Subunit D (Vma8p) of the Yeast Vacuolar H\(^{+}\)-ATPase Plays a Role in Coupling of Proton Transport and ATP Hydrolysis*

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Ting Xu and Michael Forgac‡
From the Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts 02111

To investigate the function of subunit D in the vacuolar H\(^{+}\)-ATPase (V-ATPase) complex, random and site-directed mutagenesis was performed on the VMA8 gene encoding subunit D in yeast. Mutants were selected for the inability to grow at pH 7.5 but the ability to grow at pH 5.5. Mutations leading to reduced levels of subunit D in whole cell lysates were excluded from the analysis. Seven mutants were isolated that resulted in pH-dependent growth but that contained nearly wild-type levels of subunit D and nearly normal assembly of the V-ATPase as assayed by subunit A levels associated with isolated vacuoles. Each of these mutants contained 2–3 amino acid substitutions and resulted in loss of 60–100% of proton transport and 58–93% of concanamycin-sensitive ATPase activity. To identify the mutations responsible for the observed effects on activity, 14 single amino acid substitutions and 3 double amino acid substitutions were constructed by site-directed mutagenesis and analyzed as described above. Six of the single mutations and all three of the double mutations led to significant (>30%) loss of activity, with the mutations having the greatest effects on activity clustering in the regions Val71–Gly80 and Lys209–Met221. In addition, both M221V and the double mutant V71D/E220V led to significant uncoupling of proton transport and ATPase activity, whereas the double mutant G80D/K209E actually showed increased coupling efficiency. Both a mutant showing reduced coupling and a mutant with only 6% of wild-type proton transport activity showed normal dissociation of the V-ATPase complex in vivo in response to glucose deprivation. These results suggest that subunit D plays an important role in coupling of proton transport and ATP hydrolysis and that only low rates of turnover of the enzyme are required to support in vivo dissociation.

The vacuolar H\(^ {+}\)-ATPases (or V-ATPases)\(^ {1}\) are ATP-dependent proton pumps that function in both intracellular membranes and, in certain cell types, the plasma membrane (1–7). V-ATPases within intracellular compartments function in such processes as receptor-mediated endocytosis, intracellular targeting of lysosomal enzymes, protein processing and degradation, viral entry, and coupled transport of small molecules, such as neurotransmitters (1–7). Within the plasma membrane, V-ATPases function in such processes as bone resorption (8), renal acidification (9), pH homeostasis (10), and K\(^{+}\) secretion (11).

The V-ATPases are multisubunit complexes composed of two domains (1–7). The V\(_{0}\) domain is a 570-kDa peripheral complex composed of eight different subunits (subunits A–H) of molecular mass 70–14 kDa that is responsible for ATP hydrolysis. The V\(_{1}\) domain is a 260-kDa integral complex composed of five different subunits (a, d, c, c\(_{9}\), and c\(_{0}\)) of 100 to 17 kDa that is responsible for proton translocation. The V-ATPases are structurally and evolutionarily related to the F-ATPases of mitochondria, chloroplasts, and bacteria that normally function in ATP synthesis (12–17). Thus sequence homology can be detected between the nucleotide-binding subunits of the V-ATPase (A and B) and the nucleotide-binding subunits of the F-ATPase (\(\beta\) and \(\alpha\)) (18, 19). Sequence homology also exists between the proteolipid c subunits of the two classes (13, 20), although no other sequence similarities have been identified in the remaining subunits.

An important question is the mechanism of coupling of proton transport and ATP hydrolysis in the V-ATPases. For the F-ATPases, the availability of high resolution structural data (21–24) together with a series of studies demonstrating rotation within the F-ATPase complex (25–28) have led to the rotary model for coupling (14, 29, 30). In this model, ATP hydrolysis within the \(\alpha_{i}\beta_{j}\) head of the F\(_{1}\) domain drives rotation of a central \(\gamma\) subunit, which is tightly linked to a ring of c subunits in the F\(_{0}\) domain. Rotation of the ring of c subunits relative to the a subunit of F\(_{0}\) (which is held fixed relative to the \(\alpha_{i}\beta_{j}\) head by a peripheral stator) in turn leads to unidirectional proton transport. Central to this coupling mechanism is the role of the \(\gamma\) subunit, which has been shown to rotate relative to \(\alpha_{i}\beta_{j}\) hexamer (25–27). In addition, mutations have been identified in the \(\gamma\) subunit that lead to uncoupling of ATP hydrolysis and proton transport by the F-ATPases (31, 32).

Whereas no subunits in the V-ATPase complex show homology to the \(\gamma\) subunit, two subunits (D and E) are predicted from sequence analysis to have a similarly high \(\alpha\)-helical content (33, 34). To investigate the possible role of subunit D in coupling within the V-ATPase, random mutagenesis of the gene encoding subunit D in yeast (VMA8 (35)) was performed. Mutations that lead to significant loss of V-ATPase function in yeast lead to a conditional growth phenotype in which cells are unable to grow at neutral pH (36–38). Sequencing and analysis of mutants isolated by this procedure together with analysis of subsequent mutations constructed by site-directed mutagenesis have led us to suggest that subunit D plays a role in coupling of proton transport and ATP hydrolysis by the V-
**Subunit D Functions in Coupling of the V-ATPase**

ATPases. In addition, we have further analyzed the activity and coupling requirements for in vivo dissociation of the V-ATPase, a process believed to play an important role in regulation of V-ATPase activity in the cell (39, 40).

**EXPERIMENTAL PROCEDURES**

**Materials**—Leupeptin, aprotinin, and pepstatin were obtained from Roche Molecular Biochemicals. Tras-5S-label was purchased from ICN Biochemicals. Zymolase 100T was from Sekagaku America Inc. Concanamycin A was purchased from Fluka Chemical Corp. Yeast extract, dextrose, peptone, and yeast nitrogen base were from Difco. Molecular biological reagents were from New England Biolabs, Promega, and Life Technologies, Inc. 9-Amino-6-chloro-2-methoxyacridine (ACMA) was from Molecular Probes, Inc. All other chemicals were of analytical grade and most were from Sigma.

**Strains and Plasmids**—Yeast strain KHY105 (leu-2, 112, ura-3-2, ade6, his4-519, vma8Δ::LEU2) was the generous gift of Dr. Tom Stevens, Institute of Molecular Biology, University of Oregon. Plasmid pKH3203 containing the VMA8 gene subcloned into the shuttle vector plRS316 was also from the Stevens laboratory.

**Mutagenesis**—Random mutagenesis of VMA8 gene was performed using a PCR-based method as described previously (41). Briefly, the entire encoding region of VMA8 flanked by additional 5′- and 3′-non-coding regions (approximatively 500-600 bp) was amplified by PCR under mutagenic conditions. The first 10 cycles of PCR were done in the presence of 0.25 mM Mg2+ and 4.25 mM Mg2+. The product was then diluted 1:100 into the same buffer but containing 3 mM Mg2+ in the absence of Mn2+ and amplified for another 30 cycles. The PCR products were purified using a QIAGEN purification kit.

Site-directed mutagenesis of the VMA8 gene was performed using the Altered Sites II in vitro mutagenesis kit (Promega) following the manufacturer’s protocol. The following mutagenesis oligonucleotides were employed to introduce the indicated mutations: R198G, GATGAGTTGGAGGACGAGAATTTT; L149V, TTTAGTTGAAGTAGCCTCT- TT; M221V, GGATGAGTTGAAGTAGAATA; V71D, GTTCGCGAGTTCTCATCTGA; E105M, GTTGCGAGTTCTCATCTGA; P179S, CACGCGAGTTCTCATCTGA; D218V, TGCAAAATTGGTTGCTGAGATGA; and I188N, AATTGAACAAATGA; G80D, GAAAAGACCAATTGGTTGCTGAGA; V104E, AAGATTGCTGACCTGGTTGCTGAGA; G73R, AGGTGAGTTGAAGTAGAATA; G80D, GAAAACATTGACTATCAAGTG; N100I, CGTCGACTTAAATAGATTCAAGAA; and N100I, AATGGTCCATTACAACTGATGAT. All mutations (underlined) were confirmed by direct DNA sequencing.

**Western Transformation and Selection**—The PCR products of VMA8, which were produced under the manufacture’s protocol, were used for co-transforming yeast strain KHY105 using an in vivo recombination method as described previously (41). Unique BamHI and XbaI sites flanking the VMA8-coding sequence were introduced into pKH203 by site-directed mutagenesis. pKH203 was cleaved with BamHI and XbaI and the large 6.6-kb base pair fragment lacking the VMA8 gene was purified by agarose gel electrophoresis. The mutagenized PCR products (0.2 μg) and the 6.6-kilobase pair vector fragment (1 μg) were mixed and used to co-transform the KHY105 strain using the lithium acetate method (42). The transformants were then selected on Ura plates. To screen for VMA8 mutants leading to defective V-ATPase function, colonies were replica-plated on YPD plates buffered by 50 mM KH2PO4 and succinate to pH 5.5 and 7.5. Mutants unable to grow at pH 7.5 were isolated, and the mutant plasmids were recovered and used to retransform KHY105 to verify that the observed phenotype was due to a mutation in VMA8. The mutant forms of VMA8 constructed by site-directed mutagenesis were subcloned into plRS316 and transformed into KHY105 followed by selection of transformants on Ura plates.

**Analysis of Subunit D Expression and V-ATPase Activity**—Whole cell lysates were prepared from overnight cultures in Ura medium as described previously (43), and the proteins were separated by SDS-PAGE on 10% acrylamide gels. The expression of subunit D was detected by Western blotting using a polyclonal antibody raised against Vma8p (a generous gift of Dr. Tom Stevens). Analysis of the V-ATPase activity was assessed by measurement of the amount of subunit A present on isolated vacuolar membranes and by immunoprecipitation of the V-ATPase complex from whole cell lysates. Vacular membrane vesicles were isolated as described previously (44). Following separation of the proteins on 12% acrylamide gels, Western blotting was performed using the monoclonal antibody 8B1-F3 against the A subunit (obtained from Molecular Probes, Inc.). It has previously been shown that the V1 domain (including the A subunit) only associates with the vacuolar membrane if V-ATPase assembly is normal. Western blots were developed using a horseradish peroxidase-conjugated secondary antibody and visualized by a chemiluminescent detection system from Kirkegaard & Perry Laboratories.

**In Vivo Dissociation of the V-ATPase in Response to Glucose Depiparation**—Dissociation of the V-ATPase in response to glucose deprivation was detected as described previously (39) with some modifications. The Δvma8 yeast strain expressing the wild-type or mutant forms of VMA8 was grown overnight to an absorbance at 600 nm of 0.6–0.8. The cells were then converted to spheroplasts and incubated in YEP media or YEP media containing 2% glucose for 40 min at 30 °C. Spheroplasts were pelleted, washed, and lysed in PBS (135 mM NaCl, 2 mM KC1, 10 mM sodium phosphate, pH 7.4) with 1% C4E5 and 1 mM DTT. The V-ATPase complex was then immunoprecipitated using the antibody 8B1-F3 and protein A-Sepharose followed by separation of proteins by SDS-PAGE on 12% acrylamide gels and autoradiography.

**RESULTS**

To evaluate the functional role of subunit D in the V-ATPase complex, PCR-based random mutagenesis of the VMA8 gene encoding subunit D in yeast was performed. Disruption of V-ATPase function leads to a conditional growth phenotype in yeast in which cells are unable to grow at pH 7.5 but retain the ability to grow at pH 5.5 (36–38). Following mutagenesis and transformation, transformants were selected for the inability to grow at pH 7.5. Screening of approximately 10,000 transformants resulted in the selection of 29 mutant colonies that grew at neutral pH. These mutants were then further screened for their ability to express VMA8p by Western blotting of whole cell lysates using a polyclonal antibody specific for the yeast subunit D. Of the initial isolates, 15 colonies showed nearly normal levels of subunit D in whole cell lysates. Plasmids from these mutants were recovered, amplified, and sequenced in Escherichia coli, and reintroduced into the vma8-deficient strain. Fourteen of the mutant plasmids still led to the vma- phenotype and were sequenced. All of the mutant plasmids contained multiple point mutations. Seven plasmids, containing either 2 or 3 mutations, were subjected to further analysis. Previous attempts to isolate mutants in VMA8 causing the vma- phenotype had also resulted in only mutants containing multiple point mutations.2

2 T. Xu and M. Forgac, unpublished observations.
Subunit D Functions in Coupling of the V-ATPase

TABLE I

| Mutant | Mutations in vma8 | V-ATPase activitya | ACMA quenchingb |
|--------|------------------|-------------------|-----------------|
| 2      | G80D/E220V/M221V | 10 (±5)           | 6 (±3)          |
| 4      | L149V/E182D/D249G| 35 (±7)           | 40 (±10)        |
| 7      | K210E/D218V      | 42 (±5)           | 40 (±20)        |
| 10     | I186N/R198G      | 15 (±3)           | 25 (±15)        |
| 2      | V71D/E220V/M221V | 7 (±2)            | 0               |
| 3      | G80D/K209E       | 15 (±4)           | 34 (±8)         |
| 4'     | I186N/N173N/A232T| 20 (±2)           | 15 (±8)         |

a V-ATPase activity was defined as the ATPase activity of isolated vacuoles (10 μg of protein) that was inhibitable by 0.2 μM concanamycin. Values are expressed relative to that measured for vacuoles isolated from the Δvma8 strain expressing the wild type VMA8 gene (1.3–1.5 μmol of ATP/min/mg protein). Typically, concanamycin inhibited approximately 90–95% of the ATPase activity in isolated vacuoles. ATP hydrolysis was measured using a coupled spectrophotometric assay as described previously. Values represent the average of at least two independent determinations with the numbers in parentheses corresponding to the average deviation from the mean.

b Proton transport of isolated vacuoles (2.5 μg of protein) was measured as ATP-dependent quenching of ACMA fluorescence as previously described (47). Values are expressed relative to that measured for vacuoles isolated from the Δvma8 strain expressing the wild type VMA8 gene (defined as 100%). Vacuoles isolated from the Δvma8 strain expressing the vector alone showed no ATP-dependent quenching of ACMA fluorescence. Values represent the average of at least two independent determinations with the numbers in parentheses corresponding to the average deviation from the mean.

Fig. 1. Effect of random mutations of VMA8 on the expression and stability of subunit D. Whole cell lysates (40 μg of protein) were prepared from the Δvma8 strain expressing the wild-type VMA8 gene in pRS316 (WT), the pRS316 vector alone (Vector), or the mutant forms of VMA8 in pRS316 (with the amino acid substitutions in each mutant indicated in Table I). The proteins were separated by SDS-PAGE on 10% acrylamide gels and transferred to nitrocellulose. Western blotting was then performed using a polyclonal antibody against subunit D as described under “Experimental Procedures.”

Fig. 2. Effect of random mutations of VMA8 on the association of subunit A with the vacuolar membrane. Vacular membranes (10 μg of protein) were prepared from the Δvma8 strain expressing the wild-type VMA8 gene in pRS316 (WT), the pRS316 vector alone (Vector), or the mutant forms of VMA8 in pRS316 (with the amino acid substitutions in each mutant indicated in Table I). The proteins were separated by SDS-PAGE on 12% acrylamide gels and transferred to nitrocellulose. Western blotting was then performed using the monoclonal antibody 8B1-F3 against subunit A as described under “Experimental Procedures.”

To determine whether loss of V-ATPase activity was due to a disruption of assembly of the V-ATPase complex, Western blot analysis of purified vacuoles was performed using a monoclonal antibody directed against the A subunit of the V1 domain. As has previously been shown (43), disruption of assembly of the V-ATPase complex leads to release of the entire V1 domain from the vacuolar membrane, resulting in the loss of A subunit staining by Western blot. As can be seen in Fig. 2, all of the mutants showed nearly normal levels of A subunit on the vacuolar membrane. As a further test of assembly and as a measure of stability of the V-ATPase complex, the mutant strains were also metabolically labeled with Tran35S-label followed by cell lysis, detergent solubilization, and immunoprecipitation of the V-ATPase complex using the monoclonal antibody 8B1-F3 against the A subunit. As can be seen in Fig. 3, most of the mutants showed normal stability of the V-ATPase complex, displaying the full complement of V-ATPase subunits. Mutant 2′, however, showed a dramatic reduction in stability of the V-ATPase complex, as indicated by the near complete absence of V1 subunits (i.e. subunits a and c) in the immunoprecipitate. Mutant 2 also showed somewhat reduced levels of subunit d in the immunoprecipitate. Because these mutants showed either slight or no reduction in the levels of A subunit present on vacuolar membranes (Fig. 2), these mutations in the D subunit most likely led to reduced stability of the V-ATPase complex (i.e. inability to survive detergent solubilization and immunoprecipitation) rather than to a defect in assembly of the V-ATPase.

Because all of the mutants isolated from the initial screen contained multiple point mutations within the VMA8 gene, it was necessary to determine what effect each of the single mutations would have on activity of the V-ATPase. Fourteen single mutations were constructed in the VMA8 gene by site-directed mutagenesis. In addition, three double mutations, containing two of the three point mutations in mutants 2, 4, and 2′ were also constructed. The yeast strain disrupted in the VMA8 gene was then transformed with the wild-type VMA8 gene and each of the mutant plasmids and the transformants analyzed as described above. All of the single mutants and the G80D/E220V and L149V/D249G double mutants showed a wild-type growth phenotype (i.e. normal growth at pH 7.5), whereas the V71D/E220V double mutant showed a partial growth defect (that is it grew slower at pH 7.5 than the wild-type strain but not as slowly as the deletion strain). Western blot analysis of whole cell lysates (Fig. 4) indicated that all of the mutants showed nearly wild-type levels of Vma8p. Western blot analysis of
vacuoles isolated from each of the mutant strains also showed approximately wild-type levels of the A subunit, indicating normal assembly of the V-ATPase in each of the mutants (Fig. 5).

Measurement of ATPase activity and ACMA quenching in vacuoles isolated from each of the mutant strains (Table II) revealed that many of the mutations were without effect on either ATPase activity or proton transport. Six of the single mutations, however, reduced ATPase activity by greater than 35% and proton transport activity by greater than 30%. Of these, two mutations (V71D and M221V) had particularly large effects on activity, with V71D reducing ATPase activity and pumping by 70 and 58%, respectively, whereas M221V reduced ATPase activity and pumping by 50 and 70%, respectively. All of the double mutants constructed (including G80D/E220V, L149V/D249G, and V71D/E220V) also showed dramatically reduced activity, with the V71D/E220V mutant showing the lowest activity (20% of wild-type ATPase activity and no measurable proton transport). In two cases (M221V and the V71D/E220V double mutant), the reduction in proton transport activity was significantly greater than the reduction in ATP hydrolysis, suggesting a partial uncoupling of these two activities. It is also interesting to note that for the G80D/K209E double mutant isolated in the first round, the greater reduction in ATPase activity than proton transport suggests an increase in coupling efficiency for this mutant. These results suggest that subunit D plays a role in coupling of proton transport and ATPase activity by the V-ATPase.

To determine the effect of these mutations on stability of the V-ATPase complex, cells were metabolically labeled, and the V-ATPase was solubilized and immunoprecipitated as described above. As can be seen in Fig. 6, all of the single and double mutants showed the normal complement of V-ATPase subunits except for two. Both V71D and the V71D/E220V double mutant showed greatly reduced stability relative to the wild-type enzyme. The L149V/D249G double mutant also showed slightly reduced levels of V$_D$ subunits in the immunoprecipitate. As noted above, however, these mutations are more likely to have reduced stability rather than assembly of the V-ATPase complexes.
Finally, dissociation of the V-ATPase complex has been shown to occur in yeast in response to glucose deprivation (39) and has been suggested to play a role in regulation of V-ATPase activity both in yeast (39) and higher eukaryotes (40). Glucose-dependent dissociation of the V-ATPase in yeast has been shown to require catalytic activity (49, 50), but the level of activity required for dissociation has not been investigated. In addition, because no uncoupled mutants of the V-ATPase have previously been isolated, it has not been possible to assess the requirement for tight coupling of the in vivo dissociation process. To address these questions, a mutant possessing very low activity (mutant 2) and a partially coupled mutant (M221V) were incubated in the presence or absence of glucose followed by detergent solubilization and immunoprecipitation of the V-ATPase complex using the monoclonal antibody 8B1-F3 against the A subunit (Fig. 3). The V-ATPase complex was then immunoprecipitated using the monoclonal antibody 8B1-F3 followed by SDS-PAGE and autoradiography as also described.

Interestingly, all of the vma8 mutants isolated that displayed a mutation phenotype (i.e., inability to grow at neutral pH) contained multiple point mutations in the VMA8 gene. These mutants displayed 7–42% of wild-type ATPase activity and 0–40% of catalytic activity required for dissociation has not been investigated. In addition, because no uncoupled mutants of the V-ATPase have previously been isolated, it has not been possible to assess the requirement for tight coupling of the in vivo dissociation process. To address these questions, a mutant possessing very low activity (mutant 2) and a partially coupled mutant (M221V) were incubated in the presence or absence of glucose followed by detergent solubilization and immunoprecipitation of the V-ATPase complex using the monoclonal antibody 8B1-F3 against the A subunit (Fig. 3). The V-ATPase complex was then immunoprecipitated using the monoclonal antibody 8B1-F3 followed by SDS-PAGE and autoradiography as also described.
wild-type proton transport activity. To identify the amino acid changes responsible for the observed effects on activity, a series of single point mutations were constructed by site-directed mutagenesis of the VMA8 gene. Of these, two had the most dramatic effects on activity. V71D near the N terminus reduced both pumping and ATPase activity by 60–70%, whereas M221V near the C terminus showed only 50% of wild-type ATPase activity and 30% of wild-type proton pumping. This partial uncoupling of proton transport and ATP hydrolysis was also observed for the V71D/E220V double mutant, which had 20% residual ATPase activity but no proton transport. These mutants represent the first “uncoupled” mutants identified for the V-ATPases and suggest that subunit D plays a role in coupling of proton transport and ATP hydrolysis. That the double mutant G80D/K209E shows a greater reduction in ATPase activity than proton transport suggests that the wild-type enzyme may not be as tightly coupled as possible.

Interestingly, many of the mutations affecting activity are clustered in the regions Val71–Gly80 near the N terminus and Lys209–Met221 near the C terminus (the latter region being 40–50 residues from the C terminus). In addition, several mutants bearing mutations in both of these regions show more than an additive reduction in activity (for example, the G80D/K209E mutant has a much lower activity than predicted from the effects of each of the single mutations). One possibility is that these regions interact in the folded structure of the protein. Such an interaction between the N- and C-terminal portions of the protein would be predicted if subunit D, like the γ subunit of the F-ATPase, forms an extended helical rod that folds back on itself. Alternatively, each region may represent an important contact region between subunit D and other subunits in the V-ATPase complex. Subunit γ of the F-ATPases makes important contacts with the β subunit in four regions of the protein. Residues in the region 80–90 amino acids from the N terminus are in close contact with the “DELSEQU” sequence of β (21), and introduction of cysteine residues at these sites can be used to cross-link β and γ (25, 58). Mutations at both Met23 as well as Asn269 and Thr273 cause uncoupling of proton transport and ATP hydrolysis (31, 32), whereas mutations in the region 236–246 suppress the uncoupling phenotype (64).

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Subunit D Functions in Coupling of the V-ATPase