Mutational Analysis of the Catalytic Subunit of the Yeast Vacuolar Proton-Translocating ATPase†

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ABSTRACT: In order to generate a set of tools for probing structure−function relationships in the catalytic subunit of the yeast vacuolar H^+-ATPase, the gene encoding this subunit (*VMA1*) was randomly mutagenized. Mutant plasmids unable to complement the growth defects of yeast cells lacking an intact *VMA1* gene were isolated and sequenced. Eight different mutant alleles of *VMA1* were examined for levels of the catalytic subunit and other subunits of the enzyme, assembly of the ATPase complex, targeting to the vacuolar membrane, and concanamycin A-sensitive ATPase activity. The mutations S811P and E740D resulted in mutant enzymes that assembled fully but were incapable of ATP hydrolysis, and the mutation E785G generated a similar but somewhat less severe phenotype (17% of the ATPase activity of wild-type vacuoles). When MgATP-dependent stripping of the peripheral subunits by 100 mM KNO_3 was examined in these three mutants, only the E785G mutant exhibited significant stripping, suggesting that ATP hydrolysis, even at relatively low levels, generates a conformation susceptible to dissociation. Plasmids containing the mutations E751G and F752S partially complemented the growth defects and resulted in partial defects in ATPase activity that appear to reflect reduced catalytic efficiency. Partial defects in growth and ATPase activity were also seen in the Y797H mutant, but this mutation caused an assembly defect manifested as a preferential loss of two of the peripheral subunits of the enzyme. The phenotypes of these mutants are interpreted in the context of homologies with other V-type and F-type ATPases.

Eukaryotic vacuolar proton-translocating ATPases (H^+-ATPases)[1] couple hydrolysis of cytoplasmic ATP to proton transport across intracellular membranes, resulting in acidification of lysosomes, endosomes, the Golgi apparatus, and other organelles (Forgac, 1996). V-type ATPases from fungi, plants, and animals are very similar, both in overall structure and in the sequences of individual subunits. All contain a peripheral membrane (V_1) domain that contains ATP-binding catalytic and regulatory subunits of approximately 70 and 60 kDa, respectively, and several additional peripheral subunits of 14−54 kDa. This peripheral domain is attached to an integral membrane (V_o) domain consisting of a 17 kDa proteolipid and, in most cases, subunits of approximately 36 and 100 kDa. The bovine clathrin-coated vesicle V-ATPase contains three copies each of the catalytic and regulatory ATP-binding subunits, six of the 17 kDa proteolipids, and single copies of each of the other subunits (Arai et al., 1988); subunit stoichiometries of other V-ATPases are probably similar if not identical.

The yeast vacuolar H^+-ATPase consists of at least 11 different subunits, and the genes for each of these subunits have been cloned and sequenced (Kane & Stevens, 1992; Anraku et al., 1992; Ho et al., 1993; Graham et al., 1994, 1995; Nelson et al., 1994; Supekova et al., 1995; Manolson et al., 1992). The catalytic subunit, responsible for ATP hydrolysis, is encoded by the *VMA1* (also called *TFP1*) gene (Hirata et al., 1990; Shih et al., 1988). The *VMA1* gene product undergoes an unusual post-translational protein-splicing event that removes a 50 kDa protein from the center of the 119 kDa translation product and splices together the N- and C-terminal portions of the protein to form the 69 kDa subunit of the vacuolar H^+-ATPase (Hirata et al., 1990; Kane et al., 1990). The 50 kDa protein was subsequently shown to be a homing endonuclease and designated VDE (VMA1-derived endonuclease) (Gimble & Thorner, 1992). The regions of the gene encoding the 69 kDa subunit are 62−73% identical to the catalytic subunits of other V-type ATPases and also show significant homology with the catalytic subunits of the F-type ATPases, particularly in regions previously implicated in ATP binding and catalysis (Zimniak et al., 1988; Bowman et al., 1988). Deletion of the chromosomal copy of the *VMA1* gene results in complete loss of vacuolar acidification and ATPase activity in isolated vacuoles (Hirata et al., 1990; Kane et al., 1990) and also yields a set of growth phenotypes characteristic of mutants lacking vacuolar ATPase activity, including the ability to grow in medium buffered to pH 5, but not medium buffered to pH 7.5, and sensitivity to high (≥100 mM) calcium concentrations (Nelson & Nelson, 1990; Ohya et al., 1991; Yamashiro et al., 1990). Precise deletion of the VDE-

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[1] Abbreviations: H^+-ATPase, proton-translocating ATPase; YEPD, yeast extract−peptone−2% dextran medium; SD, supplemented synthetic dextrose medium; PCR, polymerase chain reaction; CEN-plasmid, low-copy yeast shuttle plasmid containing a centromere sequence; VDE, VMA1-derived endonuclease; Mes, 4-morpholinoethanesulfonic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid (sodium salt).

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encoding portion of the *VMA1* gene does not result in any of these phenotypes, indicating that the 69 kDa subunit can be synthesized in an active form in the absence of protein splicing (Kane et al., 1990).

Preliminary studies of catalysis by the vacuolar-type ATPases have utilized a variety of biochemical approaches. The catalytic subunit of the yeast vacuolar ATPase has been labeled by the photoaffinity label 8-azido-ATP and by NBD-Cl in an ATP-protectable manner (Uchida et al., 1988). The regions of the clathrin-coated vesicle ATPase catalytic subunit important for ATP binding have been investigated in more detail by affinity labeling with 2-azido-ATP (Zhang et al., 1995) and by identification of the amino acids involved in ATP-protectable inhibition by *N*-ethylmaleimide (Feng & Forgac, 1992). These labeling studies strongly implicate the 69 kDa subunit (or the equivalent subunit in other vacuolar-type ATPases) as the site of ATP hydrolysis and provide a preliminary picture of the regions that may be responsible for ATP binding. Initial studies of the catalytic mechanism of the yeast vacuolar H^+-ATPase have suggested that the positive cooperativity in catalysis that is characteristic of the F_1F_o-ATPases may also be operative in the vacuolar ATPases. Uchida et al. (1988) showed an acceleration in the rate of ATP hydrolysis at a single catalytic site under conditions where excess ATP is available to bind to the other catalytic sites, and ^{18}O exchange measurements suggest that binding of ATP to a second catalytic site promotes product release from the enzyme (Kasho & Boyer, 1989). All of these experiments, coupled with the structural and sequence similarities described above, suggest that catalysis by the vacuolar (V-type) and F-type ATPases share many common features. There are also fundamental differences between these two classes of enzymes, however. F-type ATPases function predominantly as ATP synthases *in vivo*, although the peripheral F_1 domain is frequently studied as a soluble ATPase *in vitro*. In contrast, V-type ATPases are ATPases (and proton pumps) *in vivo*, and the peripheral V_1 domain is incapable of ATP hydrolysis when removed from the membrane (Bowman et al., 1989; Kane et al., 1989; Moriyama & Nelson, 1989; Puopolo & Forgac, 1990; Ward et al., 1991; Parra & Kane, 1996). In eukaryotic cells, F-type ATPases function in the mitochondria and the chloroplast in plant cells, but V-type ATPases appear to hydrolyze cytoplasmic ATP and acidify multiple intracellular compartments (Forgac, 1996). In keeping with the different roles and potentially different regulatory requirements of these two classes of enzymes, there is no obvious homology between the F-type and V-type ATPase subunits other than the catalytic and regulatory subunits and the proteolipid (Forgac, 1996).

Studies of the catalytic mechanism of the F-type ATPases have benefited greatly from the application of genetic techniques, predominantly in *Escherichia coli*, in combination with biochemical approaches [reviewed in Penefsky and Cross (1990), Duncan and Cross (1992), Futai et al. (1989), and Senior (1990)]. These genetic approaches range from random and site-directed mutagenesis to identification of targeted suppressors. The yeast *Saccharomyces cerevisiae* represents a comparable genetic model for studying structure and function of the eukaryotic vacuolar H^+-ATPases that has only been exploited to a limited extent. The phenotypes accompanying loss of V-ATPase activity have been elucidated in strains containing a chromosomal deletion of the subunit genes. Point mutations in the *VMA1* and *VMA3* genes have been made previously with the goal of looking at subunit structure−function relationships in the yeast vacuolar H^+-ATPase (Noumi et al., 1991; Supek et al., 1994; Taiz et al., 1994), but the levels of mutated subunit protein and other subunits of the enzyme, the targeting of the enzyme to the vacuole, and the state of assembly of the ATPase complex were not fully assessed. In this paper, we describe the first attempt to randomly mutagenize a large portion of a vacuolar ATPase subunit gene in order to dissect the partial functions of the subunit protein and the regions of the protein responsible for those functions. We have analyzed eight different mutants for the levels of the catalytic subunit produced, the assembly and targeting of the vacuolar ATPase, and the catalytic activity. The results provide new insights into the functional roles of the catalytic subunit, and the methods used should also be widely applicable to characterization of the structural and functional roles of all of the vacuolar ATPase subunits.

EXPERIMENTAL PROCEDURES

Materials and Strains. Zymolyase 100T and Trans[^{35}S]-label were purchased from ICN. Dithiobis(succinimidyl propionate) was obtained from Pierce. ^{14}C-labeled molecular mass markers and prestained molecular mass markers (high range) were obtained from Life Technologies. Concanamycin A was obtained from Wako Chemicals, and oligonucleotides were obtained from Genosys. All other biochemical reagents were from Sigma, and molecular biology reagents were from Boehringer Mannheim Biochemicals and New England Biolabs.

Yeast strain SF838-1Dα vma1Δ−8 (*MAT*α, *leu2−3,112, ura3−52, ade6, pep4−3, vma1*Δ*::LEU2*; Kane et al., 1990) was used in screening the mutant plasmids. For the integrations and subsequent biochemical characterization, wild-type strain SF838-5Aα (*MAT*α, *leu2−3,112, ura3−52, ade6*; Ito et al., 1983) and SF838-5Aα vma1Δ-8 (same as SF838-5Aα with *vma1*Δ*::LEU2*; Kane et al., 1990) were used. Yeast cells were grown in rich medium (yeast extract−peptone−dextrose; YEPD) or supplemented synthetic dextrose medium (SD) (Sherman et al., 1982); preparation of buffered medium for selection of ATPase mutants was done as described in Yamashiro et al. (1990).

Mutagenesis. Two different mutagenesis protocols, both relying on conditions that reduced the fidelity of Taq polymerase in the polymerase chain reaction, were employed. Primers 5′-GGCCTGCTCCCGCCGCTGC-3′, corresponding to nucleotides 2063−2081 of the open reading frame, and 5′-CACCCCTGCTTGGTTCAAA-3′, corresponding to nucleotides 3124−3142 (antisense) of the open reading frame of the *VMA1* gene, were obtained from Genosys and used with the uncut pFP4 plasmid as a template (at a 0.4−1.2 μg/mL final concentration) to amplify a 1000 bp region of the *VMA1* gene. For the high-frequency mutagenesis protocol of Leung et al. (1989), the amplification buffer included final concentrations of 0.5 mM $MnCl_2$, 6.1 mM $MgCl_2$, 1 mM each for dGTP, dCTP, and dTTP, and 0.4 mM dATP throughout the amplification reaction. For the low-frequency mutagenesis protocol [modified from Iwamoto et al. (1993)], the amplification buffer included final concentrations of 6.1 mM $MgCl_2$, 1.0 mM three dNTPs, and 1.0 μM for the fourth dNTP for the first two PCR cycles

and an additional 1.0 mM for the fourth dNTP for the subsequent cycles. Under both protocols, PCR was carried out in an MJ Research minicycler using the same program, which included a 2 min incubation at 95 °C, followed by 30 cycles of 1 min at 94 °C, 2 min at 59 °C, and 3 min at 72 °C, with an additional 5 min at 72 °C at the end of the final cycle. For the low-frequency protocol, the reaction was interrupted after completion of the second cycle to allow addition of the dNTP that was present at a lower concentration. Four reactions, each initiated with a 1 μM concentration of a different dNTP, were performed under the low-frequency protocol, and then the reaction mixtures were combined after the reactions were complete.

Plasmids and Mutations. The pFP4 plasmid contained a *Bam*HI-*Sal*I fragment of pDW21 (Kane et al., 1990) ligated to the *Bam*HI and *Sal*I sites of pRS316 (Sikorski & Hieter, 1989) in which the polylinker *Sac*II site had been destroyed. The 1 kb mutagenized PCR products were purified and digested with *Sac*II and *Sph*I and then ligated to the 9 kb *Sac*II-*Sph*I fragment of pFP4. The ligation mixture was used to transform *E. coli*, and plasmid DNA was obtained from the combined transformants and used to transform yeast strain SF838-1Dα vma1Δ-8 (Kane et al., 1990). Yeast transformants identified by uracil prototrophy were replica-plated to YEPD buffered to pH 5.0, YEPD buffered to pH 7.5, and YEPD containing 100 mM $CaCl_2$. Strains containing a mutated *VMA1* gene incapable of complementing the *vma1*Δ*−8* mutation were identified by their ability to grow at pH 5.0 but failure to grow at pH 7.5 or in 100 mM $CaCl_2$. Plasmids that allowed production of a stable protein reactive with the 8B1 antibody on immunoblots were rescued into *E. coli* (Strathern & Higgins, 1991), purified, and used to retransform the *vma1*Δ*−8* mutant. In those plasmids that again failed to complement the growth phenotypes of the mutant, the 1 kb mutated fragment was sequenced by the dideoxyribonucleotide chain termination method (Sanger et al., 1977) using Sequenase 2.0 (U.S. Biochemical).

The plasmids constructed in these experiments are listed in Table 1. Plasmids pJL134, pJL135, and pJL137 were obtained directly from the low-frequency mutagenesis protocol. Regions containing a limited number of mutations were obtained from a number of the plasmids mutagenized at high frequency using standard molecular biology methods (Sambrook et al., 1989). To obtain the pJL131, pJL146, and pJL153 mutant plasmids, the 1.05 kb *Hin*dIII fragment was purified from plasmids carrying the mutated *VMA1* genes. The purified fragments were then ligated to a 9 kb *Hin*dIII fragment of plasmid pJL124 to reconstruct the *VMA1* gene. Plasmid pJL124 contained a silent mutation in *VMA1* that destroyed the 3′ *Hin*dIII site of the C-domain; this mutation was constructed by mutating nucleotide A2451 to G using oligonucleotide 5′-CAAGATGGGCTGAGGCTTTGAGA-GAAAT-3′ (Kunkel et al., 1987). Plasmids pJL157 and pJL164 were constructed by subcloning the 96 bp *Hin*dIII fragment from plasmids containing the indicated mutant allele into the *Hin*dIII-cut plasmid pJL142. The pJL142 plasmid was constructed from plasmid pPK17, which lacks the entire VDE domain, by restricting the plasmid with *Hin*dIII and religating in the absence of the 96 bp *Hin*dIII fragment.

Integrating plasmids were constructed by cloning the 5.0 kb *Bam*HI-*Sal*I fragments containing the wild-type and mutant *VMA1* alleles into *Bam*HI-*Sal*I-cut integrating plasmids YIp5 (Struhl et al., 1979) or pJL166. pJL166 consisted of YIp5 with a 15 bp linker containing an *Xba*I site (constructed by annealing oligonucleotides 5′-CTGTCTAGAG-GGGCC-3′ and 5′-CCTCTAGACAGGGCC-3′) inserted at the *Apa*I site of YIp5. Integration of either *Apa*I-digested YIp5 or *Xba*I-digested pJL166 enabled SF838-5Aα vma1-Δ8 cells to grow in medium lacking uracil. Integration of the mutant alleles at the *URA3* locus was achieved by cutting the YIp5-based plasmids with *Apa*I and the pJL166 plasmids with *Xba*I, transforming yeast strain SF838-5Aα vma1−Δ8 with the linearized plasmids by the spheroplast transformation technique (Hinnen et al., 1978), and selecting for uracil prototrophy.

Table 1: Plasmids Used and Constructed

plasmid	description	reference
pFP4	*Bam*HI-*Sal*I fragment of *VMA1* inserted into *Bam*HI-*Sal*I sites of pRS316 lacking the polylinker *Sac*II site	this study
pPK17	*VMA1* lacking the entire VDE cloned into pSEYC68	Kane et al., 1990
pJL124	pFP4 with *Hin*dIII site at nucleotide 2450 destroyed	this study
pJL142	*Bam*HI-*Sal*I fragment from pPK17 with 96-base pair *Hin*dIII fragment removed cloned into *Bam*HI-*Sal*I sites of pRS316	this study
pJL134	*vma1−20* allele in pFP4	this study
pJL135	*vma1−21* allele in pFP4	this study
pJL137	*vma1−22* allele in pFP4	this study
pJL157	*vma1−40* allele in pPK17	this study
pJL164	*vma1−41* allele in pPK17	this study
pJL131	*vma1−42* allele in pFP4	this study
pJL146	*vma1−43* allele in pFP4	this study
pJL153	*vma1−44* allele in pFP4	this study
pJL166	YIp5 with *Xba*I adaptor inserted at *Apa*I site	this study
pJL179	*vma1−20* allele in YIp5	this study
pJL173	*vma1−21* allele in pJL166	this study
pJL172	*vma1−22* allele in pJL166	this study
pJL178	*vma1−40* allele in pJL166	this study
pJL177	*vma1−41* allele in pJL166	this study
pJL182	*vma1−42* allele in pJL166	this study
pJL184	*vma1−43* allele in pJL166	this study
pJL173	*vma1−44* allele in pJL166	this study

Biochemical Characterization. Vacuoles were isolated as described previously (Uchida et al., 1985), except that the vesicles were resuspended and stored in transport buffer [15 mM Mes-Tris (pH 7.0) and 4.8% glycerol (v/v)]. Protein concentrations were determined by Lowry assay (Lowry, 1951). ATPase activities were measured in a coupled enzyme assay (Lotscher et al., 1989) at 37 °C. The rates were measured within the first 10 min; however, under the conditions used here for the ATPase assays (including 25 mM KCl), the rate of hydrolysis remained linear for at least 15 min. In determinations of concanamycin A sensitivity, vacuoles were preincubated with 100 nM concanamycin A for 15−20 min on ice before activity was measured. For the kinetic analysis, ATPase activity in wild-type and mutant vacuolar membranes was measured in the presence of varied concentrations of ATP while the $MgCl_2$ concentration was maintained at 1 mM above the ATP concentration (Arai et al., 1989). Activity was measured in the presence and absence of 100 nM concanamycin A for each ATP concentration in order to determine the concanamycin A-sensitive activity. The concanamycin-sensitive ATPase activity at 50 μM to 3 mM ATP was measured for two different vacuole preparations, and the averaged results are shown in Figure 5. The data from these kinetic studies were fit to the

Michaelis−Menten equation using SigmaPlot (Marquardt−Levenberg least squares algorithm with no weighting of the data; Marquadt, 1963) to obtain values for K_m and V_{max} corresponding to the fits shown in Figure 5.

Nitrate stripping of vacuolar vesicles was performed essentially as described (Kane et al., 1989). Vacuolar vesicles (0.4 mg) were washed three times in 10 mM Tris-HCl and 1 mM EDTA (pH 7.5) as described by Uchida et al. (1985) and then divided into four equal aliquots of approximately 50 μg each. For the wild-type and each of the mutant vacuoles, the four samples were incubated with the following: (1) 100 mM KCl, (2) 100 mM KNO_3, (3) 100 mM KNO_3 + 5 mM $MgCl_2$ + 5 mM ATP, and (4) 100 mM KNO_3 + 5 mM EDTA + 5 mM ATP for 30 min at 30 °C. After incubation, the samples were centrifuged for 15 min at 100000*g* in a Beckman air-driven ultracentrifuge at room temperature and separated into supernatant and pellet fractions. The supernatant fraction was removed, and protein was precipitated with 10% trichloroacetic acid, followed by solubilization of the precipitated protein in 50 μL of cracking buffer [50 mM Tris-HCl (pH 6.8), 5% SDS, 8 M urea, 1 mM EDTA, and 5% β-mercaptoethanol]. The pellet fractions were directly solubilized in 50 μL of cracking buffer.

For immunoblot analysis, whole cell lysates and solubilized vacuoles were prepared, and SDS−polyacrylamide gel electrophoresis and Western blotting were performed, as described previously (Kane et al., 1992). Western blots were probed with mouse monoclonal antibodies 7D5 (against the 69 kDa subunit), 10D7 (against the 100 kDa subunit), 13D11 (against the 60 kDa subunit), and 7A2 (against the 42 kDa subunit) or rabbit polyclonal antisera against the 27 kDa subunit, followed by alkaline phosphatase-conjugated second antibody (Promega). Polyclonal antiserum against the 27 kDa subunit was a generous gift from M. N. Ho and T. H. Stevens. Quantitative immunoblotting was done by loading sequential dilutions of solubilized vesicles from the wild-type and mutant strains (ranging from 0.25 to 2.0 μg of vacuolar protein) on SDS−polyacrylamide gels, subjecting the gels to Western blotting as described above, but developing the Western blots using horseradish peroxidase-conjugated second antibodies and a chemiluminescence detection system (both from Kierkegard and Perry). The blots were exposed to film, and the intensities of the 69 kDa bands were analyzed by densitometry using a Shimadzu DS-9000 densitometer. Ratios of wild-type to mutant proteins were determined for three different concentrations of vacuolar protein, falling into the linear range of detection based on densitometry measurements.

Immunoprecipitations were performed as described (Kane, 1995) using the 8B1 and 13D11 monoclonal antibodies followed by protein A-Sepharose. Immunoprecipitated samples were analyzed by SDS−polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Isolation and Characterization of vma1 Mutant Alleles. We generated random mutations in the *VMA1* gene by two protocols, both involving PCR amplification of a region of the gene under conditions of reduced fidelity for the polymerase followed by incorporation of the mutated region into a plasmid-borne copy of the *VMA1* gene (Kane et al., 1990). The scheme for PCR mutagenesis is illustrated in Figure 1A. The region of the *VMA1* gene targeted for random mutagenesis encompasses most of the C-terminal half of the 69 kDa subunit. Yeast strains carrying the *vma1*Δ*−8* mutation cannot produce the 69 kDa subunit and exhibit typical Vma$^-$ growth phenotypes, including failure to grow on medium buffered to pH 7.5, and most of these phenotypes can be complemented by a plasmid-borne copy of the wild-type gene. Transformants carrying mutated copies of the *VMA1* gene that were incapable of complementing the growth phenotypes of the *vma1*Δ*−8* mutant cells were selected for further characterization on Western blots. The high-frequency mutagenesis protocol (see Experimental Procedures) yielded approximately 40% phenotypically mutant transformants of 800 screened, and the low-frequency mutagenesis protocol yielded approximately 2% phenotypically mutant transformants of 3000 screened. Growth of representative transformants at pH 5 and 7.5 is shown in Figure 1B.

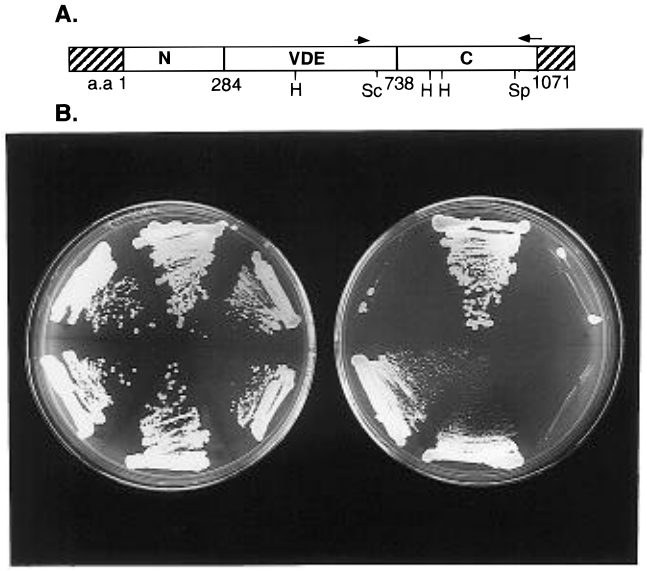

FIGURE 1: Scheme for mutagenesis and identification of mutant plasmids. (A) Arrows mark the positions of oligonucleotides used for mutagenic PCR of the indicated region of the *VMA1* gene. Restriction sites used for subcloning of the mutated region included the following: Sc, *Sac*II; Sp, *Sph*I; and H, *Hin*dIII. (B) Growth of wild-type and mutant cells on YEPD buffered to pH 5 (left) and YEPD buffered to pH 7.5. The same strains have been streaked on both plates and are as follows (clockwise from top): SF838-5Aα and SF838-5Aα vma1-Δ8 transformed with pJL134 (CEN-*vma1-20*), pJL137 (CEN-*vma1-22*), pJL146 (CEN-*vma1-43*), pFP4 (CEN-*VMA1*), and no plasmid.

Mutations that severely destabilize the *VMA1* gene product are not useful for structure−function studies. Therefore, we prepared whole cell lysates from the phenotypically mutant strains, subjected them to SDS−polyacrylamide gel electrophoresis, and probed for the presence of a stable product reactive with monoclonal antibody 7D5 against the 69 kDa subunit on immunoblots (Figure 2). A relatively high percentage (44%) of transformants derived from the high-frequency mutagenesis showed very little or no immunoreactive protein, but approximately 90% of the transformants mutagenized by the low-frequency protocol had protein recognized by the 8B1 antibody. At this step, we were also able to eliminate mutations that resulted in full or partial protein-splicing defects (resulting in a 119 kDa or 119 and 69 kDa products; Kane et al., 1990; Hirata & Anraku, 1992) and identify those that yielded a spliced but nonfunctional

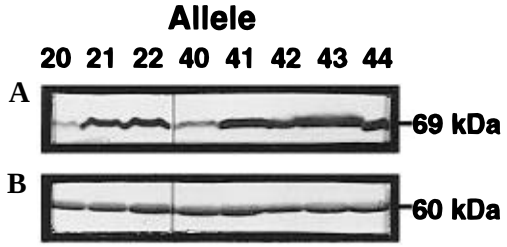

FIGURE 2: Identification of the 69 kDa subunit in mutant cells. Whole cell lysates were prepared from vma1−Δ8 mutant cells containing plasmid-borne copies of the indicated *vma1* mutant alleles as described in Experimental Procedures and subjected to SDS−polyacrylamide gel electrophoresis and Western blotting. Lysate derived from 0.75 OD_{600} unit of cells was loaded for each sample. Blots were probed with (A) monoclonal antibody 7D5 (anti-69 kDa subunit) and (B) monoclonal antibody 13D11 (anti-60 kDa subunit).

69 kDa subunit. Comparison with lysates from a wild-type strain indicated that all of the mutants shown in Figure 2 expressed nearly wild-type levels of the 69 kDa subunit (data not shown).

Plasmids were rescued into *E. coli* from mutants that produced a stable 69 kDa subunit, purified, and reintroduced into a *vma1*Δ*−8* mutant strain. Those plasmids that again conferred the mutant phenotype were sequenced, and a subset of those plasmids is characterized here. Three of the mutated plasmids obtained from low-frequency mutagenesis (pJL134, pJL135, and pJL137; Table 1) that had only one or two mutations in the 1000-base pair mutagenized sequence were chosen for further characterization. The high-frequency mutagenesis generally yielded a very high number of mutations (as many as one or two per 100 base pairs), particularly near the N- and C-termini of the mutated region. However, we were able to subclone regions carrying a more limited number of mutations from several of the mutant plasmids, and five that represented a variety of different phenotypes were chosen for further characterization. For two of the plasmids that appeared to have no effect on protein splicing (containing mutations *vma1−40* and *−41*), a mutated region in the C-domain was cloned into a *VMA1* gene lacking the VDE domain to allow further characterization of the effects of point mutations in the 69 kDa subunit. Previous work has shown that an allele of the *VMA1* gene lacking the VDE domain generates a 69 kDa catalytic subunit indistinguishable from the subunit obtained after protein splicing (Kane et al., 1990). Figure 2 shows a Western blot of whole cell lysates from cells carrying plasmid-borne copies of the mutant alleles listed in Table 1. The immunoblots were also probed with antibody against the 60 kDa vacuolar ATPase subunit (Figure 2B); loss of the 69 kDa subunit does not appear to affect the cellular levels of this or other subunits of the enzyme that have been characterized (Kane et al., 1992; Noumi et al., 1991; Umemoto et al., 1990). Four of the subcloned mutations yielded mutant *vma1* alleles that partially complemented the pH 7.5 growth defect of the *vma1*Δ*−8* strain (Table 2). The complementation by one of these mutant alleles (*vma1−43*) is shown in Figure 1B.

Biochemical Characterization of Mutant Alleles Producing a Stable Catalytic Subunit. Although the plasmid-borne copy of the wild-type VMA1 gene complemented the growth phenotypes of *vma1*Δ strains well enough to allow us to screen for mutants, closer examination revealed that the complementation was not complete and that the vacuolar

Table 2: Mutant Alleles of the *VMA1* Gene

allele	69 kDa subunit mutation	growth at pH 7.5
vma1−20	F906S/M940V	−
vma1−21	S811P	−
vma1−22	E740D	−
vma1−40	Y797H	±
vma1−41	I792T/S812T	+
vma1−42	E785G	−
vma1−43	F752S	+
vma1−44	E751G	±
VMA1	−	++

[a] Growth of strains carrying plasmid-borne copies of the mutant alleles was assessed after replica plating from SD medium lacking uracil to YEPD buffered to pH 7.5 and incubating for approximately 24 h at 30 °C.

Table 3: Vacuolar ATPase Activities in Vacuolar Vesicles Purified from Mutants[a]

allele	vacuolar ATPase activity ± SD (*n*)
VMA1	0.83 ± 0.07 (5)
vma1−20	0.044 ± 0.006 (2)
vma1−21	0.048 ± 0.018 (2)
vma1−22	0.031 ± 0.001 (2)
vma1−40	0.25 ± 0.01 (3)
vma1−41	0.69 (1)
vma1−42	0.14 ± 0.02 (3)
vma1−43	0.57 ± 0.06 (3)
vma1−44	0.36 ± 0.02 (3)
*vma1*Δ	0.035 ± 0.025 (4)

[a] Concanamycin A-sensitive ATPase activity [μmol min^{-1} (mg of protein)$^{-1}$] was measured in vacuolar vesicles isolated from cells containing either the wild-type (*VMA1*) or the indicated mutant allele integrated at the *URA3* locus (as described in Experimental Procedures). Assays were performed at 37 °C with or without preincubation of the vacuoles with 100 nM concanamycin A to determine concanamycin A sensitivity. Assays contained 2 mM MgATP and an ATP regenerating system.

ATPase activity in isolated vacuoles from *vma1*Δ strains carrying *VMA1* on a low-copy plasmid [approximately 0.25 μmol min^{-1} (mg of protein)$^{-1}$] was about 10-fold lower than the activity in wild-type vacuoles from a comparable strain expressing *VMA1* from its own chromosomal locus [2.5−3 μmol min^{-1} (mg of protein)$^{-1}$]. This was surprising because a number of other vacuolar H^+-ATPase subunit genes have been shown to fully complement the corresponding mutations when expressed from low-copy plasmids (Graham et al., 1994; Liu et al., 1996). The wild-type *VMA1* gene appeared to give better complementation when it was integrated into the genome of the *vma1*Δ strain at the *URA3* locus, showing much better growth at pH 7.5 and yielding vacuoles with a specific activity of 0.83 μmol min^{-1} (mg of protein)$^{-1}$ (Table 3). In order to characterize the functional defects of the mutants containing a stable 69 kDa subunit more thoroughly and to avoid the problems caused by extrachromosomal expression of the *VMA1* gene, the mutant alleles were integrated at the *URA3* locus. Although expression of the mutant alleles from the wild-type *VMA1* locus might be preferable, integration of the mutant alleles at this locus was technically much more difficult, making it rather impractical for comparison of a number of alleles. After integration of the mutant alleles at *URA3*, the mutant strains gave the same pattern of growth phenotypes as we had seen in expression of the alleles from a CEN plasmid (Table 2), and the mutant *vma1* genes were stably maintained whether cells were grown under selective or nonselective conditions.

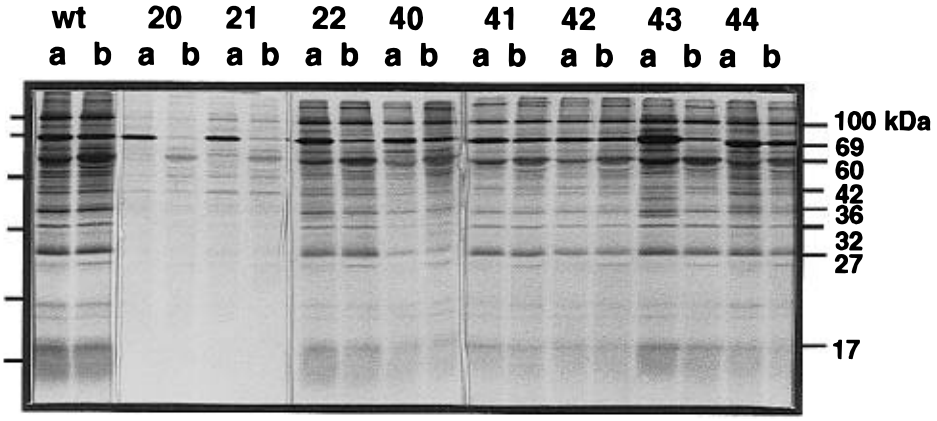

FIGURE 3: State of assembly of the vacuolar ATPase in cells carrying integrated copies of various *vma1* mutant alleles. Cells carrying the indicated mutant alleles integrated at the *URA3* locus were converted to spheroplasts and labeled with Trans[^{35}S]label for 60 min. Fully and partially assembled complexes were immunoprecipitated from the spheroplasts solubilized under nondenaturing conditions using monoclonal antibody 8B1 (a) or 13D11 (b). Immunoprecipitated proteins were visualized by autoradiography. Molecular mass markers are identified on the left (from top; 200, 97, 68, 43, 29, 18.4, and 14.3 kDa), and previously identified H^+-ATPase subunits are indicated on the right.

We first determined whether the 69 kDa subunits produced from the mutant alleles were competent for assembly into the vacuolar H^+-ATPase complex. Cells lacking the *VMA1* gene product produce all of the other subunits of the vacuolar H^+-ATPase complex but appear not to assemble any of the V_1 subunits at the vacuolar membrane (Kane et al., 1992; Noumi et al., 1991; Umemoto et al., 1990) or in the cytosol (Doherty & Kane, 1993). We immunoprecipitated the vacuolar H^+-ATPase from the mutant cell lines with monoclonal antibodies 8B1 and 13D11, against the 69 and 60 kDa subunits, respectively, and the results are shown in Figure 3. Both antibodies are able to immunoprecipitate the intact complex from cells containing the wild-type allele of *VMA1* [Figure 3 and Doherty and Kane (1993)]. The *vma1−20* and *vma1−21* strains grew very poorly in minimal medium lacking methionine and also labeled very poorly with [^{35}S]-methionine. The strain containing the *vma1−20* mutant allele appeared to have an almost complete assembly defect by immunoprecipitation, while the *vma1−21* allele showed at least partial assembly. In all of the other mutants, the previously identified subunits were coprecipitated, although to differing degrees. The *vma1−40* mutant, in particular, appeared to show selective loss of the 32 and 42 kDa subunits by immunoprecipitation as well as reduced levels of the other peripheral subunits. The immunoprecipitation of complexes shown in Figure 3 provides a sensitive test of whether any complexes can form in the cell with the mutated 69 kDa subunits and indicates that most of the mutations do not perturb the structure so drastically that assembly is prevented.

Vacuoles were isolated from strains containing mutant alleles of the *VMA1* gene integrated at the *URA3* locus in order to assess the level of targeting to the vacuole and assembly at the vacuolar membrane as well as the enzymatic activity of the mutant enzymes. Western blots of the isolated vacuolar membranes are shown in Figure 4. As expected, all of the mutant vacuolar membranes contain the 100 kDa V_o subunit. [This subunit is frequently observed as a 75 kDa proteolytic product as in this experiment (Kane et al., 1992).] Vacuoles from the *vma1−20* mutant strain have very low levels of the peripheral subunits at the vacuolar membrane, consistent with the severe assembly defect seen by immunoprecipitation. Significantly, these vacuoles have levels of the 100/75 kDa integral membrane subunit comparable to the other strains, indicating that the lowered levels

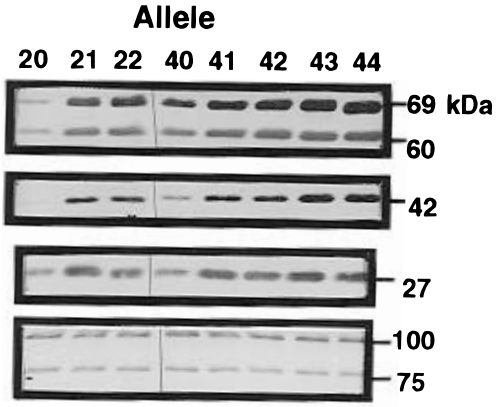

FIGURE 4: Levels of integral and peripheral vacuolar H^+-ATPase subunits present in vacuolar vesicles isolated from cells containing integrated copies of various *vma1* mutant alleles. Vacuolar membranes were isolated from SF838-5Aα vma1−Δ8 mutants carrying integrated copies of the mutant alleles indicated as described in Experimental Procedures, solubilized, and transferred to nitrocellulose for immunoblotting. The blots were probed for the presence of the 69, 60, 42, 27, and 100 kDa subunits as described in Experimental Procedures. Vacuolar protein (7.5 μg) was loaded in each lane for detection of the 100 kDa subunit, and 3.75 μg was loaded for detection of the other subunits.

of the peripheral subunits cannot be accounted for by overall poor yield or purity of the vacuoles from the mutant strain. The *vma1−40* mutant has somewhat lower levels of the peripheral subunits, particularly the 42 kDa subunit, consistent with the immunoprecipitation results. All of the other mutants, including *vma1−21*, which was difficult to evaluate from the immunoprecipitations, showed wild-type levels of the peripheral subunits, indicating that assembly and targeting to the vacuole were comparable to that seen in wild-type cells.

The specific concanamycin A-sensitive ATPase activities (measured at 2 mM MgATP) of the vacuoles containing mutated forms of the 69 kDa subunit are shown in Table 3. Concanamycin A is a highly specific inhibitor of vacuolar ATPase activity (Drose et al., 1993). The mutants exhibited vacuolar H^+-ATPase activities ranging from 3.7 to 83% of that obtained with the integrated wild-type gene in the presence of 2 mM MgATP, with the *vma1−20*, *−21*, and *−22* mutants showing levels of activity comparable to that of the *vma1*Δ strain lacking any 69 kDa protein. The *vma1−*

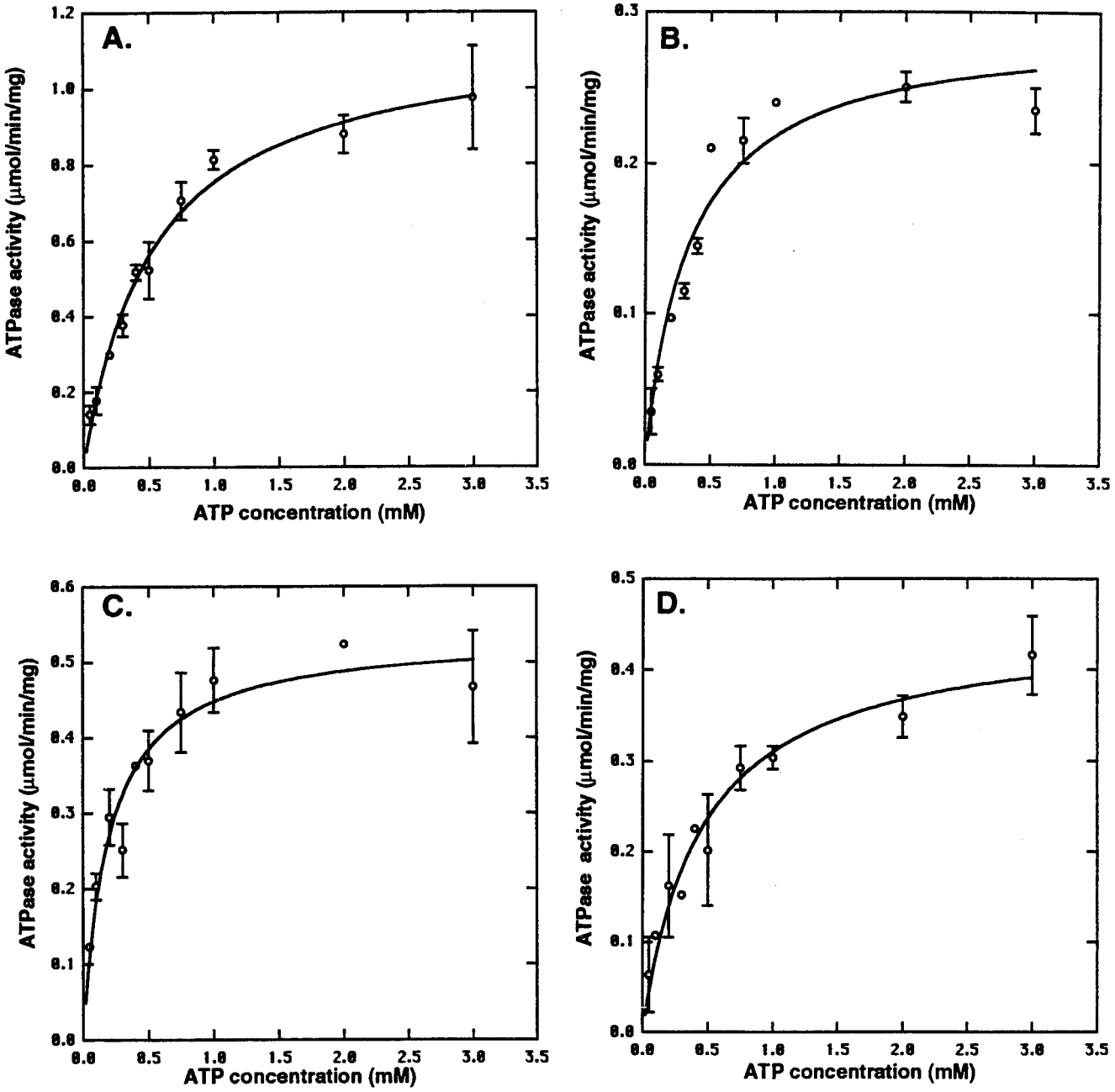

FIGURE 5: Kinetic analysis of mutants exhibiting partial vacuolar H^+-ATPase activity. The rate of concanamycin A-sensitive ATP hydrolysis [μmol min^{-1} (mg of protein)$^{-1}$] in wild-type and mutant vacuolar membranes was measured for ATP concentrations ranging from 0.050 to 3.0 mM, while the $MgCl_2$ concentration was maintained at 1 mM above the ATP concentration. Error bars represent the range of rates obtained for two independent vacuole preparations from each strain. The line represents the best fit of the Michaelis−Menten equation to the data, obtained as described in Experimental Procedures, and the kinetic constants (apparent K_m and V_{max}) corresponding to each fit are given in the text. Plots are shown for vacuoles purified from cells carrying the integrated wild-type *VMA1* gene (A), *vma1−40* allele (B), *vma1−43* allele (C), and *vma1−44* allele (D).

40, *−41*, *−42*, *−43*, and *−44* alleles showed partial ATPase activity, and the level of ATPase activity correlated well with the extent of partial complementation of the growth phenotypes of a *vma1−Δ8* mutant.

In order to better understand the catalytic defects in mutants showing partial activity, we measured the concanamycin-sensitive ATPase activity at varied ATP concentrations in vacuoles from strains carrying the wild-type *VMA1* gene and the *vma1−40*, *−43*, and *−44* mutant alleles at the *URA3* locus. The results are shown in Figure 5. Data points represent averaged results from two different vacuole preparations with error bars representing the range of values obtained for the two preparations. The data were fit to the Michaelis−Menten equation, and the best fit for each mutant is superimposed on the data points in Figure 5. The data for all of the mutants, except perhaps the *vma1−40*-containing mutant, were fit fairly well by assuming Michaelis−Menten kinetics, so a single apparent K_m and V_{max} value was obtained for each set of vacuoles. Vacuoles from the strain containing the integrated wild-type gene (Figure 5A) exhibited an apparent K_m of 0.528 ± 0.054 mM, in the range of those previously reported for solubilized and membrane-bound vacuolar ATPases (see Discussion), and a V_{max} of 1.15 ± 0.047 μmol min^{-1} mg^{-1}. The *vma1−40* mutant (Figure 5B) exhibited an apparent K_m of 0.334 ± 0.075 mM and a V_{max} of 0.291 ± 0.021μmol min^{-1} mg^{-1}, the *vma1−43* mutant (Figure 5C) exhibited an apparent K_m of 0.196 ± 0.039 mM and a V_{max} of 0.535 ± 0.028 μmol min^{-1} mg^{-1},

and the *υma1−44* mutant (Figure 5D) exhibited an apparent K_m of 0.452 ± 0.080 mM and a V_{max} of 0.451 ± 0.029 μmol min^{-1} mg^{-1}. The catalytic defects shown in Table 3 thus can be attributed primarily to differences in the V_{max} values, because only the *υma1−43* mutant exhibited a K_m value significantly different from that for the strain carrying the wild-type gene, and the K_m for this mutant was actually lower than that obtained in the presence of the wild-type Vma1 protein. We also determined the relative levels of the 69 kDa subunits in the vacuolar membranes isolated from wild-type, *υma1−40*, *υma1−43*, and *υma1−44* mutant cells by quantitative Western blotting. The *υma1−40* mutant contained 102% of the amount of 69 kDa subunit present in wild-type, the *υma1−43* mutant contained 128% of the wild-type amount, and the *υma1−44* mutant contained 107% of the wild-type amount. The activity in the vacuoles of the *υma1−42* mutant was too low to allow a good kinetic analysis. We were not able to test the ATP dependence of proton transport in any of the mutant vacuoles because vacuoles from this wild-type strain exhibited very little transport. We did look at quinacrine uptake by the *υma1−40*, *−42*, *−43*, and *−44* mutant cells, however, as a rough measure of proton flux *in vivo* (Rothman et al., 1989). We observed different levels of uptake that were qualitatively similar to the extent of growth of the cells at pH 7.5 and the ATPase activity in isolated vacuoles (data not shown) and in all cases higher than the fluorescence signal from *υma1*Δ cells, indicating that none of the mutations resulting in partial ATPase activity causes complete uncoupling of ATP hydrolysis and proton transport.

One of the original defining characteristics of vacuolar-type ATPases was sensitivity to low concentrations of chaotropic anions such as nitrate, isothiocyanate, and iodide. Inhibition by these anions is accompanied by full or partial removal of the peripheral subunits from the membrane, and a variety of studies have indicated that conditions optimal for ATP hydrolysis enhance the stripping of the peripheral subunits from the membrane, suggesting that catalysis induces a conformation that is particularly susceptible to stripping by chaotropic anions (Kane et al., 1989; Moriyama & Nelson, 1989; Ward et al., 1991). These data were later interpreted in the context of a two-state model for the vacuolar ATPase, in which ATP binding induced a conformation of the enzyme susceptible to oxidation by nitrate (and the other ions) that was loosely associated with the membrane (Dschida & Bowman, 1995). We tested the ability of several of the mutants exhibiting different levels of ATPase activity to dissociate in the presence of 100 mM potassium nitrate alone or in combination with MgATP, the catalytic substrate. As shown in Figure 6A, vacuoles from cells carrying the wild-type *VMA1* allele incubated at 30 °C in the presence of 100 mM KCl, 100 mM KNO_3 alone, or 100 mM KNO_3 + ATP (5 mM) + EDTA (5 mM) showed very little dissociation of the 69, 60, 42, or 27 kDa peripheral subunits from the membrane but showed approximately 50% dissociation (estimated by comparision of supernatant and pellet fractions on the immunoblot) in the presence of 100 mM KNO_3 and 5 mM MgATP. The cells carrying the *υma1−42* allele (Figure 6B), which had only 17% of the ATPase activity of wild-type cells (Table 3), exhibited a similar pattern of dissociation, except that there was slightly more dissociation under conditions that did not dissociate the wild-type enzyme and slightly less dissociation in the presence of MgATP (depletion of the subunits from the pellet fraction in lane 3 is less obvious). More dramatic differences are seen with the *υma1−21* (Figure 6C) and *υma1−22* (Figure 6D) mutant vacuoles. Although both sets of mutant vacuoles show some dissociation under all of the conditions examined, there is little enhancement in the presence of the catalytic substrate (supernatant, lane 3). The dissociation that does occur with nitrate + MgATP also appears to be less of a "coordinated removal" of the V_1 subunits; the 27 kDa subunit appears to be preferentially removed from the *υma1−21* mutant vacuoles (C), but the 42 and 69 kDa subunits appear to show slightly more dissociation from the *υma1−22*

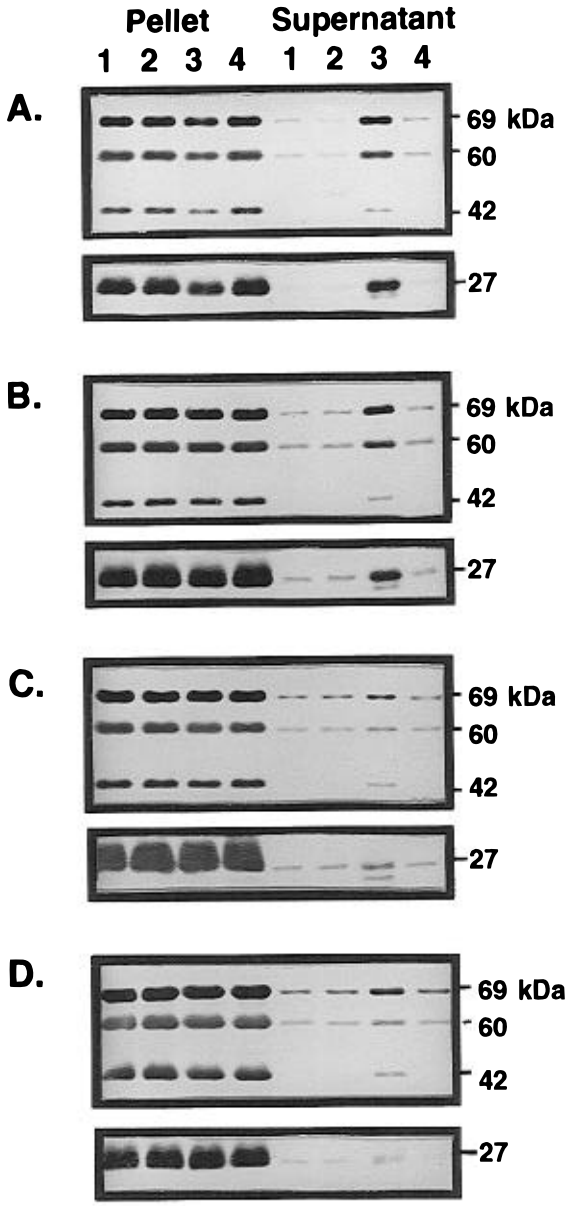

FIGURE 6: MgATP-dependent nitrate stripping of wild-type and mutant vacuolar membranes. Vacuolar membranes from cells containing the wild-type *VMA1* (A), *υma1−42* (B), *υma1−21* (C), or *υma1−22* (D) allele integrated at the *URA3* locus were incubated for 30 min at 30 °C in the presence of 100 mM KCl (1), 100 mM KNO_3 (2), 100 mM KNO_3 + 5 mM MgATP (3), or 100 mM KNO_3 + 5 mM ATP + 5 mM EDTA (4). The mixtures were separated into supernatant and pellet fractions by centrifugation as described in Experimental Procedures, and the distribution of peripheral subunits of the vacuolar H^+-ATPase was visualized by probing immunoblots as described for Figure 5. The supernatant and pellet fractions were derived from approximately 1 μg of washed vacuoles in parts A and B and 2 μg washed vacuoles in parts C and D.

vacuoles (D). These results support previous studies indicating that ATP hydrolysis induces a conformation of the enzyme that is susceptible to nitrate stripping of the V_1 sector and suggest that the enzyme in the *υma1−21* and *υma1−22* vacuoles may not be able to access this conformation.

DISCUSSION

Using random mutagenesis in combination with a genetic screen for mutants showing impaired vacuolar H^+-ATPase activity, we have generated an initial collection of mutants in the catalytic subunit of the yeast vacuolar H^+-ATPase exhibiting a range of different phenotypes. Our initial biochemical characterization provides a basis for categorizing these eight mutants that can be further interpreted in light of homologies to other V- and F-type ATPases.

The immunoprecipitation data shown in Figure 3 combined with the immunoblots of vacuolar vesicles in Figure 4 provide a good assessment of V-ATPase assembly in the different mutant strains. We would anticipate that different types of ATPase mutants might show assembly phenotypes ranging from a total loss of enzyme assembly to wild-type assembly, with possible intermediate phenotypes including partial complexes either in the cytosol (if there is a defect in membrane attachment of the V_1 as a result of the mutation) or at the vacuole (if interactions of a subunit or subunits was affected). The data indicate that only the *υma1−20* allele appears to cause an almost total loss of assembly. The sequence of the *υma1−20* allele would not necessarily suggest a severe assembly defect. The M940V change is a rather conservative mutation in a poorly conserved region of the V-type and F-type ATPases, and the F906S mutation occurs at a residue conserved as phenylalanine in many different V- and A-type ATPases (Bowman et al., 1988; Zimniak et al., 1988; Puopolo et al., 1991; Penefsky & Cross, 1993). However, mutation of the corresponding *E. coli* F_1 β-subunit Y331 to phenylalanine or serine does not affect assembly, although the serine mutation does severely compromise activity (Wise, 1990). We have not yet determined if one of the individual mutations of the *υma1−20* allele can account for the assembly defect. The *υma1−40* allele (Y797H) appears to cause an intermediate assembly phenotype. Although this mutant shows partial complementation of growth phenotypes and some ATPase activity, there appears to be a reduction in the levels of the peripheral subunits at the vacuole, and a selective loss of the 32 and 42 kDa subunits. These data do not distinguish whether the 32 and 42 kDa subunits fail to assemble with the mutant enzyme or are loosely attached and therefore lost during purification, but they do suggest that the *υma1−40* mutation alters the interactions of these two subunits with the ATPase complex. The kinetic data for the *υma1−40* mutant were not fit as well by a single apparent K_m and V_{max} as data for the other mutants were. One possible explanation of this behavior would be the existence of different partial complexes in the vacuolar membrane with different kinetic properties, but further resolution of the complexes will be necessary to confirm this. Most V-, F-, and A-type ATPases have tyrosine conserved at this position (Penefsky & Cross, 1990), although the *E. coli* F_1 has a lysine. The function of this sequence is not yet clear (Penefsky & Cross, 1990), but our data would suggest that in the vacuolar ATPases this region of the catalytic subunit might be either directly or indirectly involved in interactions with the smaller peripheral subunits and perhaps through these interactions in maintaining the stability of the complex.

All of the other mutants appear to assemble the vacuolar ATPase well, so we categorize the mutations as "catalytic" mutations, with full or partial defects in ATP hydrolysis. The *υma1−21* (S811P) and *υma1−22* (E740D) alleles showed both the most profound growth defects at pH 7.5 and an almost total loss of ATP hydrolysis (Table 3). The E740D mutation (*υma1−22*) affects an amino acid that is absolutely conserved among V- and F-type ATPases (Bowman et al., 1988; Zimniak et al., 1988; Puopolo et al., 1991; Penefsky & Cross, 1993), and we isolated another allele containing an E740G mutation that also failed to support growth at pH 7.5 (data not shown). The X-ray crystal structure of the bovine F_1-ATPase (Abrahams et al., 1994) indicates a direct role for the carboxylic acid of the glutamate at this position in the activation of the water molecule involved in ATP hydrolysis. If the catalytic center in the vacuolar ATPase 69 kDa subunit resembles that of the F_1 β-subunit, as proposed, we would anticipate a phenotype similar to the one we observe for the E740D mutation, in which assembly of the complex appears to be unaffected but ATP hydrolysis is totally abolished. Although the aspartate carboxyl could theoretically provide the hydrogen bond required for water activation, it would not be surprising if the structural requirements at the catalytic site were too restrictive to accommodate such a change. Further studies will be necessary to determine whether this mutant enzyme can bind ATP, as the model would predict. The S811P mutation (*υma1−21*) changes a serine in a region that is very highly conserved among V- and A-type ATPases but not with F-type ATPases (Bowman et al., 1988; Zimniak et al., 1988; Puopolo et al., 1991; Penefsky & Cross, 1993). The severe catalytic defect caused by this mutation makes this amino acid an interesting candidate for further site-directed mutagenesis to explore the effects of more conservative amino acid changes. The same is true of the *υma1−42* allele (E785G) which gives a less severe phenotype but also occurs at an amino acid highly conserved among V- and A-type ATPases but not with F-type ATPases (Bowman et al., 1988; Zimniak et al., 1988; Puopolo et al., 1991; Penefsky & Cross, 1993).

The kinetic behavior of the mutants exhibiting partial catalytic defects indicates that all of the mutations appeared to generate more significant effects on the V_{max} than on the apparent K_m. Under the conditions used for these assays, a reduced V_{max} can be accounted for by either a lower level of the mutant ATPase in the vacuolar membrane or a reduction in catalytic efficiency, but quantitative Western blotting of the isolated vacuolar membranes indicated that the mutants make and assemble as much or more 69 kDa subunit as the wild-type cells, so the differences in V_{max} reflect reduced catalytic efficiency. The clathrin-coated vesicle ATPase has been shown to have a low K_m site of 80 μM and a high K_m site of 800 μM ATP (Arai et al., 1989), but most other V-type ATPases appear to exhibit a single apparent K_m falling between these two values (Bowman & Bowman, 1982; Wang & Gluck, 1990; Randall & Sze, 1986). Reported apparent K_m values for the purified yeast vacuolar H^+-ATPase range from approximately 250 μM ATP (Kibak et al., 1993; Uchida et al., 1985) up to 1.3 mM ATP in the presence of sulfite (Kibak et al., 1993). The single apparent K_m value (528 $\pm$ 54 μM ATP) reported here for the membrane-bound yeast

vacuolar H^+-ATPase in the presence of 1 mM $MgCl_2$ is relatively consistent with the previously reported values. On the basis of the criteria of Uchida et al. (1988), the wild-type and mutant enzymes are being assayed under steady-state conditions at the concentrations of nucleotide and enzyme used here. We have not yet isolated sufficient quantities of the mutant enzymes to explore their catalysis under unisite conditions. The rather modest kinetic changes seen for the partially active mutants might suggest that they retain the potential for catalytic cooperativity, but it is still possible that the *vma1−21*, *−22*, or *−42* mutants are affected in the transition from single-site to multisite catalysis, resulting in very low catalytic activities.

The E751G mutation (*vma1−44*) occurs at a position corresponding to a conserved glutamate that is labeled by dicyclohexylcarbodiimide in a number of F-type ATPases (Penefsky & Cross, 1990; Duncan & Cross, 1992) and is present as glutamate or aspartate in vacuolar ATPase catalytic subunits as well (Bowman et al., 1988; Zimniak et al., 1988; Puopolo et al., 1991; Penefsky & Cross, 1993). The crystal structure of the F_1-ATPase suggests that this amino acid is in a "conical tunnel" traveled by the nucleotide as it moves from the surface of the enzyme to the nucleotide binding site (Abrahams et al., 1994). The E751G mutation found here in the yeast V-ATPase has a partial effect on cell growth at pH 7.5 and ATPase activity, but if this amino acid plays a similar role in V-type ATPases as it appears to in F-type ATPases, it might not be unreasonable that introduction of a smaller amino acid could still allow passage of nucleotide and ATPase activity. The mutation in the *vma1−43* allele (F752S) lies adjacent to this position and is conserved among V-type but not F-type ATPases (Bowman et al., 1988; Zimniak et al., 1988; Puopolo et al., 1991; Penefsky & Cross, 1993). Given its proximity to E751, this mutation could also affect entry and/or release of nucleotide, either of which could cause the partial defects and moderate lowering of the K_m that is observed.

We still do not fully understand the basis of the differences in specific activity between vacuoles isolated from cells expressing wild-type *VMA1* from a low-copy plasmid, the chromosomal *URA3* locus, and its own chromosomal locus. We have reconfirmed that the sequence of the wild-type *VMA1* allele we have been using matches that reported by Hirata et al. (1990), indicating that there are no secondary mutations, and we have found similar differences in activity when a wild-type *VMA1* allele that lacks the VDE domain is expressed from a *CEN*-plasmid and from an integrated allele (data not shown), indicating that the differences are not linked to the protein-splicing event. Differences in genetic behavior between a low-copy plasmid-borne copy of a gene and an integrated copy can indicate that the cell is very sensitive to expression levels of the gene (Ohya & Botstein, 1994), and it has been suggested previously that overexpression of *VMA1* can have deleterious effects on cell growth (Shih et al., 1988). We are further investigating these issues, but for these studies, integration of the wild-type and mutant alleles at the *URA3* locus allowed us to compare the different forms of the 69 kDa subunit relatively easily and to obtain genetically and biochemically consistent results.

The initial characterization of these mutants has both suggested many new avenues for further biochemical characterization and site-directed mutagenesis and allowed us to re-examine some of the older models for structure and catalysis by this enzyme from new angles. The nitrate-stripping experiments shown in Figure 6 are an example of the latter. Previous experiments similar to those shown in Figure 6A have suggested that vacuolar ATPases undergo a conformational change during catalysis that renders the peripheral domain more susceptible to removal by chaotropic anions such as nitrate, iodide, or isothiocyanate (Kane et al., 1989; Bowman et al., 1989; Moriyama &Nelson, 1989; Dschida & Bowman, 1995). Using mutant enzymes that are inactive in ATP hydrolysis but fully capable of assembly, we provide support for the models indicating that ATP hydrolysis is necessary for stripping at low concentrations of nitrate but also show that a relatively slow rate of hydrolysis (17% of wild-type) is sufficient to allow fairly efficient removal of the peripheral subunits.

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