Site-directed Mutagenesis of the Yeast V-ATPase B Subunit (Vma2p)*

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The B subunit of the vacuolar (H⁺)-ATPase (V-ATPase) has previously been shown to participate in nucleotide binding and to possess significant sequence homology with the α subunit of the mitochondrial F-ATPase, which forms the major portion of the noncatalytic nucleotide binding sites and contributes several residues to the catalytic sites of this complex. Based upon the recent x-ray structure of the mitochondrial F₆-ATPase (Abrahams, J. P., Leslie, A. G., Lutter, R., and Walker, J. E. (1994) Nature 370, 621-628), site-directed mutagenesis of the yeast VMA2 gene has been carried out in a strain containing a deletion of this gene. VMA2 encodes the yeast V-ATPase B subunit (Vma2p). Mutations at two residues postulated to be contributed by Vma2p to the catalytic site (R301S and Y352S) resulted in a complete loss of ATPase activity and proton transport, with the former having a partial effect on V-ATPase assembly. Interestingly, substitution of Phe for Tyr-352 had only minor effects on activity (15-30% inhibition), suggesting the requirement for an aromatic ring at this position. Alteration of Tyr-370, which is postulated to be near the adenine binding pocket at the noncatalytic sites, to Arg, Phe, or Ser caused a 30-50% inhibition of proton transport and ATPase activity, suggesting that an aromatic ring is not essential at this position. This finding shows the nucleotide binding sites located near the interfaces of the α subunit (H180K, H180G, H180D, N181V) also inhibited proton transport and ATPase activity by approximately 30-50%. The catalytic sites of residues in the region corresponding to the P-loop of the α subunit (H180K, H180G, H180D, N181V) also inhibited proton transport and ATPase activity by approximately 30-50%. None of the mutations in either the putative adenine binding pocket nor the P-loop region had any effect on the ability of Vma2p to correctly fold nor on the V-ATPase to correctly assemble. The significance of these results for the structure and function of the nucleotide binding sites on the B subunit is discussed.

Vacuolar acidification plays an important role in a number of cellular processes, including receptor-mediated endocytosis, intracellular targeting of lysosomal enzymes, macromolecular processing, and degradation of the coupled transport of small molecules (for review, see Forgac (1993)). Vacuolar acidification is carried out by the vacuolar family of (H⁺)-ATPases (or V-ATPases),¹ which have been purified from a variety of sources, including clathrin-coated vesicles (Arai et al., 1987), chromaffin granules (Moriyama and Nelson, 1987), renal microsomes (Gluck and Caldwell, 1987), the central vacuoles of yeast (Uchida et al., 1985; Kane et al., 1989), Neurospora (Bowman et al., 1989), and plants (Parry et al., 1989; Ward and Sze, 1991), and the apical membranes of insect midgut cells (Schwefel et al., 1989).

The V-ATPase complex from clathrin-coated vesicles, like other members of this class, is composed of two functional domains (for review, see Forgac (1992)). The V₆ domain, which has the structure A₃B₄p, 34, 33 (Arai et al., 1988), is a 500-kDa peripheral complex that is responsible for ATP hydrolysis, with both the 73-kDa A subunit and 58-kDa B subunit participating in nucleotide binding. The integral V₀ domain, which has the structure 100,38,19,19, C₉ (Zhang et al., 1992), is a 250-kDa complex responsible for proton translocation. The structure of the yeast V-ATPase (Kane et al., 1989) is very similar to that of the bovine coated vesicle enzyme, with the exception that several subunits of the yeast V-ATPase, including the 54-kDa product of the VMA13 gene (Ho et al., 1993), the 14-kDa product of the VMA7 gene (Graham et al., 1994; Nelson et al., 1994), and the 13-kDa product of the VMA10 gene (Supekova et al., 1995) have not yet been identified in the mammalian enzyme.

Both structural analyses (Arai et al., 1988; Adachi et al., 1990b) and sequence homology (Zimniak et al., 1988; Bowman et al., 1988a, 1988b; Manolson et al., 1988; Mandel et al., 1988; Hirata et al., 1990; Puopolo et al., 1991, 1992) indicate an evolutionary relationship between the V-ATPases and the F-ATPases of mitochondria, chloroplasts, and bacteria (Senior, 1990; Penevsky and Cross, 1993; Pedersen and Amtz, 1993). Thus the A and B subunits of the V-ATPases are homologous to the β and α subunits of the F-ATPases, respectively, indicating that these proteins are derived from an ancestral nucleotide binding protein. The recent x-ray crystal structure of the F₆ domain of the mitochondrial F-ATPase (Abrahams et al., 1994) shows the nucleotide binding sites located near the interfaces of the α and β subunits, with the catalytic sites located principally on the β subunits and the noncatalytic sites located principally on the α subunits, in agreement with extensive mutagenesis and chemical modification data (Senior, 1990; Penevsky and Cross, 1991; Futai et al., 1994).

Several lines of evidence suggest that the catalytic sites of the V-ATPase are located on the A subunit. First, ATP protectable labeling of this subunit is observed using the inhibitors N-ethylmaleimide and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (see Forgac et al. (1989) for references), and modification of Cys-254 of the A subunit with sulphydryl reagents has been shown to lead to inactivation (Feng and Forgac, 1992). Second,
labeling of the A subunit by 2-azido-[32P]JATP correlates well with inhibition of ATPase activity, with complete inhibition observed upon modification of a single A subunit per complex (Zhang et al., 1995). Finally, the A subunit possesses all of the consensus sequences, including the glycine-rich loop region, which appear critical for ATP hydrolysis by the F-ATPase β subunit (Zimniak et al., 1988; Bowman et al., 1988b; Hirata et al., 1990; Puopolo et al., 1991).

The V-ATPase B subunit has also been shown to participate in nucleotide binding. Thus, the B subunit is modified by the photoaffinity analog 3-O-(4-benzoyl)benzoyl-ATP (Manolson et al., 1985) and by 2-azido-[32P]JATP (Zhang et al., 1995). Interestingly, modification of the B subunit by 2-azido-[32P]JATP occurs only at rapidly exchangeable sites, under which conditions the A subunit is also modified. These results suggest that, as with the F-ATPase, the catalytic sites are also at the interface between the A and B subunits. Unlike the α, β and A subunits, the B subunit lacks the glycine-rich loop consensus sequence which, from the crystal structure of F1, appears to be in close proximity to the triphosphates of ATP (Abrahams et al., 1994). The importance of this consensus sequence for ATP hydrolysis (Senior, 1990; Penefsky and Cross, 1991; Futai et al., 1994) supports the idea that the nucleotide binding site on the B subunit is noncatalytic.

To determine the effect of modifying specific residues in the B subunit on activity of the V-ATPase, we have carried out site-directed mutagenesis of the yeast VMA2 gene in a strain lacking this gene. The VMA2 gene encodes the yeast V-ATPase B subunit (Vma2p). Our results reveal that changes in residues predicted to be contributed by the B subunit to the catalytic nucleotide binding sites dramatically affect activity, whereas those predicted to affect the noncatalytic site on the B subunit itself have significant but less marked effects on activity.

### EXPERIMENTAL PROCEDURES

**Materials and Strains—** Zymolyase 100T was obtained from Seikagaku America, Inc. Baflofinycin A1 is a kind gift from Dr. Karlheinz Altenhof, University of Osnabruck, and concanamycin A was purchased from Fluka Chemical Corp. 9-Amino-6-chloro-2-methoxyacrine as described previously (Feng et al., 1990) and was a kind gift from Dr. Tom Stevens, University of Oregon.

**Construction of Mutants—** Various mutants were constructed using Altered Sites II in vitro mutagenesis systems (Promega). The full-length VMA2 gene was cloned into pBluescript as described in Yamashiro et al. (1990) and was a kind gift from Dr. Tom Stevens, University of Oregon.

**VMA2 Mutagenesis**

Deletion of genes encoding the yeast V-ATPase subunits results in a conditional lethal phenotype (Nelson and Nelson, 1990). Such strains are unable to grow at neutral pH but are able to grow at acidic pH. As demonstrated previously (Nelson and Nelson, 1990), we observe that a strain lacking functional Vma2p is able to grow at pH 5.5 but grows very poorly relative to the wild-type strain at pH 7.5. Of the Vma2p mutations tested, only R381S and Y352S showed defective growth at neutral pH, whereas the remaining mutations grew normally under these conditions (results not shown). It has previously been observed that strains containing as little as 20% of the wild-type V-ATPase activity show relatively normal growth at pH 7.5. It is thus necessary to directly test the activity of the V-ATPase in the mutant strains to determine whether these mutations caused loss of less than 80% of the wild-type activity.

FIG. 1A shows ATP-dependent proton transport activity in isolated vacuoles from the mutant and wild-type strains, while Fig. 1B shows bafilomycin-sensitive ATPase activity in isolated vacuoles. As can be seen, both R381S and Y352S completely eliminated proton transport activity and reduced V-ATPase activity in the vacuole by greater than 90%. This is consistent with the growth phenotype observed for these mutants described above. Both of these residues correspond to α subunit residues contributed to the catalytic site of the F-ATPases (Abrahams et al., 1994). Interestingly, substitution of Phe for Tyr-352 had a relatively small effect on activity (15–20%), suggesting that the aromatic ring rather than the hydroxyl group is important at this position.

By contrast, mutations at other positions had significant but much less dramatic effects on proton transport and V-ATPase activity. Thus, mutation of Tyr-370, to Arg, Phe, or Ser generally inhibited activity by 40–50%, although the Y370F mutation showed only a 20% inhibition of ATPase activity. Tyr-370 corresponds to an Arg residue located in the adenine binding pocket of the noncatalytic nucleotide binding site of F1 (Abrahams et al., 1994). Similarly, mutation of residues that correspond to the glycine-rich loop region of the F-ATPase α subunit, including H180K, H180G, H180D, and N181V, inhibited ATPase and proton transport activity by 30–50%, although the H180D mutant again gave only a 20% inhibition of ATP hydrolysis. It is possible in the cases where proton transport is inhibited to a greater extent than ATPase activity (as with Y370F and H180D) that the mutations are causing some uncoupling of proton transport from ATP hydrolysis.

Because mutations in the homologous α subunit of F1 have in some cases been shown to affect assembly and/or stability of the

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2 P. Kane, unpublished observations.
F-ATPase complex (Maggio et al., 1987; Soga et al., 1989; Weber et al., 1993; Jounouchi et al., 1993), it is necessary to determine the effect of each of the Vma2p mutations constructed on the assembly of the yeast V-ATPase. Vacuoles were isolated from each strain, and the V-ATPase was solubilized with detergent and isolated by density gradient sedimentation as described under “Experimental Procedures.” Fig. 2 shows the SDS-PAGE profile for the fractions containing the peak of V-ATPase activity, and Fig. 3 shows the V-ATPase activity in the peak fraction. As can be seen, nearly normal assembly of the V-ATPase was observed in all of the mutants with the exception of R381S, where a partial loss of assembly is observed. In addition, for most of the mutations, there is generally good agreement between the level of V-ATPase activity observed for the isolated enzyme and the level of bafilomycin-sensitive ATPase activity in the native vacuole, with the following exceptions. For the Y370F mutation, only 53% of control activity was observed for the isolated V-ATPase as compared with 80% in the native vacuole. Conversely, for H180K, nearly normal activity was observed for the isolated V-ATPase as compared with 40% inhibition in the vacuole. In these cases, the observed differences are unlikely to be due to a change in stability of the isolated V-ATPase since nearly normal assembly was observed for these mutants by SDS-PAGE (Fig. 2). In fact, of the mutations tested, only R381S had any significant effect on assembly or stability of the V-ATPase complex.

DISCUSSION

While photochemical labeling studies indicate that the V-ATPase B subunit participates in nucleotide binding (Manolson et al., 1985; Zhang et al., 1995), the structure and function of the nucleotide binding sites on the B subunit remain uncertain. In the case of the homologous F-ATPases, the α subunit forms the major portion of the noncatalytic nucleotide binding sites and contributes several residues to the catalytic sites, which are located principally on the β subunits (Abrahams et al., 1994). The effects of mutagenesis of the Escherichia coli α subunit residues on F-ATPase activity together with the data on mutagenesis of yeast Vma2p residues presented in this paper are summarized in Table I.

The x-ray crystal structure of mitochondrial F$_1$ reveals two residues, Arg-373 and Ser-344, which are contributed by the α subunit to the catalytic nucleotide binding sites, with both residues near the terminal phosphate of ATP (Abrahams et al., 1994). The positively charged guanidinium group of Arg-373 is postulated to stabilize the negative charge, which develops on the γ phosphate during the transition state of ATP hydrolysis, and mutation of this Arg to Cys in the E. coli enzyme results in a total loss of ATPase activity (Soga et al., 1989). Mutation of
the corresponding Arg residue to Ser in Vma2p has a similarly dramatic effect on activity, suggesting that this residue also plays a critical role in the V-ATPases. The partial loss of assembly may indicate that this residue also forms an important contact between the A and B subunits.

Mutagenesis of the E. coli residue corresponding to Ser-344 (Ser-347), has not been reported, but this residue has not been conserved as a serine residue in the V-ATPase B subunit. It is, instead, a tyrosine residue in the B subunits from Neurospora (Bowman et al., 1988a), plants (Manolson et al., 1988), yeast (Nelson et al., 1989), and bovine brain (Puopolo et al., 1992), as well as the archaebacterial ATPases from Sulfolobus (Denda et al., 1988) and methanobacteria (Inatomi et al., 1989). Interestingly, it is a phenylalanine residue in the B subunit from bovine kidney (Nelson et al., 1992). Mutagenesis of this residue to Ser in yeast totally abolishes V-ATPase activity, whereas substitution of Phe at this position has a relatively minor effect (Table I). This result, consistent with the presence of a phenylalanine at this position in the bovine kidney sequence, suggests that an aromatic residue rather than a hydroxyl group is important at this position, making a direct interaction between this residue and the terminal phosphates of ATP unlikely in the V-ATPases.

Combined mutagenesis, photochemical labeling, and fluorescence energy transfer studies indicate that Arg-365 is near the adenine ring of nucleotides bound at the noncatalytic sites of the E. coli a subunit (Weber et al., 1993). Thus, substitution of Tyr at this position results in significant fluorescence quenching of lin-benzo-ATP bound at the noncatalytic site. This mutation resulted in a 30% decrease in ATPase activity, while substitution of Phe at this position caused approximately 40% loss of activity (Weber et al., 1993). By contrast, the corresponding residue in the V-ATPase B subunit from all species (Bowman et al., 1988a; Manolson et al., 1988; Nelson et al., 1989; Puopolo et al., 1992), as well as the β subunit of the archaebacterial ATPases (Denda et al., 1988; Inatomi et al., 1989) is a tyrosine. Consistent with the results obtained for the E. coli a subunit, replacement of Tyr-370 with Arg, Phe, or Ser inhibited proton transport and ATPase activity by only 30–50% (Table I), suggesting that tyrosine is not absolutely required at this position. Other mutations in the vicinity of Arg-365, including S373F, G351D, and S375F, have been shown to impair catalysis (Maggio et al., 1987), and the x-ray crystal structure of F₁ confirms that this arginine residue is near the adenine binding pocket on α.

Considerable mutagenesis has been carried out on residues in the P-loop region of the E. coli α subunit (Jounouchi et al., 1993), which from the crystal structure lies in close proximity to the triphosphates of ATP bound to the noncatalytic sites of F₁ (Abrahams et al., 1994). Thus replacement of Lys-α175 with either His or Gly results in 42 or 92% inhibition of activity, respectively, while replacement with Phe or Trp abolishes assembly (Jounouchi et al., 1993). By contrast, replacement of the corresponding Vma2p residue (His-180) with Lys, Gly, or Asp had somewhat less dramatic effects on activity, inhibiting 20–50% of the activity. Similarly, for the E. coli α subunit, T176V and T176L showed 23 and 31% of wild-type activity, respectively, whereas replacement of the corresponding Vma2p residue (Asn-181) with Val gave 50–70% of wild-type activity (Ta-

![FIG. 3. Effect of Vma2p mutations on ATPase activity of purified V-ATPase.](image)

**Table I**

| Site                      | [F-ATPase α Subunit](#) | [V-ATPase Vma2p](#) |
|---------------------------|------------------------|---------------------|
|                           | Bovine mitochondria | E. coli Mutant       | ATPase activity | Yeast Mutant | Proton pumping activity | Bafilomycin-sensitive ATPase activity | Purified ATPase activity |
| Catalytic site            | Arg-373               | Arg-376 Cys<sup>a</sup> | ~0               | Arg-381 Ser   | 0 ± 1             | 14 ± 2                     | 72 ± 11               |
|                           | Ser-344               | Ser-347             |                  | Tyr-352 Ser   | 9 ± 1             | 3 ± 2                      | 79 ± 11               |
|                           |                       |                    |                  | Phe           | 85 ± 13           | 79 ± 11                    | 79 ± 11               |
| Noncatalytic site (adenine ring pocket) | Arg-362               | Arg-365 Tyr<sup>b</sup> | 66%            | Tyr-370 Arg   | 64 ± 3             | 64 ± 39                   | 84 ± 59               |
|                           |                       | Phe                | 59%             | Phe           | 64 ± 3             | 80 ± 10                   | 61 ± 6               |
|                           |                       | Ser               |                  | 61 ± 5        | 66 ± 25           | 66 ± 3                    |                     |
|                           |                       |                    |                  |               |                   |                           |                     |
| Noncatalytic site (P-loop region) | Lys-175               | Lys-175 His<sup>c</sup> | 58%            | His-180 Lys   | 51 ± 2             | 59 ± 9                    | 84 ± 6               |
|                           |                       | Gly               | 8%              | Gly           | 55 ± 1             | 71 ± 9                    | 67 ± 3               |
|                           |                       | Phe               | Not assembled   | Asp           | 54 ± 4             | 81 ± 12                   | 63 ± 7               |
|                           |                       | Trp               |                  | Asn-181 Val   | 47 ± 4             | 65 ± 16                   | 77 ± 3               |
|                           |                       | Leu               | 31%             |                |                   |                           |                     |
|                           |                       | Val               | 23%             |                |                   |                           |                     |

<sup>a</sup> Soga et al. (1989).
<sup>b</sup> Weber et al. (1993).
<sup>c</sup> Jounouchi et al. (1993).
ble 1). In fact, the glycine-rich loop region has not been conserved in the V-ATPase B subunit sequence, although this region (Ser-174 to Asn-181 in yeast) is nearly perfectly conserved in all the available V-ATPase B subunits. Thus, despite extensive amino acid substitution between the a and B subunit sequences, this region has retained an important role in maintaining maximal ATPase activity. Interestingly, a positively charged residue at position 180 of the yeast B Vma2p does not appear to be essential, suggesting that this residue may not be interacting directly with the triphosphates of ATP at the non-catalytic sites or that binding of ATP to the noncatalytic sites of the V-ATPase is not absolutely required for activity.

These studies thus represent a first step in the use of site-directed mutagenesis for the molecular dissection of the structure and function of the V-ATPase B subunit.

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