Mutational Analysis of the Nucleotide Binding Sites of the Yeast Vacular Proton-translocating ATPase*

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To further define the structure of the nucleotide binding sites on the vacuolar proton-translocating ATPase (V-ATPase), the role of aromatic residues at the catalytic sites was probed using site-directed mutagenesis of the VMA1 gene that encodes the A subunit in yeast. Mutations were made at three positions (Phe452, Tyr532, and Phe538) that correspond to residues observed in the crystal structure of the homologous β subunit of the bovine mitochondrial F-ATPase to be in proximity to the adenine ring of bound ATP. Although conservative substitutions at these positions had relatively little effect on V-ATPase activity, replacement with nonaromatic residues (such as alanine or serine) caused either a complete loss of activity (F452A) or a decrease in the affinity for ATP (Y532S and F538A). The F452A mutation also appeared to reduce stability of the V-ATPase complex. These results suggest that aromatic or hydrophobic residues at these positions are essential to maintain activity and/or high affinity binding to the catalytic sites of the V-ATPase.

Site-directed mutations were also made at residues (Phe479 and Arg486) that are postulated to be contributed by the A subunit to the noncatalytic nucleotide binding sites. Generally, substitutions at these positions led to decreases in activity ranging from 30 to 70% relative to wild type as well as modest decreases in Km for ATP. Interestingly, the R483E and R483Q mutants showed a time-dependent increase in ATPase activity following addition of ATP, suggesting that events at the noncatalytic sites may modulate the catalytic activity of the enzyme.

The vacuolar proton-translocating ATPases (or V-ATPases) are a class of enzymes that couple the hydrolysis of ATP to the transmembrane movement of protons. This proton movement results in the generation of a pH gradient and the acidification of intracellular compartments (1–9) or, for certain specialized cells, the extracellular space (10–12). The V-ATPases are encoded by at least 14 genes, including VMA1, which encodes the 69-kDa A subunit (14, 15), and VMA2, encoding the 60-kDa B subunit (16). Sequence homology indicates that the A and B subunits of the V-ATPases (14–22) are related to the F-ATPases, function in mitochondria, chloroplasts, and bacteria to synthesize ATP (23–28). The level of sequence identity between these proteins is only 20–25%, however, and the A subunit contains a large (100-amino acid) insertion not present in β, suggesting that significant structural and functional differences may exist between these proteins.

From the x-ray crystal structure of the F1 domain from bovine heart mitochondria it was confirmed that the nucleotide binding sites are at the interface between the β and α subunits, with the catalytic site primarily on β and the noncatalytic site primarily on α (29). Previous biochemical experiments on the V-ATPase suggest that the catalytic nucleotide binding site is located on the A subunit while the noncatalytic site resides on the B subunit. For the catalytic site, these data include ATP-protectable labeling of the A subunit by reagents such as N-ethylmaleimide and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, which correlates with inhibition of activity (for references, see Forgac (30)). In addition, modification of a single A subunit cysteine residue (Cys352 of the bovine A subunit) by N-ethylmaleimide, cystine, or by disulfide bond formation with Cys332 leads to inactivation of the enzyme (31–33). Also, labeling of the A subunit by the photoaffinity ATP analog 2-azido-[32P]ATP correlates well with inhibition by this reagent (34). Finally, the A subunit, unlike the B subunit, contains several consensus sequences that appear to be critical for ATP hydrolysis in the homologous β subunit (35–37), including the glycine-rich loop.

There is limited information concerning the structure of the nucleotide binding sites on the V-ATPase, although mutagenesis studies in yeast have begun to identify residues important for V-ATPase activity (38–40). To investigate residues involved in formation of the nucleotide binding sites, site-directed mutagenesis of the VMA1 gene in yeast was performed. Based on the crystal structure of the F1 β subunit and sequence alignment of the β and A subunits, mutations were made in several residues proposed to form part of the adenine binding pocket at the catalytic site, including Phe452, Tyr532, and Phe538 (corresponding to Tyr345, Phe419, and Phe324, respectively, of the...
bovine mitochondrial β subunit) (29). In addition, mutations were constructed at two residues, Phe ⁷⁷⁹ and Arg ⁴⁸³ (corresponding to Tyr ³⁶⁸ and Arg ³⁷⁷ of the bovine F₇ β) (29), which are postulated to be contributed by the A subunit to the non-catalytic nucleotide binding sites. The effects of these mutations on activity, kinetics and assembly of the V-ATPase complex were assessed.

**EXPERIMENTAL PROCEDURES**

**Materials and Strains—** Zymolyase 100T was obtained from Seikagaku America, Inc. ConcanaAMycin A was obtained from Fluka Chemical Corp. Transcript labeling was purchased from ICN Biomedicals. Leupeptin, aprotinin, and pepstatin were all purchased from Boehringer Mannheim. Yeast extract, dextrose, peptone, and yeast nitrogen base were purchased from Difco. Twiztergent 3–14 was purchased from Calbiochem-Novabiochem Corp. Molecular biology reagents were from Promega and New England Biolabs. ATP and most other chemicals were purchased from Sigma.

Yeast strain SF388–5Aa vma1ΔΔ (MATa, leu-3-2, 112, ura3-52, ade6, vma1ΔΔ/LEU2), used for integrations and subsequent biochemical characterization, was a kind gift from Dr. Patricia Kane, Department of Biochemistry and Molecular Biology, SUNY, Syracuse. The plasmid pPK17-7, containing VMA1 lacking the entire VMA1-derived endonuclease spacer region, was a gift from Dr. E.L. Boger, Department of Chemistry, Boston College. The yeast shuttle vector pSEYC68 was a kind gift from Dr. J. Barr and M. Gietz. The yeast integration vector YIp5 was obtained from New England Biolabs. ATP and most other chemicals were purchased from Sigma.

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**Transformation—** Wild type VMA1 cDNA used in the altered Sites II in vitro mutagenesis system (Promega) following the manufacturer’s protocol. The full-length VMA1 cDNA lacking the endonuclease spacer space region (15) was cloned into pAlter-1 using BamHI and SalI sites. The mutagenesis oligonucleotide was as follows with the substitution sites underlined: F452Y, GAAAGCATCTGCTGTTTTAGAAGATCGTATGAAG; R483Q, GAATTTCCTGTTTTACATAGAG; F479Y, CAATTACCTGTTTTACATAGAG; F452A, CAAAGAAAGCATGCCCCATCTATCAAC; F538Y, CACTTATGAAAGATCGTATGAAG; Y532S, CAACAAAATGGTTCCTCCACTTATGATG; F538Y, CACTTATGAAAGATCGTATGAAG; R483Q, GAATTTCCTGTTTTACATAGAG; F479Y, CAATTACCTGTTTTACATAGAG; F452A, CAAAGAAAGCATGCCCCATCTATCAAC; F538Y, CACTTATGAAAGATCGTATGAAG; Y532S, CAACAAAATGGTTCCTCCACTTATGATG.

**RESULTS**

**Growth Phenotypes for vma1 Mutants—** Site-directed muta-
tions of the VMA1 cDNA encoding the yeast V-ATPase A subunit were constructed as described under “Experimental Procedures.” Three of the mutants described, Phe ⁶⁵², Tyr ³³² and Phe ³⁵³, are located in the C-terminal domain of the A subunit and are candidates for contributing to the adenine binding pocket of the catalytic nucleotide binding site. Two additional A subunit residues, Phe ⁷⁷⁹ and Arg ⁴⁸³, are postulated on the basis of sequence alignment and the crystal structure of F₁, to form part of the noncatalytic nucleotide binding site located on the B subunit. The mutant vma1 cDNAs were subcloned into the yeast integration vector YIp5 and expressed in a vma1Δ strain in which the VMA1 gene was deleted. The deletion of the introns encoding subunits of the V-ATPase results in a conditional lethal phenotype (14, 15, 52), which is also used to screen for mutants defective in vacuolar acidification. These strains are able to grow on medium buffered to acidic pH (5.0–5.5) but are unable to grow at pH 7.5. To isolate secondary antibody (Bio-Rad). Immunoblot was developed using a chemiluminescent detection method following the manufacturer’s protocol from Kurkegaard & Perry Laboratories.

**Metabolic Labeling and Immunoprecipitation of the V-ATPase—** Yeast strains WT-VMA1, vma1Δ Δ/Δ, and vma1Δ Δ/Δ were grown in synthetic dextrose-methionine-free medium overnight, converted to spheroplasts, and metabolically labeled with Tran³⁵S-label (50 μCi/5 x 10⁶ spheroplasts) for 60 min at 30 °C. Spheroplasts were pelleted, washed, and lysed in solubilization buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10% glycerol) with C₅₀, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin). The V-ATPase was cross-linked using dithiothreitol (dissolved im) and immunoprecipitated using the monoclonal antibody SBI-F3 against the yeast A subunit and protein A-Sepharose (Pharmacia Biotech Inc.). Samples were subjected to SDS-PAGE on a 12% acrylamide gel, fixed in 30% methanol and 7.5% acetic acid for 1 h, incubated in Enlightening solution (NEN Life Science Products) for 30 min, dried, and analyzed by autoradiography.

**Immunoblot Analysis—** Whole cell lysates and solubilized vacuoles were prepared using 50 mM Tris-HCl, pH 6.8, 8 M urea, 5% SDS, 1 mM EDTA, and 5% β-mercaptoethanol, as described previously (46). Samples were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with the monoclonal antibody SBI-F3 against the yeast V-ATPase A subunit (Molecular Probes, Inc.), followed by horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Immunoblots were developed using a chemiluminescent detection method following the manufacturer’s protocol from Kurkegaard & Perry Laboratories.
growth phenotype similar to the *uma1Δ* strain. F538W was unable to grow at pH 7.5 in the presence of high calcium. The remaining mutants, including those at the noncatalytic site, showed relatively normal growth at pH 7.5. It has previously been determined that 20% of V-ATPase activity is sufficient to rescue the growth phenotype (39). These results thus suggest that the F452A and F538W mutants are deficient in vacuolar acidification, but that the remainder of the mutant strains have a V-ATPase activity that is at least 20% of wild-type. It was next necessary to determine whether these mutations directly caused a loss of V-ATPase activity.

**ATPase Activities in Purified Vacuoles from vma1 Mutants**—Fig. 1 shows the V-ATPase activities of the wild type WT-*VMA1*, *uma1Δ/YIp5*, and *vma1* mutant strains. Fig. 1A shows the relative ATPase activities for the strains containing mutations at the catalytic site, and Fig. 1B the activities for the strains with the proposed noncatalytic site mutations. V-ATPase activities were measured in isolated vacuoles using a coupled spectrophotometric assay at 1 mM ATP and 37 °C as described under “Experimental Procedures.” Activities are expressed relative to the WT-*VMA1* strain, defined as 100%, which had a specific activity of 0.19 (± 0.04) μmol of ATP/min/mg protein.2 The negative control, *uma1Δ/YIp5* strain, had no measurable activity. F452Y, Y532F, F538Y, and F538A had nearly wild type levels of activity, whereas F452W and Y532S had approximately 50% and F538W had 20% of wild type levels. F452A had less than 5% of wild type activity and was comparable to the deletion strain. Mutations at the noncatalytic site all appear to reduce activity by 30–70% as shown in Fig. 1B. These data are consistent with the growth phenotypes.

**A Subunit Expression and V-ATPase Assembly in vma1 Mutants**—It was next necessary to determine whether the observed decrease in activity was due to decreased protein levels or to decreased V-ATPase assembly. Fig. 2 shows the effects of the mutations on A subunit stability and the presence of the A subunit on the vacuolar membrane, which provides an initial measure of V-ATPase assembly. Fig. 2 is an immunoblot using the monoclonal antibody 8B1-F3 against the yeast A subunit to determine levels of the A subunit in either whole cell lysates (CELL) or isolated vacuoles (VAC). The top panel shows the immunoblots for the wild type WT-*VMA1* and for *uma1Δ/YIp5*. The middle and bottom panels show the immunoblots for the strains containing mutations at the catalytic site, and the proposed noncatalytic site, respectively. Of the mutations tested, only R483E showed somewhat lower levels of A subunit

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2 The specific activity reported is that for unwashed vacuoles at 1 mM ATP. Higher specific activities are obtained for washed vacuoles at saturating ATP concentrations.
in the whole cell lysate compared with the wild type. However, all of the mutated proteins, including R483E, showed normal levels of A subunit on the vacuolar membrane.

As a further test of assembly, the V-ATPase was immuno-

precipitated from metabolically labeled cells. Cells were con-

verted to spheroplasts and labeled with Tran35S-label for 60 min at 30 °C followed by cell lysis, detergent solubilization, and immunoprecipitation using the monoclonal antibody 8B1-F3 as described under “Experimental Procedures.” As can be seen in Fig. 3A, most of the mutants at the catalytic site showed normal assembly relative to wild type. An exception is the mutant F452A, which shows very little intact V-ATPase complex by immunoprecipitation. Because this mutant showed normal levels of A subunit on the vacuolar membrane (Fig. 2) and normal assembly following detergent solubilization and density gradient sedimentation (data not shown), it appears that this mutation has resulted in a destabilization of the complex such that it can no longer survive immunoprecipitation. Fig. 3B shows immunoprecipitation of the V-ATPase from the noncatalytic site mutants. As can be seen, none of the mutations dramatically reduces assembly, although F452W and R483E show somewhat lower labeling in the B subunit region. The possible significance of these results is discussed below.

Kinetic Analysis of vma1 Mutants—To determine if any of the mutations resulted in changes in $K_m$ or $V_{max}$ for ATP hydrolysis, kinetic analysis on the purified enzymes was performed (Table II). Vacuoles were isolated from wild type WT-VMA1 and vma1 mutant strains and the V-ATPases were solubilized and purified by glycerol density gradient sedimentation as described under “Experimental Procedures.” ATPase activities of the purified enzymes were measured over a range of ATP concentrations from 100 μM to 2.5 mM ATP, while the MgCl₂ was maintained at 1 mM above the ATP concentration. $K_m$ and $V_{max}$ values were calculated from double reciprocal plots of ATP concentration versus ATPase activity. WT-VMA1 had a specific activity of 1.8 (± 0.1) μmol/min/mg of protein at 1 mM ATP, a $K_m$ of 0.72 (± 0.19) mM ATP and a $V_{max}$ of 2.7 (± 0.1) μmol/min/mg of protein. As shown in Table II, the $K_m$ and $V_{max}$ for F452Y and F538Y were not significantly different from wild type, whereas F452W, F538W, and Y532F had a decreased $K_m$, suggesting an increase in substrate affinity. $k_{cat}/K_m$ values for the former two mutations were reduced by about 2-fold, whereas for the Y532F mutation, a small increase in $k_{cat}/K_m$ was observed. F538A had a $K_m$ value approximately 2 times that of WT-VMA1, suggesting a small decrease in ATP affinity.
VMA1 Mutagenesis and Nucleotide Binding Sites

TABLE II

| VMA1 mutant | $K_m$ (mM ATP) | $V_{max}$ (μmol/min/mg) | $k_{cat}/K_m$ (s⁻¹) |
|-------------|----------------|------------------------|---------------------|
| WT-VMA1     | 0.72           | 2.7                    | 5.1 × 10⁻⁴          |
| Catalytic site |                |                        |                    |
| F452Y       | 1.0            | 2.5                    | 3.4 × 10⁻⁴          |
| F452W       | 0.39           | 0.50                   | 2.8 × 10⁻⁴          |
| F452A       | 0              |                        |                     |
| Y532F       | 0.45³          | 2.7                    | 8.2 × 10⁻⁴          |
| Y532S       | >5             |                        |                     |
| F538Y       | 0.60           | 1.8                    | 4.1 × 10⁻⁴          |
| F538W       | 0.45           | 0.91                   | 2.7 × 10⁻⁴          |
| F538A       | 1.5            | 1.5                    | 1.4 × 10⁻⁴          |
| Noncatalytic site |            |                        |                    |
| F479Y       | 0.60           | 1.0                    | 2.3 × 10⁻⁴          |
| F479W       | 0.25           | 0.48                   | 2.6 × 10⁻⁴          |
| F479A       | 0.33           | 1.18                   | 4.9 × 10⁻⁴          |
| R483K       | 0.27           | 0.46                   | 2.3 × 10⁻⁴          |
| R483E       |                |                        |                     |
| R483Q       |                |                        |                     |

$^{a}$ $k_{cat}$ was calculated using a molecular mass of the yeast V-ATPase complex of 830 kDa (1).

$^{b}$ At ATP concentrations above 1 mM, significant substrate inhibition was observed for this mutant.

Because the reaction rate for the Y532S mutant showed no sign of saturating, even at the highest ATP concentrations tested (2.5 mM), precise values for $K_m$ and $V_{max}$ could not be determined (data not shown). However, we estimate that the $K_m$ for ATP is greater than 5 mM, thus reflecting a significant decrease in affinity relative to the wild type. Some of the mutations proposed to be at the noncatalytic site also resulted in kinetic changes. F479W, F479A, and R483K all showed significant decreases in both $K_m$ and $V_{max}$, although values of $k_{cat}/K_m$ were either unchanged (F479A) or reduced by at most 2-fold. Interestingly, both R483Q and R483E exhibited a time-dependent increase in ATPase activity, as depicted for the R483Q mutant in Fig. 4.

**DISCUSSION**

Although mutagenesis has been employed in several studies to identify A subunit residues important for activity of the V-ATPases (39, 40, 53), there is currently no information concerning the residues that may participate in formation of the adenine binding pocket on the A subunit. In the F-ATPase β subunit, the adenine ring appears to be in close contact with a hydrophobic surface formed by aromatic rings, in particular Tyr$^{345}$ of the bovine mitochondrial enzyme (29). To determine whether aromatic rings are important in this region of the A subunit, site-directed mutagenesis was performed on Phe$^{452}$, Tyr$^{452}$, and Phe$^{538}$, located in the C-terminal domain of the A subunit. Conservative substitutions (such as Phe to Tyr and Tyr to Phe) as well as nonconservative substitutions (such as Phe to Ala or Tyr to Ser) were carried out. Substitution with a bulkier amino acid (Trp) was also performed.

From the data presented, it appears that an aromatic ring or a hydrophobic residue is important at each of these positions, particularly Phe$^{452}$. Of the catalytic site mutations tested, none significantly reduced stability of the A subunit and only one (F452A) reduced stability of the V-ATPase complex as evidenced by the inability to immunoprecipitate the intact complex from metabolically labeled cells. With respect to their effects on activity of the V-ATPase complex, the conservative mutations F452Y, Y532F, and F538Y had little effect on enzyme activity whereas the F452W mutation reduced activity by 50–70%, although this mutation also resulted in a lower value for $K_m$, suggesting an increased affinity for ATP. Interestingly, the F452A mutant, which could be isolated as an intact complex from vacuoles, was completely inactive, suggesting that an aromatic or sufficiently large hydrophobic residue at this position is crucial. Phe$^{452}$ corresponds to Tyr$^{345}$ of the mitochondrial β subunit, which appears from the crystal structure of F$_1$ to be in direct contact with the adenine ring (29). Tyr$^{345}$ of the mitochondrial β subunit is labeled by the photoaffinity analog 2-(azido-$^{32}$P)ATP (54) and substitution of tryptophan for Tyr$^{331}$ (which corresponds to Tyr$^{345}$ in the Escherichia coli β subunit) results in a reduction in both $K_m$ and $V_{max}$ by about 2-fold (55), whereas substitution with alanine reduces $V_{max}$ by 5-fold and increases $K_m$ by 5-fold (56). It should be noted that substitution of leucine for Tyr$^{311}$ in the E. coli F-ATPase results in a 7.5-fold increase in $K_m$ for ATP with little effect on $V_{max}$ (56), suggesting that a hydrophobic residue can partially substitute for the aromatic function at this position. Our data suggest that Phe$^{452}$ plays a comparably important role in nucleotide binding to the catalytic site on the V-ATPase A subunit. The inability to immunoprecipitate the intact V-ATPase from cells bearing the F452A mutation suggests that this substitution may also lead to a reduced stability of the V-ATPase complex.

Mutations at Tyr$^{352}$ and Phe$^{538}$ had rather different effects on activity. In particular, Y532S appeared to show a significantly reduced affinity for ATP, again consistent with a role of
this residue in ATP binding. This residue corresponds to Phe418 of the bovine mitochondrial β subunit which is situated near the end of the adenine binding pocket (29). Replacement of Phe418 with alanine also significantly reduced the affinity for ATP (approximately 2-fold), consistent with its interaction with the adenine ring. The corresponding residue in the F1 β subunit, Phe424, appears to interact with the adenine ring from the opposite side of Tyr345 (29). It should be noted that the effects of mutations in these β subunit residues (Phe418 and Phe424) on nucleotide binding to the F-ATPases have not been reported. These results support a model in which aromatic or hydrophobic residues in the C-terminal domain of the A subunit contribute to nucleotide binding to the catalytic site of the V-ATPase, with their absence resulting in substantial changes in either nucleotide binding or activity. These results also point up the likely similarity in structure of the catalytic nucleotide binding sites of the V and F-ATPases.

Using the recently released coordinates of the bovine heart mitochondrial F1 ATPase (29) and sequence alignment of the F-ATPase β subunit and V-ATPase A subunit, we have carried out energy minimization to arrive at a tentative model of the catalytic nucleotide binding site on the V-ATPase (Fig. 5). Shown are the aromatic residues investigated in the current study (Phe452, Tyr532, and Phe538) as well as two residues (Lys263 and Glu286) shown by previous mutagenesis studies (39, 40) to be important for activity. The bound AMP-PNP molecule is shown in dark shading; the bound Mg2+ is shown in the lightly shaded sphere, and the water molecule involved in hydrolysis is shown in the darkly shaded sphere.

FIG. 5. Proposed model for the catalytic nucleotide binding site of the yeast V-ATPase. Shown is an energy minimized model of the structure of the catalytic nucleotide binding site on the yeast V-ATPase A subunit based upon the x-ray crystal structure of the bovine mitochondrial F-ATPase β subunit (29) and sequence alignment of the A and β subunits (see “Experimental Procedures”). Shown are the aromatic residues mutated in the current study (Phe452, Tyr532 and Phe538). Also shown are two cysteine residues (Cys539 and Cys261) previously shown to form an inhibitory disulfide bond (33) as well as two residues (Lys263 and Glu286) shown by previous mutagenesis studies (39, 40) to be important for activity. The bound AMP-PNP molecule is shown in dark shading; the bound Mg2+ is shown in the lightly shaded sphere, and the water molecule involved in hydrolysis is shown in the darkly shaded sphere.

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