1 Å$_{600}$ units of the culture was loaded for detection of the 69-, 60-, and 27-kDa subunits.

![Table II. V-ATPase activity in isolated vacuolar vesicles](image-url)
mutants exhibited poor V1 sector assembly and poor ATPase (Fig. 3). To better understand the apparent enhancement of isolated vacuoles, even though the mutant Vma10 proteins are inhibit disassembly by stabilizing the V1V0 interactions. The (34, 35), and we reasoned that the Arg-25 mutations might V-ATPase reversibly disassembles upon glucose deprivation followed by restoration of glucose. The yeast V-ATPase reversibly disassembles upon glucose deprivation (34, 35), and we reasoned that the Arg-25 mutations might inhibit disassembly by stabilizing the V1V0 interactions. The results are shown in Table III. The 100- and 17-kDa V0 subunits assemble with the V2 sector to comparable levels in wild-type and mutant cells incubated with glucose throughout the labeling and chase periods (in YEPD, Table III). The mutants do appear to differ from wild-type cells in their response to glucose deprivation, however. Almost twice as much V0 sector remains bound to the V2 sectors in the R25A and R25L mutants after a 15-min glucose deprivation. The V2 sectors that disassemble in all three cell lines retain their ability to reassemble with V0. This is the first time we have observed an active V-ATPase with a defect in disassembly in response to glucose deprivation. As discussed below, the decreased disassembly of the mutant V-ATPase complexes in the absence of glucose may help explain their increased assembly in vacuolar vesicles isolated from glucose-grown cells.

**DISCUSSION**

**VMA10 Mutants Fall into Distinct Phenotypic Classes**—The VMA10 mutations described here were designed to change highly conserved positions in subunit G, particularly positions that fell on the proposed conserved face of the helix. Because of the conservation at these positions, it may not be surprising that all but the E42A mutation appear to have effects on the V-ATPase. It is intriguing, however, that the mutants appear to fall into different classes, which may provide novel insights into the functions and subunit interactions of the G subunit. The phenotypes of the *vma10* mutants described above are summarized in Table IV.

The Y46A and K55A mutations have the most severe defects of the point mutations described here. Although the mutant proteins appear to be stable, neither appears to give any complementation of the growth defect of *vma10Δ* cells, and both show clear assembly defects by immunoprecipitation and in Western blots of vacuolar proteins. Western blots of whole cell lysates indicate that both of these mutations result in destabilization of the E subunit, Vma4p, which may account for their observed assembly defects. Although loss of the G subunit (in a *vma10Δ* strain) has been shown to destabilize the E subunit (38), this is the first time that a point mutation in the G subunit has been shown to affect stability of the E subunit. This may suggest that Tyr-46 and Lys-55 lie in a region of the G subunit critical for interaction with and/or stabilization of the E subunit, but further experiments will be necessary to confirm this.

Although the K50A and E14A mutations would be predicted to be separated by 9 turns and almost 50 Å if this region of the G subunit is strictly helical, they are predicted to fall directly in line with each other in the conserved face of the helix. They also give remarkably similar phenotypes. Both mutant plasmids complement the growth phenotypes of the *vma10Δ* strain well, allow production of wild-type levels of Vma10 protein, and appear to allow assembly of the V-ATPase complex based on immunoprecipitation. Previous results suggested that mutants must have >25% wild-type V-ATPase activity to support growth at elevated pH and calcium concentrations (49). Vacuolar vesicles isolated from these mutant strains consistently show very low levels of V-ATPase activity, however, and Western blots of whole cell lysates indicate that the low activity is due to loss of V1 subunits. Because the immunoprecipitation experiments indicate that V-ATPase complexes do form in these mutants, we hypothesize that the complexes formed are unstable, and unable to tolerate the multiple steps between sphero-plast lysis and final isolation of vacuolar vesicles. (The immunoprecipitation experiment looks at freshly synthesized subunits and stabilizes complexes formed by addition of a cross-linker.) Curiously, we have observed this same instability phenotype in a number of VMA5 point mutants.3 Although the K50A mutation appears to cause a general loss of V1 subunits from the vacuolar membrane, the E14A mutation appears to cause a more specific loss of the C (Vma5p) and G subunits from the vacuole. These data indicate that Lys-50 and Glu-14 of the G subunit are essential for stable association of V1 with V0, and it is possible that Glu-14 is important for retention of the C subunit in the V-ATPase complex.

The R25A and R25L mutations, which would be predicted to lie near Glu-14 in the conserved face of the α helix, also affect stability of V-ATPase complexes, but these mutations appear to stabilize V1V0 interactions rather than destabilizing them. Both mutations result in increased V-ATPase activity in isolated vacuolar membranes that can be accounted for by in-

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3 S. Archibald and P. M. Kane, unpublished data.
creased levels of V1 subunits in the isolated membranes. The response of the mutant complexes to glucose deprivation, shown in Table II, suggests one explanation for the enhanced assembly of the V-ATPase in these mutants. Insect V-ATPases also disassemble with starvation (39), and it has been proposed that V-ATPase complexes exhibit a dynamic instability (40). In

**TABLE III**

**Disassembly and reassembly of V-ATPase in vma10 mutant strains**

| Strain          | % 100 kDa subunit assembled with V1 | % 17 kDa subunit assembled with V1 |
|-----------------|-------------------------------------|-----------------------------------|
|                 | in YEPP    | after glucose deprivation | after glucose readdition | in YEPP    | after glucose deprivation | after glucose readdition |
| VMA10-MYC       | 43%        | 12%                    | 45%          | 51%        | 19%                    | 55%          |
| R25A            | 44%        | 22%                    | 44%          | 58%        | 34%                    | 65%          |
| R25L            | 48%        | 29%                    | 55%          | 52%        | 30%                    | 63%          |

**TABLE IV**

**Summary of phenotypes of vma10 mutants**

| Mutation | Growth on YEPP, pH 7.5 + CaCl2 | V-ATPase activity | Assembly |
|----------|--------------------------------|-------------------|----------|
|          |                                |                   | On vacuolar membranes | By immunoprecipitation |
| None (wild-type) | +                              | +                 | +         | +         |
| E14A     | +                              | +                 | +         | +         |
| E42A     | +                              | +                 | +         | +         |
| Y46A     | -                              | -                 | -         | -         |
| K50A     | +                              | -                 | -         | -         |
| K55A     | -                              | -                 | -         | -         |
| R25A     | +                              | +                 | +         | +         |
| R29L     | +                              | +                 | +         | +         |
| △QD      | +                              | +/-               | +/-       | +         |
| 2xQD     | -                              | +/-               | +/-       | +         |
| △RQDK    | -                              | +/-               | +/-       | +         |
| △VS      | -                              | +/-               | +/-       | +         |
| 2xVS     | -                              | +/-               | +/-       | +         |
this context, release of \( V_i \) complexes with glucose deprivation may represent a shift in an ongoing equilibrium of disassembly and reassembly of V-ATPase complexes. The Arg-25 mutations in the G subunit may perturb this equilibrium in the absence of any change in carbon source, resulting in a net stabilization of the V-ATPase complexes.

The deletion and duplication mutations gave somewhat unexpected, but very interesting phenotypes. Importantly, none of these mutations appeared to destabilize the G, or the E, subunit extensively. The most significant result may be the retention of a considerable level of V-ATPase activity in the \( \Delta QD \) mutant. This result calls into question the model of the G subunit N terminus as a strictly helical structure with a conserved face, because a 2-amino acid deletion would be expected to introduce a break in the conserved face (for example, separating conserved residues Glu-14 and Lys-50, discussed above, by almost 180°). If the conserved face of the helix is indeed important, then the helix must be quite flexible and able to restore the “register” of the conserved residues. Alternatively, it may not be essential that the most conserved amino acids in the G subunit fall along one face of the helix, perhaps because there are separable and distinct functions performed by the conserved regions along the length of the helix.

Although the 2xQD and \( \Delta RQDK \) mutations did not allow V-ATPase activity, they are nevertheless interesting. A systematic series of deletions and insertions in the b subunit of the E. coli F1F0-ATPases was recently reported, with strikingly different results (41, 42). The b subunit was remarkably tolerant of deletions of 2–11 amino acids and of insertions of up to 14 amino acids, and, in mutants exhibiting loss of activity, assembly of the complex appeared to limit the extent of catalysis. In contrast, the 2xQD and \( \Delta RQDK \) mutant G subunits assemble into V-ATPase complexes that appear normal in immunoprecipitations or Western blots of vacuolar membranes but lack all V-ATPase activity. This suggests that the length of the G subunit is more critical for catalysis than the length of the b subunit in F1F0, but further experiments will be necessary to confirm that changes in helix length, rather than the specific amino acid changes, are responsible for the observed phenotype. The results with the 2xVS mutant are consistent with an importance for helix length in catalysis, but this mutation does appear to result in somewhat defective assembly, based on immunoprecipitation. The \( \Delta V S \) mutation results in a clear assembly defect.

Implications for Structure and Function of the G Subunit—Although there appear to be significant differences between the G subunits and the b subunit of F1F0-ATPases, the sequence similarities between the F0 b and V0 G subunits remain compelling enough to support assignment of this subunit to the peripheral or second stalk of the V-ATPase, where it may function in conjunction with the C and E subunits as part of the stator. The data described above clearly implicate the G subunit in stabilization and regulation of \( V_i V_o \) interactions and support previous genetic and biochemical data suggesting that the E, C, and G subunits interact (38, 43).

The lack of a transmembrane domain in the G subunits suggests that the stator must be composed of non-covalently linked subunits that bridge the \( V_i \) and \( V_o \) sectors, perhaps to accommodate reversible disassembly of the V-ATPase. Defining which subunits provide the bridge from \( V_i \) to \( V_o \) is thus very important. A common structure for the F0 b and V0 G subunits would place the N terminus of the G subunit, including amino acid Glu-14, near the membrane, but as yet, there is no direct evidence that the G subunit interacts with any \( V_o \) subunit. The E14A mutation appeared to show a specific loss of the C subunit, which has been implicated in both stabilization of \( V_i V_o \) interactions and reversible disassembly of V-ATPase complexes in response to glucose deprivation (34, 44, 45). It is thus possible that the C subunit forms all or part of the bridge between \( V_i \) and \( V_o \) in the peripheral stalk. The R25A and R25L mutations would also be predicted to lie on the membrane-proximal end of a G subunit helix, and this may help explain their ability to modulate the stability of the V-ATPase.

A final unresolved issue is the stoichiometry of the G subunit in the \( V_i V_o \) complex, and this may prove to be relevant to some of the mutations described here. The b subunit is a dimer in E. coli F0 (20), and dimerization of the b subunit appears to be essential for assembly with F1 (46). Mutations at position Ala-79 of the b subunit, which aligns with Tyr-46 of the yeast G subunit, disrupt b subunit dimerization and enzyme assembly. The G subunit has been reported to be present in multiple copies in the V-ATPases of yeast and bovine cells (21, 47). More recently, quantitative amino acid analysis has indicated that bovine clathrin-coated vesicle enzyme contains roughly two copies of subunit G per V-ATPase complex (43). It is not yet clear whether these G subunits dimerize, but it is an intriguing possibility that the Y46A mutation might disrupt G subunit dimerization and that dimerization of the G subunits might be linked to E subunit stability. Future experiments will address this possibility.

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