Resolution of Subunit Interactions and Cytoplasmic Subcomplexes of the Yeast Vacular Proton-translocating ATPase*

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The vacuolar proton-translocating ATPase is the principal energization mechanism that enables the yeast vacuole to perform most of its physiological functions. We have undertaken an examination of subunit-subunit interactions and assembly states of this enzyme. Yeast two-hybrid data indicate that Vma1p and Vma2p interact with each other and that Vma4p interacts with itself. Three-hybrid data indicate that the Vma4p self-interaction is stabilized by both Vma1p and Vma2p. Native gel electrophoresis reveals numerous partial complexes not previously described. In addition to a large stable cytoplasmic complex seen in wild-type, ∆vma3 and ∆vma5 strains, we see partial complexes in the ∆vma4 and ∆vma7 strains. All larger complexes are lost in the ∆vma1, ∆vma2, and ∆vma8 strains. We designate the large complex seen in wild-type cells containing at least subunits Vma1p, Vma2p, Vma4p, Vma7p, and Vma8p as the definitive V1 complex.

The V-type proton-translocating ATPase is the cornerstone of the yeast vacuole. This multisubunit membrane-bound enzyme converts the energy of ATP into a proton electrochemical gradient that is essential for the majority of vacuolar functions, including ion homeostasis, accumulation of amino acids, and the correct targeting of vacuolar resident proteins (1-3). Enzymes of the V-ATPase class are found throughout the biological world serving many functions, oftentimes at different subcellular locations or with tissue-dependent activities (4).

Many of the genes encoding ATPase subunits, as well as genes necessary for vacuolar acidification, have been identified (4, 5). Null mutations in the VMA (vacuolar membrane ATPase) genes result in a conditional phenotype characterized by several different traits. Growth of vma mutants is severely inhibited in media buffered to pH 7.0 or higher; best growth is obtained in media buffered to pH 5.5 (6). These mutants are sensitive to high concentrations of Ca²⁺ (≥50 mM) in the media (7). In vma strains that are also ade2, the reddish color caused by accumulation of fluorescent amino-imidazole ribotide conjugates in the vacuole is diminished, providing a convenient visual screen for ATPase mutants (8). Deacidification of the vacuolar lumen, which is normally maintained at pH 6 in wild-type cells, can be determined by direct fluorescent ratio measurements (9) and has been used as a screen for vacuolar pH mutants (5). Screens utilizing these characteristics have revealed the genes catalogued in Table I. Each of these genes is essential for vacuolar ATPase activity and most are required for proper assembly of the complete V-ATPase complex.

Biochemical analyses have begun to elucidate the assembly and regulation of the enzyme (10, 11). The vacuolar ATPase is divided into two subcomplexes: a membrane-bound V0 complex, which is responsible for the translocation of protons, and a peripheral membrane V1 complex, which contains the ATP-hydrolyzing subunits. In yeast, more extensive biochemical studies have lagged behind the more readily achieved genetic analyses. It has been shown that the ATPase can form the soluble, cytoplasmic V1 complex independent of the vacuolar membrane components (V0) and that this complex requires at least Vma1p, Vma2p, and Vma4p, but not Vma5p (12). Studies on the homologous complex from bovine clathrin-coated vesicles have shown that the ATPase can be disassembled and reassembled in vitro (13). The homologues to Vma1p, Vma2p, Vma4p, Vma8p, but not the Vma5p homologue, are essential for reassembly and activity (14). The subtleties of the interactions between these core subunits, however, have not been examined. Furthermore, the role of other peripheral subunits for which genes have been cloned from yeast have not yet to be determined.

Our laboratory has traditionally studied the biogenesis of the yeast vacuole and its role in cellular metabolism. We became interested in the ATPase due to the substantial influence of ATPase mutants on the targeting of proteins to the vacuole (2, 3, 15). Because of its central role in most vacuolar functions, we have begun to explore the structure/function relationship and regulation of the ATPase. We initiated our studies using the two-hybrid method for examining interactions between subunits (16). To further explore interactions we developed a native gel system for looking at subcomplexes of the V1 complex. From these studies we have discovered a surprising number of cytoplasmic subcomplexes which we predict will be assembly intermediates on the pathway to the complete enzyme.

Experimental Procedures

Reagents, Antibodies, Strains, and Plasmids—All reagents were from Sigma unless otherwise stated. HEPES buffer was from Research Organics, Inc. Media components were from Difco. All strains were derived from the wild-type strain W303-1B (MATa leu2 his3 ade2 trpl ura3) with the exception of the ∆vph1, ∆vph2, and ∆vph1Δvph2 strains (17, 18), which were a gift from M. Mandson (Hospital for Sick Children, Toronto). All W303-1B-based ∆vma strains were a gift from N. Nelson (Tel Aviv University), except for the ∆vma4 strain, which was prepared in W303-1B using the vma4:LEU2 disruption construct described previously (15), and confirmed by Western blot. The two- and three-hybrid assays were conducted in strain SFY526(MATa ura3 his3 ade2 lys2 trpl1 leu2 gal4 gal80 URA3::GAL1-1ac22) (19). To produce antisera to Vma1p, Vma2p, and Vma8p, synthetic peptides were made (Multiple Peptide Systems, San Diego, CA) based on the deduced amino acid sequence of the VMA1, VMA2, and VMA8 genes. Peptides corresponding to amino acid residues 4-24 and 932-951 for Vma1p, 386-400 and 482-499 for Vma2p, and 191-205 and 209-232 for Vma8p were separately conjugated at their C termini to
keyhole limpet hemocyanin. Standard procedures were used to generate
eruminant in New Zealand White rabbits. Antiserum to Vma4p has
been described previously (15).

The two-hybrid vectors, pGAD424 and pGBT9, which contain the
GAL4 DNA-binding domain and DNA binding domain, respectively,
were purchased from Clontech. The multiple cloning sites (MCS)1 in
these vectors were modified by digestion with EcoRI and PstI to elim-
inate the existing sites, and ligated with the complementing oligonu-
clotides 5'-AATTCGGAATTCTGGAAATGCTGCTGCGGCGGTCGCA-3'
and 5'-GGCCGGTGATGATCTGGAGATGTGGAAGATGTCGCGG-3', to create pA
and pB, respectively. This altered the reading frame of the sites, and added a
unique Spel site into each of the vectors. The VMA11deor vector
was obtained by polymerase chain reaction from plasmid pVMAD16vde, a gift from F.
Gimble (Texas A & M University), using the primers 5'-
ATCGG-3' and 5'-GCTCGAGATATCTTAA-3', to create pA
and pB, respectively. Likewise, the fragments were
cloned into the BamHI and XhoI sites of plasmid pYF12 from nitrocellulose to generate pGA1 and pGA2, respectively. These are galactose inducible and were used to demonstrate
that the polymerase chain reaction products could complement
their respective deletion strains. The BamHI-Xhol fragments were subcloned from pGA1 and pGA2 into the BamHI and SalI sites of pA'
to create pA'1 and pA'2, respectively. Likewise, the fragments were cloned into pB' to create pB'1 and pB'2. The VMA4 ORF and terminator
were cloned as a BamHI fragment from pYF12VMA4 (15), which can
complement the Δvma4 strain, and into pA' and pB' to make pA'4 and pB'4, respectively.

The three-hybrid plasmid was constructed by eliminating the two-
hybrid portion of pGAD424, which lies between two HindIII sites, then leaving the alcohol dehydrogenase promoter and terminator intact. We
constructed a gift from T. Stevens (University of Oregon) and has been described previ-
ously (33).

Two- and Three-hybrid Assays—The two- and three-hybrid con-
structs were transformed into strain SYF526 and plated onto synthetic
medium with glucose supplemented with appropriate amino acids. Colon-
ies were patched to fresh plates, grown, and transferred onto What-
man filter paper #50 discs which had been layered onto synthetic
medium with glucose plates. The patches were allowed to grow over-
night and then lysed by two freeze-thaw cycles in liquid nitrogen. The filters were then placed on additional filters soaked in Z-buffer (100 mM
sodium phosphate, pH 7.0, 10 mM KCl, 1 mM MgSO4, 0.3% b-mercapto-
ethanol) with 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (0.34
mg/ml) and incubated at 30°C for 36 h. The filters were dried and scanned.

For liquid assays, cells were grown to late logarithmic growth phase
(A600 = 1.0), and then lysed in Z-buffer containing SDS (0.005%) and
chloroform (5%). o-Nitrophenyl-b-D-galactopyranoside was added to a final
concentration of 0.4 mg/ml and the samples incubated at 30°C for 30 min, then quenched with Na2CO3. 300 mM final concentration. Color
development was measured by absorbance at 420 nm and the activity
calculated according to the formula of Miller (20). Multiple assays were
conducted on several independent transformations of each cross.

Preparation of Extracts for Native Gel Electrophoresis—Wild-type
deleterious strains described above were grown to late log in YPD (1% yeast extract, 2% bactopanone, 2% glucose) buffer to pH 5.5 with 50
mM HEPES and 50 mM MOPS buffers. The cells were pelleted and treated
with 100 mM Tris-SO4, pH 9.4, 10 mM diethy ether for 5 min at 30°C.
These were pelleted and resuspended in osmotically supportive media
containing 1% glucose and 1.5 mg of oxalylase (Enzogenetics)/ml and incubated
for 40 min at 30°C. The resulting spheroplasts were pelleted gently and washed once with supportive media lacking oxalylase. The cells were then pelleted and lysed on ice in native sample buffer (10 mM Tris acetate, pH 6.9, 5 mM potassium acetate, 1
mM EDTA, 2 mM phenylmethanesulfonyl fluoride (Boehringer Mann-
heim), 10% glycerol, 0.01% bromphenol blue, 0.01% xylene green) at
a concentration of 5 mg/ml total protein, based on an estimate of 200
μg of protein/A600/ml of cells. These samples were quickly aliquoted on ice,
then frozen in liquid nitrogen, and stored at −80°C until use. For native
gels, the samples were thawed on ice, centrifuged briefly to pellet
particle matter, and loaded immediately.

Native Gel Electrophoresis—Native gels employed a continuous
buffer system of HEPES and imidazole at pH 7.4. For qualitative first
dimensions and two dimensional gels, 6% acrylamide (37.5:1 acryl:bis;
Boehringer Mannheim) gels were used. For Hewick-Smith analyses,
varrying percentages from 6 to 8.25% were used. Gels were prerun at a constant
current of 150 V (25 V/cm) at 4°C for 2 h until the current was
steady. Buffer was changed before sample loading, and the gels were run
for 100 min at 150 V. For qualitative first dimensions and Hewick-
Smith analyses, the gels were electrophorized onto Immobilon PVDF
membrane (Millipore) overnight in Tris/glycine transer buffer at 60 mA
constant current. For two-dimensional gels, the first dimension was cut
into strips and soaked in Laemmli buffer for 1 h at 37°C. These were
loaded onto the 3% stack of a 10.5% SDS-PAGE preparative gel and run at 100 V constant until the dye front was at the bottom of the gel. These gels were then transferred to PVDF as described above.

| Table 1 | AT Pase subunits |
|---------|------------------|
| Gene    | Molecular mass  |
| VMA1    | 68 kDa           |
| VMA2    | 57 kDa           |
| VMA3    | 16 kDa           |
| VMA4    | 27 V6           |
| VMA5    | 42 V6           |
| VMA6    | 40 V6           |
| VMA7    | 13 V6           |
| VMA8    | 28 V6           |
| VMA10   | 13 V6           |
| VMA11   | 16 V6           |
| VMA12   | 25 V6           |
| VMA13   | 5 V6            |
| VMA21   | 8 V6            |
| VMA22   | 21 V6           |
| VPH1    | 96 V6           |
| STV1    | 102 V6          |

| Subcomplex | Function | Stoichiometry |
|------------|----------|---------------|
| ATP hydrolysis | 3         | 22, 23, 24    |
| ATP binding/regulatory | 3        | 25, 26        |
| Proton translocator | a        | 27, 28        |
| Vp assembly | ?        | 1             |
| Vp assembly | ?        | 29, 30        |
| Vp assembly | ?        | 31            |
| Vp assembly | >1       | 32, 33        |
| Vp assembly | >1       | 34, 35        |
| Vp assembly | ?        | 36            |
| Vp assembly | ?        | 37, 38        |
| Vp assembly | ?        | 39, 40        |
| Vp assembly | ?        | 41            |
| Vp assembly | ?        | 42            |
| Vp assembly | ?        | 53            |
| Targeting | 1        | 17            |
| Targeting | 1        | 18            |

a Vma3p and Vma11p are proteolipids, of which there are a total of six per complex.
b Vma12p, Vma21p, and Vma22p are assembly factors and not found in the complete complex.
c Vph1p and Stv1p are homologues and presumably are not found together in a single complex.

1 The abbreviations used are: MCS, multiple cloning site; HA, hemag-
glutinin; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-mor-
pholino)propanesulfonic acid; NLS, nuclear localization sequence; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinyl difluoride; SV40, simian virus 40.
Fig. 1. Two-hybrid filter assay of interactions between VMA genes. Full-length ORFs of VMA1Δvde (1), VMA2 (2), and VMA4 (4) were cloned behind the GAL4 trans-activating domain (pA′) or the GAL4 DNA binding domain (pB′) and transformed into the yeast two-hybrid detector strain SFY526. The filter was soaked in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-containing buffer for 30 h at 30 °C. The dark spots, indicating an interaction between the respective VMA gene products, are blue in the original data.

Fig. 2. Three-hybrid liquid assay of interactions between VMA genes. The combination of VMA genes in the three-hybrid cross are indicated beneath the respective columns. The N-terminal fusion is indicated on the bottom left. The data are the product of four independent experiments (n = 4).

RESULTS

Interactions Detected by Two-hybrid Assay—To begin our examination of interactions between V1 subunits, we cloned VMA1Δvde, VMA2, and VMA4 into the two-hybrid vectors pA′ and pB′ and examined all possible crosses in yeast strain SFY526, which contains the β-galactosidase gene under control of the GAL1–10 promoter integrated into the genome. The VMA1 gene codes for a self-splicing protein (54), and the VMA1Δvde version has the VDE coding region deleted from the ORF (55).

An interaction between ATPase subunits would be detected as a β-galactosidase activity on filters in the two-hybrid system (blue color). Fig. 1 shows a filter assay for β-galactosidase activity of yeast patches containing all possible pairs of two-hybrid constructs. For a positive control we crossed the SV40 large T-antigen and murine p53 proteins (data not shown), whose interaction has been demonstrated by independent methods. Signals from the VMA1 × VMA2 crosses are clearly evident in both possible combinations on the filter, indicating that Vma1p and Vma2p may interact directly. Surprisingly, we also discovered that Vma4p can interact with itself. This result was not expected, since Vma4p has a proposed stoichiometry of one per ATPase complex based on comparison with the V-ATPase complex from bovine clathrin-coated vesicles (43). We found no interactions between VMA1 × VMA4 and VMA2 × VMA4 in the two-hybrid assay, although all three proteins are necessary for formation of V1 complexes (12, 13).

All signals from the filter assays were distinctly above background, yet required from one to 2 h to develop on the filter at 30 °C. The positive control, by contrast, developed a strong signal within 30 min. Liquid assays for β-galactosidase activity gave low signals (1–2 Miller units) from the VMA4 × VMA4 interaction, and no detectable signals from the VMA1 × VMA2 crosses were obtained. Again, the positive control gave a reproducible signal of 80–100 Miller units. Western blots indicated that the full-length fusion constructs were being stably expressed, albeit at low levels (data not shown). We suspect that the Vma1p-Vma2p interaction is transient and requires stabilization by other subunits for a strong interaction.

Three-hybrid Interactions—We conjectured that two-hybrid interactions could be stabilized by the presence of a third ATPase subunit targeted to the nucleus. Therefore, we constructed the three-hybrid vector which fuses the SV40 nuclear localization sequence to the N terminus of a protein so that it is targeted to the nucleus for potential interaction with the two-hybrid fusion products. It has been previously demonstrated that this signal is sufficient to target passenger proteins to the yeast nucleus (44). We envisioned three possible interactions: 1) a bridge interaction, where two proteins which did not show an interaction previously would now give a positive signal in the β-galactosidase assay; 2) an enhancement interaction, where the third protein would stabilize the two-hybrid products and increase the signal from the assay; and 3) a competitive interaction, where the third protein would diminish a pre-existing signal.

Results from all possible three-hybrid crosses were somewhat equivocal. In certain instances a three way interaction (i.e. including Vma1p, Vma2p, and Vma4p, each in a different fusion vector) gave detectable signals on filter assays. However, these signals were not detectable by liquid assay, and the interactions did not show reciprocal relationships. The one clear result which did emerge involved the Vma4p self-interaction (Fig. 2). In this case we saw a significant and reproducible enhancement of the β-galactosidase activity in liquid assays when NLS-Vma1p or NLS-Vma2p was included with the Vma4p fusion constructs. In contrast, when the NLS-Vma4p fusion is present we see a competitive diminishment of the self-interaction, further indicating that the interaction is specific. The enhancement by NLS-Vma1p and NLS-Vma2p suggest that they interact with Vma4p. Why Vma4p does not show an interaction with Vma1p or Vma2p in a direct two-hybrid cross is uncertain, but we hypothesize that the site of interaction in Vma1p and Vma2p may be masked by the two-hybrid fusions, yet accessible in the third hybrid fusion construct.

Two-dimensional Analysis of V1 Complexes—Vexed by the results of our two- and three-hybrid results, we developed a native gel analysis to identify cytoplasmic complexes more directly, and by extension subunit interactions. The first lane of Fig. 3A shows an immunoblot of the first dimension from our wild-type strain of yeast, probed with an antibody to Vma1p. Several bands are visible, one near the top of the gel and an indeterminate smear further down. It must be remembered that although native gels separate in part on molecular size and shape, separation depends substantially upon the charge/mass ratio and that therefore a higher band does not necessar-
The immunoblots were stripped of antibodies and reprobed with antibodies to Vma2p, Vma4p, and Vma8p, shown in the second, third, and fourth lanes, respectively, of Fig. 3A. The actual order of blotting is 2p, 1p, 4p, 8p. Stripping of the blot is sometimes incomplete, but the second dimensions for Vma2p, Vma4p, and Vma8p shown in Fig. 3B clarify the situation (order of probing: 4p, 8p, 2p, 1p). The antibodies to Vma2p and Vma4p have strong nonspecific signals which proved to be useful as reference points for comparison of strains. What can clearly be seen is that all four proteins run in the higher band of the first dimension, designated complex II, and also run at second, separate locations lower in the first dimension. These data strongly suggest that 1p, 2p, 4p, and 8p form a complex together. Vma4p also runs at an anomalously high location and was designated complex III for reasons given below. We have designated all the complexes from this study with Roman numerals, counting down from the top of the native gel as indicated in Fig. 3. Complex I does not appear in detectable quantities in wild-type extracts and is therefore not indicated in Fig. 3.

To confirm that complex II was dependent on all four proteins, extracts from ∆vma1, ∆vma2, ∆vma4, and ∆vma8 strains were prepared and resolved on two-dimensional gels. Fig. 4A shows the first dimension from these strains. Complex II (Fig. 3) is completely missing from all four deletion strains. The Vma4p-containing complex III, which does not contain detectable amounts of either Vma1p or Vma2p, also disappears from the ∆vma1 and ∆vma2 strains, but is clearly evident in the ∆vma8 strain. We have noticed that this complex is unstable in the ∆vma1 and ∆vma2 strains, visible only after long exposure of the immunoblot. Analysis of our extracts by SDS-PAGE and immunoblot shows that Vma4p is being degraded during spheroplasting and lysis, rather than disassembling from complex III (data not shown). However, steady state levels of Vma4p in ∆vma1 and ∆vma2 strains are similar to wild-type (data not shown). We have been unable to detect Vma1p, Vma2p, Vma7p, or Vma8p in complex III, and this complex is present and stable in ∆vma7 or ∆vma8 deletion strains (see below). We think complex III may be multimerized Vma4p, which is somehow stabilized by Vma1p and Vma2p. This corroborates our observation from the three-hybrid system, that Vma4p can form a complex with itself, and that this complex is stabilized by Vma1p and Vma2p.

In ∆vma4, complex II is missing, but several new intermediate complexes have now appeared that are detectable with antiserum to Vma1p and/or Vma2p. Two of these have been designated complexes IV and V. Two-dimensional analysis reveals that complex IV contains Vma1p, Vma2p, and Vma8p (Fig. 4B). Complex V contains Vma2p, but not Vma1p or Vma8p. This seemed unusual, since complex V does not appear in either deletion strain. Long exposures of our blots have revealed very small amounts of this form of Vma2p in wild-type and deletion strains (data not shown). This suggests that this form of Vma2p is rapidly altered in a Vma4p-dependent manner, either by simple association or perhaps by direct or indirect modification of Vma2p. In any event, Vma4p is necessary for assembly of complex II, suggesting that complexes IV and V are intermediates leading to complex II.

Calculation of Molecular Weights—We wanted to know how molecular masses in kilodaltons are given to the left of each blot. The arrow at the right of the blot indicates the position of the protein in the second dimension. The arrows on the blot indicate the position of complex I and lower complexes and smears of the given subunit. Although the blots were stripped between antibodies, some residual antibody from the previous blot(s) does remain. Complex I is not detectable in wild-type extracts.

Fig. 3. ATPase complexes in wild-type yeast. Extracts were prepared and resolved on two-dimensional gels. Fig. 4A shows the first dimension from these strains. Complex II (Fig. 3) is completely missing from all four deletion strains. Complex II is missing, but several new intermediate complexes have now appeared that are detectable with antiserum to Vma1p and/or Vma2p. Two of these have been designated complexes IV and V. Two-dimensional analysis reveals that complex IV contains Vma1p, Vma2p, and Vma8p (Fig. 4B). Complex V contains Vma2p, but not Vma1p or Vma8p. This seemed unusual, since complex V does not appear in either deletion strain. Long exposures of our blots have revealed very small amounts of this form of Vma2p in wild-type and deletion strains (data not shown). This suggests that this form of Vma2p is rapidly altered in a Vma4p-dependent manner, either by simple association or perhaps by direct or indirect modification of Vma2p. In any event, Vma4p is necessary for assembly of complex II, suggesting that complexes IV and V are intermediates leading to complex II.
large these complexes are so that we might estimate the subunit composition. We used the Hedrick-Smith method (45) to calculate the molecular weights (Fig. 5 and Table II). Native gels ranging from 6 to 8.25% were run under identical conditions, and the relative mobilities of the complexes were measured. Standard proteins were run on the same gels, blotted, and stained with Amido Black. The relative mobilities were plotted as a function of gel percent (Fig. 5A, inset) and the negative slopes plotted as a function of molecular weight (Fig. 5A). The relative mobilities of our complexes were likewise plotted (Fig. 5B) and the molecular weights calculated from the standard curve. The molecular weights calculated from multiple experiments are compiled in Table II. Since Vma1p and Vma2p have proposed stoichiometries of three each per complex, this would indicate that complex II (calculated molecular mass of 576 ± 97 kDa) is large enough to be a 1p32p3 hexamer (predicted molecular mass of 375 kDa) with additional subunits, including Vma4p and Vma8p. Complex IV (calculated mass 317 ± 49 kDa) from the Dvma4 strain is too small to encompass a 1p32p3 hexamer, but may be a 1p22p2 tetramer (predicted weight of 249 kDa) with accessory subunits, including Vma8p. We have noticed a trend for our calculated molecular masses to be higher than expected. Calculations made on lower bands give no molecular mass closer than one and a half times the predicted mass of the subunit examined. Further experiments examining these lower molecular mass intermediates are underway. These results strongly suggest that Vma4p is necessary for assembly to a 1p32p34p8p XY complex (where X and Y indicate other as yet unidentified subunits).

Complex III, which contains Vma4p and perhaps other unidentified subunits, migrates with a molecular mass of 96 ± 28 kDa. If this is exclusively Vma4p, then this would indicate a complex of three subunits (predicted mass of 81 kDa). Although we cannot rule out other subunits at this time, we have seen no effect on this complex in other deletion strains tested (see below) and cannot detect the presence of Vma1p and Vma2p in the complex, even though its stability is dependent upon these subunits. The apparent weight of complex III further supports our hypothesis that Vma4p forms a homomultimer complex.

![Figure 4](http://www.jbc.org/)

**Fig. 4.** ATPase complexes in strains deleted for Vma1p, Vma2p, Vma4p, and Vma8p. A, immunoblot of single dimension native gel with extracts from Δvma1, Δvma2, Δvma4, and Δvma8 strains. Matching lanes are shown probed with antibodies to Vma1p, Vma2p, Vma4p, and Vma8p. The order of blotting was 2p, 1p, 4p, 8p, resulting in some residual signals in the Δvma4, Vma4p lanes. B, two-dimensional immunoblots of Δvma4 with Vma1p, Vma2p, and Vma8p antibodies. The order of blotting was: 4p (not shown), 8p, 2p, 1p. Some residual signals remain on the Vma1p blot.

![Figure 5](http://www.jbc.org/)

**Fig. 5.** Hedrick-Smith plot for calculation of complex sizes. A set of the standard proteins indicated were run on separate native gels, each of a different percent acrylamide. A, relative mobilities were measured and plotted as a function of gel percent (inset) and the negative slopes plotted as a function of molecular weight. B, extracts from wild-type, Δvma3, and Δvma4 strains were run on the same gels as in A, immunoblotted, and the relative mobilities plotted as a function of gel percent. The average molecular weights of multiple experiments are shown in Table II.
TABLE II

| Complex | Molecular mass (kDa) | Strains detected in | Detected by antibodies against |
|---------|---------------------|---------------------|-------------------------------|
| I       | Not determined      | Δvma3/Δvma7         | Vma1p, Vma2p, Vma4p, Vma8p    |
| II      | 576 ± 97 (7)*       | WT, Δvma3, Δvma5    | 1p, 2p, 4p, 7p, 8p            |
| III     | 96 ± 28 (4)         | All except Δvma4    | Vma4p                         |
| IV      | 317 ± 49 (6)        | Δvma4               | Vma1p, Vma2p, Vma7p, Vma8p   |
| V       | 156 ± 49 (4)        | Δvma4               | Vma2p                         |

*The values in parentheses are n, the number of experimental measurements.

\( V_0 \) is unnecessary for cytoplasmic assembly—We wished to know if complex II required a membrane sector for assembly. To answer this question, we examined a Δvma3 strain, which lacks the \( V_0 \) in our two-dimensional system. Fig. 6 shows immunoblots of the first dimension. Both complex II and the poorly resolved lower bands are present. Hedrick-Smith analysis indicates that this complex II is the same size as seen in the wild type. Two-dimensional analysis revealed the same composition as in wild-type extracts (data not shown). In the Δvma3 strain, all of the complex II signals are stronger than in wild-type, even though the total protein load was identical. We believe this is due to loss of membrane-bound complexes from the wild-type extracts, which are centrifuged to eliminate particulate matter before being loaded onto the native gel. We also see strong signals at the position of the gel-well from wild-type extracts, suggesting that membrane-bound material does not enter the gel. Since no complexes are membrane-bound in Δvma3, there is a larger pool of cytoplasmic complexes detected in this strain. This result also suggests that complex II does not contain any of the membrane subunits.

We further examined extracts from Δvph1, Δstv1, and the Δvph1Δstv1 double deletion strains. VPH1 and STV1 code for 96- and 102-kDa integral membrane proteins, respectively, which are part of the membrane complex and are believed to be responsible for targeting of the ATPase to specific organelar membranes (17, 18). In the absence of either or both of these proteins, complex II is seen in the native dimension (data not shown). Therefore, \( V_0 \) is unnecessary for assembly of complex II.

Involvement of Other Peripheral Subunits—We have examined several other peripheral subunits to see if they are necessary for formation of the large cytoplasmic complex. Fig. 7 shows immunoblots of the first dimension for extracts from Δvma5 and Δvma7. Vma5p is unnecessary for formation of complex II, although it has been demonstrated to be necessary for assembly of \( V_0 \) on the membrane. In the Δvma7 strain complex II vanishes, but is replaced by a weakly detectable complex I, higher in the gel, and a broad smear of Vma1p, which runs immediately below this complex. Because of the weak signal from complex I, we were unable to get complete data sets for Hedrick-Smith analysis. However, two-dimensional analysis reveals that it contains Vma1p, Vma2p, Vma4p, and Vma8p (data not shown). Hence, we suspect that complex I is a loose hexamer which is stabilized by Vma7p to form complex II.

To further examine complexes for the presence of Vma7p, we expressed an HA epitope-tagged version of Vma7p in our wild-type, Δvma3, and Δvma4 strains. This construct has been demonstrated to complement the Vma7p null mutation and hence to be competent for proper assembly into complexes (33). Fig. 8 shows immunoblots of extracts from Δvma3 and Δvma4 strains expressing the tagged Vma7p (order of blotting: HA, 4p, 1p, 8p, 2p). The signals from wild type were similar to Δvma3, but weaker due to loss of membrane-bound material (data not shown). The tagged protein can be seen with both complex II and complex IV, as indicated by the subsequent blot against Vma2p. Complex V is obscured by a nonspecific band which was seen in extracts not expressing the HA construct. No other bands were seen in the absence of the HA construct (data not shown).

DISCUSSION

The assembly of multisubunit enzymes from numerous different proteins is a fascinating and complex problem. We have
ATPase subunits are components of the complexes we have explored, and they are associated with Vma2p in this band. While our results do not exclude the possibility of interactions with other large proteins or protein complexes (e.g., chaperones), we have applied Occam’s razor and assumed that only these subunits, which we have not yet examined, are components of the complexes we have observed.

Based on our results, as well as those from other laboratories, we propose that the initial V1 assembly event is an interaction between the Vma1p and Vma2p proteins. Vma1p has been implicated as the ATP-hydrolyzing subunit of the ATPase complex (23, 24), while Vma2p is a nucleotide-binding protein, which is believed to be involved in regulation of the ATPase (25, 26). Both subunits have a stoichiometry of three per complex, based on comparison with the bovine clathrin-coated vesicle ATPase (43). From our two-hybrid results, it appears that these two subunits can interact with each other in the absence of other subunits. In addition, these subunits show homology to F-type ATPase/synthase subunits (46). The Vma1p is homologous to the catalytic β protein, whereas Vma2p is homologous to the α protein. From the crystal structure of the F-type enzyme, these proteins can be seen in close proximity and appear to interact (51).

The pattern exhibited by Vma2p in the native gel analysis is very complex. In addition to the complexes, including Vma2p, which we discussed in this study, we can also identify at least three species in the lower portion of the gel with strong signals and additional weak signals at several places in the gel. We believe that these are due to modifications of Vma2p which alter its charge-to-mass ratio. It has been shown that Vma2p is phosphorylated in both plant and animal systems (47, 48), and there is recent evidence that it is also phosphorylated in yeast. At least one modification may be dependent upon Vma4p, since an unusual band appears in gel-resolved extracts from the Δvma4 strain, with Vma2p as the only detectable component. It may also be the case that other subunits, which we have not explored, are associated with Vma2p in this band.

Recently, two laboratories have cloned VMA8 (34, 35), the product of which one laboratory has proposed is the homologue of the F-type γ subunit (34), although there is no significant sequence homology between these proteins. From the results of our native gel analysis, it is apparent that Vma8p is essential for the assembly of Vma1p-Vma2p into larger complexes. It may be, therefore, that Vma8p has some structural homology to γ. The issue remains open to debate.

Another candidate for γ is the Vma4p protein, which has been suggested for this role in Neurospora crassa (49). From our native gel analysis, it is clear that higher order complexes can form in the absence of this subunit. Some F-type ATPases are capable of forming higher order complexes from just α and β, but in general γ or δ is also necessary for this assembly (reviewed in Ref. 50). Therefore, by comparison with the F-type ATPases, Vma4p is an unlikely γ homologue. However, V-type ATPases are not F-type ATPases, and we believe that a search for one-to-one homologues is a faulty perspective on the situation. From crystal structures it can be seen that the F-type γ subunit has a coiled-coil as an essential structural motif (51). Coiled-coils are involved in protein-protein interactions, most notably in the leucine zipper motif. Both Vma4p and Vma8p are predicted to have high α-helical content. We envision a scenario where the structural role of γ is filled by two separate proteins, potentially Vma4p and Vma8p, each of which contribute a coil, and both of which are necessary for stable assembly of the complete Vma1p-Vma2p hexamer, which is believed to surround the coiled-coil structure.

The interaction of Vma4p with itself is supported by both our two-hybrid data and our native gel analysis, as well as the observation from other laboratories that this protein runs anomalously in glycerol gradients (12). Why Vma4p interacts with itself and if this is necessary for assembly remain interesting questions, to which we have no answers at this time. We cannot rule out the possibility that Vma4p interacts with proteins which we have not yet examined and that these proteins make up a substantial portion of complex III.

The addition of Vma7p is necessary for stabilization, but not formation, of the ATPase subcomplexes (complex II and IV). We are currently making the Δvma4vma7 double deletion to examine if complex IV has an unstable “7-less” counterpart. While complex II is stable in extract for over 90 min on ice, complex I is substantially diminished even after 30 min (data not shown, Fig. 8). Complex IV shows similar stability to complex II. The Vma7p subunit is interesting, since it has been shown to be necessary for assembly of both the V1 and the V0 complex, and it was therefore proposed that Vma7p may be involved in the attachment of V1 to V0 (33). Vma7p may serve as a multipurpose “clamp” to hold the V1 and V0 subcomplexes in stable conformations which may then interact.

As has been demonstrated previously, Vma5p is not necessary for the assembly of V0, although it has been shown to be necessary for assembly of the complete complex in yeast (12, 13). Examination of other subunits is currently under way. We suspect that Vma13p is unnecessary for the formation of complex II, since deletion of its gene does not eliminate the ability of the ATPase to assemble on the vacuolar membrane (41). Likewise Vma6p, which is a hydrophilic peripheral protein tightly associated with the integral membrane subunits and therefore regarded as a component of V0, is unlikely to be involved in the assembly of V1 (31).

Our current studies seek to characterize the components of complex II, which we consider to be the definitive V1 complex. This is the largest and most stable self-contained complex that can form independent of the membrane. Other subunits, such as Vma5p, which are soluble and cytoplasmic but not part of complex II, we would regard as accessory factors necessary for assembly.

Fig. 9. Model for cytoplasmic assembly of V1 complex. Roman numerals indicate complexes separated in the native gels. The asterisk indicates the Vma4p-dependent modified version of Vma2p seen unmodified as complex V in the native gels. A plausible alternative to this model would have the Vma4p-complex cycling with complex IV to render complex I.

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assembly or activity of the complete complex. We have also observed that our native gel system is extremely sensitive to modifications caused by glucose deprivation, which has recently been demonstrated to cause disassembly of the ATPase (52). We are initiating experiments to explore modification of the V₁ complex under stress conditions. We are also pursuing experiments to demonstrate that these complexes are not simply dead ends, but are bona fide intermediates in the assembly pathway. We hope, ultimately, to establish the order of intermediates in the assembly or activity of the complete complex. We have also recently been demonstrated to cause disassembly of the ATPase (52).

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