Early Steps in Assembly of the Yeast Vacuolar H\textsuperscript{+}-ATPase*

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Vacuolar proton-translocating ATPases are composed of a complex of integral membrane proteins, the V\textsubscript{o} sector, attached to a complex of peripheral membrane proteins, the V\textsubscript{1} sector. We have examined the early steps in biosynthesis of the yeast vacuolar ATPase by biosynthetically labeling wild-type and mutant cells for varied pulse and chase times and immunoprecipitating fully and partially assembled complexes under nondenaturing conditions. In wild-type cells, several V\textsubscript{1} subunits and the 100-kDa V\textsubscript{o} subunit associate within 3–5 min, followed by addition of other V\textsubscript{o} subunits with time. Deletion mutants lacking single subunits of the enzyme show a variety of partial complexes, including both complexes that resemble intermediates in the assembly pathway of wild-type cells and independent V\textsubscript{1} and V\textsubscript{o} sectors that form without any apparent V\textsubscript{1}V\textsubscript{o} subunit interaction. Two yeast sec mutants that show a temperature-conditional block in export from the endoplasmic reticulum accumulate a complex containing several V\textsubscript{1} subunits and the 100-kDa V\textsubscript{o} subunit during incubation at elevated temperature. This complex can assemble with the 17-kDa V\textsubscript{o} subunit when the temperature block is reversed. We propose that assembly of the yeast V-ATPase can occur by two different pathways: a concerted assembly pathway involving early interactions between V\textsubscript{1} and V\textsubscript{o} subunits and an independent assembly pathway requiring full assembly of V\textsubscript{1} and V\textsubscript{o} sectors before combination of the two sectors. The data suggest that in wild-type cells, assembly occurs predominantly by the concerted assembly pathway, and V-ATPase complexes acquire the full complement of V\textsubscript{o} subunits during or after exit from the endoplasmic reticulum.

Vacuolar proton-translocating ATPases (V-ATPases)\textsuperscript{2} are highly conserved proton pumps found in all euayctic cells (reviewed in Ref. 1). V-type ATPases couple hydrolysis of cytoplasmic ATP to transport of protons from the cytosol into internal organelles or, in certain cells, across the plasma membrane. The catalytic sites for ATP hydrolysis reside in a peripheral complex of subunits called the V\textsubscript{1} sector of the enzyme, and the proton pore appears to be contained within a complex of integral membrane and tightly bound peripheral subunits called the V\textsubscript{o} sector. V-ATPases have been implicated in constitutive physiological processes ranging from protein sorting to pH and calcium homeostasis to activation of lysosomal proteases (reviewed in Refs. 1–3).

The yeast V-type ATPase is composed of at least 13 different subunits, which have been identified by a combination of genetic and biochemical techniques (1, 4, 5). The V\textsubscript{1} sector of the yeast vacuolar ATPase is composed of a 69-kDa catalytic subunit, a 60-kDa subunit that appears to play a regulatory role, and six other peripheral subunits of relative molecular masses 54, 42, 32, 27, 14, and 13 kDa. The V\textsubscript{o} sector of the yeast enzyme consists of a 100-kDa integral membrane subunit, a tightly associated peripheral subunit of 36 kDa, and a trio of proteolipid subunits of 23, 17, and 16 kDa. The genes for all of these subunits have been cloned, and the subunit size, letter designation (for comparison to V-ATPases from other organisms), and gene names are listed in Table I. Deletion mutants lacking each of these subunits have been constructed. Deletion of any of these genes, except for the individual STV1 and VPH1 genes (6), results in a well defined set of Vma\textsuperscript{-} growth phenotypes, including failure to grow in medium containing high concentrations of calcium, pH conditional growth (the cells grow at pH 5, but fail to grow in medium buffered to above pH 7), and failure to grow on nonfermentable carbon sources (7, 8). The STV1 and VPH1 genes appear to encode functionally similar 100-kDa subunits present in different cellular locations; deletion of both genes is necessary to generate a full Vma\textsuperscript{-} phenotype (6).

The assembly of V-ATPases presents a fascinating set of problems. These enzymes are composed of a combination of membrane proteins that are believed to traverse at least portions of the secretory pathway and peripheral proteins that appear to be synthesized as cytoplasmic proteins and never enter the secretory pathway. V-ATPases reside in multiple organelles of eukaryotic cells, but correct targeting of these complexes is critical because functional assembly of a V-ATPase in an inappropriate location could fundamentally change the characteristics of that organelle by changing its internal pH. In addition, the assembly state of V-ATPases has been shown to be modified in response to extracellular conditions (9–11), indicating that V-ATPases are dynamic structures and assembly may be linked to regulation of enzyme activity.

The physical requirements for enzyme assembly have been probed by a number of different methods. The V\textsubscript{1} sector of the bovine clathrin-coated vesicle enzyme has been assembled from individual subunits derived from either heterologous expression systems or biochemical purification (12). The V\textsubscript{1}V\textsubscript{o} and V\textsubscript{1}V\textsubscript{o} complexes of the bovine enzyme have been reassembled from partial complexes obtained by dissociation of the enzyme in vitro (13–16). The yeast V-ATPase has also been reassembled from partial complexes in vitro (17, 18). Partial complexes formed in yeast mutants lacking single subunits of the enzyme have provided information about the extent of assembly possi-

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1 The abbreviations used are: V-ATPase, vacuolar proton-translocating ATPase; H\textsuperscript{+}-ATPase, proton-translocating ATPase; V\textsubscript{1}, peripheral sector of V-ATPase; V\textsubscript{o}, membrane sector of V-ATPase; ER, endoplasmic reticulum; CPY, carboxypeptidase Y; PCR, polymerase chain reaction.
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The nomenclature suggested by Stevens and Forgac (1) is followed.

| Apparent molecular mass | Gene | Letter designation |
|-------------------------|------|-------------------|
| 69                      | VMA1 | A                 |
| 60                      | VMA2 | B                 |
| 54                      | VMA3 | H                 |
| 42                      | VMA5 | C                 |
| 27                      | VMA8 |                  |
| 16                      | VMA10| G                 |
| 14                      | VMA7 | F                 |
| 100                     | VPH1 | a                 |
| 36                      | VMA6 | d                 |
| 23                      | VMA16| c                 |
| 17                      | VMA3 | c                 |
| 17                      | VMA11| c                 |

*The predicted molecular mass of Vma10p is 13 kDa, but it runs as a 16-kDa protein on SDS-polyacrylamide gels (55).

RESULTS

Materials—Zymolyase 10T and Tran[35S] label were purchased from ICN. Dithiothreitol (succinimidyl propionate) was obtained from Pierce. Molecular mass markers (high range) were obtained from Life Technologies, Inc. Restriction enzymes and other enzymes for molecular biology were purchased from New England Biolabs or Roche Molecular Biochemicals. The TA cloning system and pCRII plasmid were obtained from Invitrogen. Oligonucleotides were purchased from Genosys. All other reagents were purchased from Sigma.

Strain and Plasmid Constructions—Yeast strains used in this study and their genotypes are listed in Table II. When possible, isogenic mutant and wild-type strains were compared in the immunoprecipitations. The SF383-5Aa and SF383-1Da wild-type strains are closely related and were shown to give virtually identical results in immunoprecipitations. The vma6Δ mutant strain was constructed by first amplifying the VMA6 gene from yeast genomic DNA by PCR using oligonucleotides 5'-CGAGAAGGGGAAGGCTGAG-3' and 5'-CTCTGCAA-TAGCCACTCTAC-3' and Tq2 polymerase and inserting into the pCRII vector as directed by the manufacturer. Nucleotides 330 to +966, relative to the VMA6 open reading frame, were then replaced with the LEU2 marker by cleaving the VMA6-containing plasmid gene with SstI and ligating to an HpaI fragment containing the entire LEU2 gene. The disrupted vma6Δ::LEU2 fragment was released from the vector by digestion with Xbol and BamHI. The vma6Δ::LEU2 mutant strain was generated by the one-step gene replacement technique (21). Wild-type yeast cells were transformed with the linear vma6Δ::LEU2 fragment by the lithium acetate method (22), and transformants, initially identified by growth on minimal medium lacking leucine, were further screened for pH-sensitive growth to identify vma mutants. Disruption of the VMA12 gene was confirmed by PCR using genomic DNA from strains exhibiting a Vma- phenotype and the same oligonucleotides used for the original amplification of the VMA6 gene. Construction of the vma7Δ and vma10Δ strains was performed by similar methods. The VMA7 gene was amplified using oligonucleotides 5'-GGATCCGTAATACGG- TGGCT-3' and 5'-CGAGGCGGTCGTACGTCTCC-3'. The URA3 gene was used to replace nucleotides 39–360 of the VMA7 gene by fusion PCR, cloned into the pCRII vector, and the disrupted fragment was released from the pCRII vector by digestion with BamHI and XhoI. The VMA10 gene was amplified using oligonucleotides 5'-ACCTTTG- AATGCTTACAG-3' and 5'-GATAGTTGTAGTCCCTTGG-3'. The URA3 gene was used to replace nucleotides 3–336 of the open reading frame by fusion PCR, and the disrupted fragment was amplified and used directly for transformation. Disruption of the VMA7 and VMA10 genes in yeast was confirmed by PCR using genomic DNA as a template. For generation of the vma13Δ and vma12Δ strains, the VMA13 and VMA12 genes in plasmids pNUV4540 and pNUV410, respectively (a generous gift from Yusaku Anraku and Ryogo Hirata, University of Tokyo) were transferred to pBluescript KS+ (27). The 1085-base-pair BglII fragment of VMA13 was replaced with an HpaI fragment containing the LEU2 gene to form plasmid pYO6. A linear fragment containing the disrupted VMA13 gene was released by digestion with BamHI and SacII. The VMA12 gene was disrupted by digestion with EcoRI, treatment of the resulting fragment with T4 DNA polymerase to generate blunt ends, and ligation with an HpaI fragment containing the LEU2 gene to form plasmid pYO4. The vma12Δ::LEU2 disruption fragment was released by digestion with XhoI and SalI. Absence of the VMA13- and VMA12-encoded subunits in the deletion strains was confirmed by Western blot. A single Myc epitope tag was added to the C terminus of the VMA3 gene, immediately before the stop codon, by fusion PCR. Oligonucleotides 5'-CCCTTGAAGAATAGGTGTGGGACGAGCAACAC- TCTGGATAGGAC-3' and 5'-AGCTTTATTCGGAAGAAGCTTGA- AGCTTGAACATCTGTTTTACGTAAGC-3' were used for incorporation of the Myc tag and oligonucleotides 5'-GCAACATAAACACAGATGCC-3' and 5'-TAA- GGAGTGGATGAGGAGG-3' were used to amplify the VMA10 gene. All molecular biology manipulations were carried out as described (23).

Microscopy for growth of yeast strains was prepared as described by Sherman et al. (24). For immunoprecipitations, yeast strains were grown overnight in supplemented minimal medium lacking methionine that contained 2% dextrose. Cells were harvested, washed, converted to spheroplasts, and disrupted by osmotic shock. The spheroplasts were centrifuged at 25 °C. Spheroplasts from the sec1-1 strain were preincubated at 25 or 37 °C for 5 min before addition of the anti-Myc [35S] label and then labeled for 60 min at the same temperature. sec18-1 spheroplasts were preincubated for 5 min at 25 or 37 °C, labeled for 30 min at the indicated temperature, and then chased in the presence of excess methionine and cysteine. sec12-4 spheroplasts were preincubated for 5 min at 25 or 35 °C and then labeled for 30 min at the same temperature and chased. Immunoprecipitations were carried out using either 5 μl of purified 8B1 monoclonal antibody, 250 μl of SB1 cultured supernatant, or 250 μl of 13D11 cultured supernatant, each brought to a final volume of 500 μl with phosphate-buffered saline containing 5% bovine serum albumin, or 500 μl of 10D7 cultured supernatant. Carboxypeptidase Y (CPY) and the Myc epitope-tagged VMA3 were immunoprecipitated from labeled spheroplasts under denaturing conditions as described (27) using polyclonal antiserum against CPY (provided by Dr. Tom Stevens) and anti-Myc monoclonal antibody 9E10 (Santa Cruz Biotechnology), respectively. Immunoprecipitated samples were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described (19).

Results

Kinetics of Assembly of the Wild-type Yeast V-ATPase—We first addressed the early steps in assembly of the yeast V-ATPase by immunoprecipitating partial complexes of the ATPase following a brief pulse with Tran[35S] label and varied times of chase in the presence of excess unlabeled methionine and cysteine. Wild-type yeast cells were converted to spher-
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**TABLE II**

Genotypes of strains used in this study

| Strain          | Genotype                      | Source (Ref.) |
|-----------------|-------------------------------|---------------|
| SF838–5a       | MATα ura3-32 leu2-3,112 his4-519 ade6 | 50            |
| SF838–1D       | MATα ura3-32 leu2-3,112 his4-519 ade6 pep4-3 | 50            |
| SF838–1D ma1D  | MATα ura3-32 leu2-3,112 his4-519 ade6 pep4-3 vma1Δ::LEU2 | 51            |
| SF838–1D ma2A  | MATα ura3-32 leu2-3,112 his4-519 ade6 pep4-3 vma2A::LEU2 | 52            |
| SF838–5A ma13Δ | MATα ura3-32 leu2-3,112 his4-519 ade6 pep4-3 vma13Δ::LEU2 | This study |
| SF838–1D ma5A  | MATα ura3-32 leu2-3,112 his4-519 ade6 pep4-3 vma5Δ::LEU2 | This study |
| SF838–1D ma10Δ | MATα ura3-32 leu2-3,112 his4-519 ade6 vma10Δ::URA3 | 19            |
| SF838–5A ma7A  | MATα ura3-32 leu2-3,112 his4-519 ade6 vma7Δ::URA3 | 6             |
| MM112          | MATα ura3-32 leu2-3,112 his4-519 ade6 vma200 lyS2 Δstv1::LYS2 ade4::LEU2 | This study |
| SF838–5A ma6A  | MATα ura3-32 leu2-3,112 his4-519 ade6 vma6Δ::LEU2 | This study |
| SF838–1D ma3Δ  | MATα ura3-32 leu2-3,112 his4-519 ade6 vma3Δ::LEU2 | This study |
| SF838–5A ma12Δ | MATα ura3-32 leu2-3,112 his4-519 ade6 vma12Δ::LEU2 | This study |
| CJRY22–6Ba     | MATα ura3-32 leu2-3,112 his4-519 ade6 vma10Δ::URA3 | 37            |
| CJRY21–3Ba     | MATα ura3-32 leu2-3,112 his4-519 ade6 vma13Δ::LEU2 | 37            |
| MBY10–7A       | MATα ura3-32 leu2-3,112 his4-519 ade6 vma12Δ::LEU2 | 54            |

plasts, biosynthetically labeled for 3 min, and then solubilized under non-denaturing conditions in the presence of cross-linker as described previously (9). Partially or fully assembled complexes of the ATPase were then immunoprecipitated with monoclonal antibodies 8B1, 13D11, or 10D7, which recognize the 69-, 60-, and 100-kDa subunits, respectively (19). Previous experiments have demonstrated that the 8B1 and 13D11 monoclonal antibodies can recognize the individual subunits, partially assembled complexes, and the fully assembled V-ATPase (19). In contrast, the 10D7 monoclonal antibody recognizes a cryptic epitope that is exposed on the 100-kDa subunit alone or assembled as part of Vo complexes but is hidden when V1 subunits are bound to the Vo subunits (25). The results of the immunoprecipitations with monoclonal antibodies 8B1 and 13D11 are shown in Fig. 1. The amount of 69-kDa subunit and the amount of 60-kDa subunit immunoprecipitated by each of the specific antibodies appeared to be fairly constant during the 2–90-min chase times. During the chase time, however, the collection of V-ATPase subunits co-immunoprecipitated by the antibodies changes as subunits labeled during the 3-min pulse were incorporated into complexes with the 69-kDa subunit (Fig. 1A) and 60-kDa (Fig. 1B) subunits.

Several features of the results shown in Fig. 1 are notable. First, labeled V1 and certain Vo subunits, specifically the 100-kDa subunit, are co-immunoprecipitated to a large extent at very early times of chase, whereas the 17-kDa subunit, another Vo subunit, are co-precipitated to a large extent at very early times and then disappeared, as shown in Fig. 1. A number of other proteins are also co-precipitated by this antibody, but at present, we have focused on the subunits that are found as part of the intact V-ATPase or also co-precipitated by the anti-V1 subunit antibodies. The 10D7 antibody has been used in conjunction with the anti-V1 subunit antibodies to reveal the reversible disassembly of the yeast V-ATPase in response to changes in carbon source. In these experiments, assembled Vo sectors were co-immunoprecipitated by the antibody under conditions where the V1 and Vo sectors were disassembled, but the Vo sectors disappeared into the intact complex under conditions where the enzyme was fully assembled (9, 48). Significantly, we saw no disappearance of the V1 sectors immunoprecipitated by the 10D7 antibody during the 90-min chase time, suggesting that these V1 sectors are not being chased into V1Vo complexes.

**FIG. 1.** Kinetics of assembly of the yeast vacuolar H+-ATPase in wild-type cells. Wild-type (SF838-1D) yeast spheroplasts were biosynthetically labeled with Tran35S label for 3 min and then chased in the presence of excess unlabeled methionine and cysteine for the indicated times. The labeled spheroplasts were solubilized under non-denaturing conditions in the presence of dithiobis(succinimidyl propionate) cross-linker as described previously (9). The solubilized complexes were immunoprecipitated with subunit-specific monoclonal antibodies 8B1, which recognizes the 69-kDa subunit (A), or 13D11, which recognizes the 60-kDa subunit (B), followed by protein A-Sepharose. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The sizes of previously identified V-ATPase subunits and the 19-kDa protein discussed in the text are shown on the right.
but instead are forming an independent pool of \( V_o \) sectors.

The data in Fig. 2A suggest that the 17- and 36-kDa subunits may appear at somewhat earlier chase times in the \( V_o \) complexes immunoprecipitated by the 10D7 antibody than in the \( V_1V_o \) complexes immunoprecipitated by the 8B1 and 13D11 antibodies. We addressed this question directly for the 17-kDa subunit by comparing complexes immunoprecipitated from identical quantities of wild-type cells with the three antibodies after a 5-min pulse and 0- and 5-min chases. As shown in Fig. 2B, the anti-\( V_1 \) subunit antibodies coprecipitated the 69-, 60-, 32-, and 27-kDa \( V_1 \) subunits, the 100-kDa \( V_o \) subunit, and the 19-kDa protein after a 5-min pulse. The 17-kDa subunit appeared only after the subsequent 5-min chase. In contrast, the 10D7 antibody coprecipitated the 17-kDa subunit with the 100-kDa subunit at both chase times. These results indicate that the labeled 17-kDa subunit is assembling more rapidly with the free \( V_o \) sectors immunoprecipitated by the 10D7 antibody than with the \( V_1V_o \) complexes. Equally important, these results emphasize that the \( V_o \) subunits present in \( V_1V_o \) complexes and the \( V_o \) subunits in “free \( V_o \) sectors” immunoprecipitated by the 10D7 antibody are distinct subpopulations; the free \( V_o \) complexes do not necessarily arise by disassembly of the \( V_1V_o \) complexes during isolation.

In order to have a second probe of \( V_o \) subunit assembly, we constructed an epitope-tagged version of the \( VMA3 \) gene. This gene encodes the most abundant of three proteolipid subunits that are part of the yeast vacuolar \( H^+ \)-ATPase (45). Placement of the Myc epitope tag just before the stop codon yielded a Myc-\( VMA3 \) construct that was able to fully complement the growth defects of a \( vma3 \) mutant strain (data not shown). We were also able to immunoprecipitate a protein of slightly greater than 17 kDa, reflecting the size of the tag, using the Myc antibody under denaturing conditions (Fig. 2C). The Myc antibody did not immunoprecipitate this protein or any other \( V_1 \)-ATPase subunits under nondenaturing conditions, however. This limited the utility of this construct for assembly studies, but it did allow us to identify unambiguously the 17-kDa band seen in the non-denaturing immunoprecipitations as the \( VMA3 \) gene product. As shown in Fig. 2C, a protein of the same size as the Myc-\( VMA3 \) immunoprecipitated by the anti-Myc antibody under denaturing conditions was coprecipitated by both anti-\( V_1 \) and anti-\( V_o \) subunit antibodies under non-denaturing conditions, and the 17-kDa band disappeared under these conditions.

Assembly of Partial Complexes in Mutants Lacking One Subunit—We previously examined the extent of assembly of the yeast vacuolar \( H^+ \)-ATPase in a number of mutants lacking one subunit of the enzyme (19). The results showed that the \( V_1 \) and \( V_o \) sectors could assemble separately, leading us to speculate that assembly of the enzyme in wild-type cells might occur by combination of preassembled \( V_1 \) and \( V_o \) sectors. At the time those experiments were done, a rather limited collection of deletion mutants was available, and the immunoprecipitations were performed in the absence of cross-linker, so unstable complexes containing both \( V_1 \) and \( V_o \) subunits may not have been identified. In light of the kinetic data shown in Figs. 1 and 2, we examined whether the deletion mutants might contain partial \( V_1V_o \) complexes that were not detected previously. The extent of assembly was measured in a more complete set of deletion mutants using the conditions of the experiments shown in Fig. 1, including cross-linker to stabilize weakly bound complexes. The results of immunoprecipitation following a 60-min pulse with Tran\(^{35}S\) label and no chase are shown in Fig. 3. Panels A and B of Fig. 3 show the complexes immunoprecipitated by the 8B1 (anti-69-kDa subunit) and 13D11 (anti-60-kDa subunit) antibodies, respectively. Assembled \( V_1 \)-ATPase complexes, containing both \( V_1 \) and \( V_o \) sector subunits, were immunoprecipitated by both antibodies from wild-type cells after the 60-min pulse. The \( vph1 \Delta \) strain, \( vma3 \Delta \), \( vma6 \Delta \), \( vma7 \Delta \), and \( vma12 \Delta \) mutants appear to contain core \( V_1 \) complexes, containing minimally the 69-, 60-, 32-, and 27-kDa subunits,
Spheroplasts were prepared from wild-type cells and mutants lacking individual subunits of the V-ATPase. The 100-3A, 3A, and 6A strains lack the following V1 subunits: two 100-kDa subunit isoforms (100A, 100B), one of the 17-kDa subunits (vma3A), and the 36-kDa subunit (vma6A), respectively. The 4A, 5A, 7A, 13A, 10A, 8A, 2A, and 1A strains lack the 27-, 42-, 14-, 54-, 16-, 32-, 60-, and 69-kDa Vo subunits, respectively. The 12A strain lacks a 25-kDa assembly factor that is not part of the final V-ATPase complex. Full genotypes of all of the strains are given in Table II. The wild-type strain shown is SF838-1D, but wild-type strain SF838-5A gave virtually identical results. The spheroplasts were labeled for 60 min with Tran35S label and then solubilized under non-denaturing conditions and cross-linked with dithiobis(succinimidyl propionate). Partial complexes were immunoprecipitated with monoclonal antibodies 8B1, recognizing the 69-kDa subunit, and 13D11, recognizing the 60-kDa subunit. Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The positions of ATPase subunits are indicated.

without attached Vo subunits. The vma10Δ mutant, which lacks the 13-kDa V1 subunit and as a result has lowered levels of the 27-kDa subunit (27), contains a partial complex consisting of the 100-, 69-, 60-, and possibly the 32-kDa subunits (the 32-kDa subunit appears to be coprecipitated only by the 8B1 antibody). The vma6AΔ mutant, which lacks the 32-kDa V1 subunit, may contain a similar complex containing the 27-kDa subunit (again immunoprecipitated by only one of the anti-V1 subunit antibodies) instead of the 32-kDa subunit. The vma6AΔ mutant, which lacks the 27-kDa V1 subunit, contains an even smaller complex that appears to include the 69- and 60-kDa subunits but lacks the 100- and 32-kDa subunits. The vma5AΔ and vma13AΔ mutants, which lack the 42- and 54-kDa V1 subunits, respectively, show some level of assembly of V1 with all of the Vo subunits, as indicated by co-precipitation of the 100-, 36-, and 17-kDa subunits with the V1 subunits. These complexes may be rather unstable because the V1 subunits appear to be at low levels in the immunoprecipitations from vma13AΔ cells, and only the 13D11 antibody could co-precipitate the V1 subunits from vma5AΔ cells. These results are consistent with previous experiments (18, 19, 26, 27) in that a number of the mutants can assemble a core V1 sector (30, 31). Taken together, these results indicate that V1 and Vo sectors can assemble independently in mutants lacking one subunit, even though assembly does not appear to occur via independent assembly of the two sectors when all of the subunits are present (Figs. 1 and 2).

Figs. 1 and 2 show interactions between V1 and Vo sector subunits at very early time points. We reasoned that some of the mutant strains might form similar complexes at early stages of assembly but later dissociate these complexes because absence of a subunit prevents assembly from proceeding to formation of a stable, fully assembled complex. This question was addressed by examining the complexes formed in the deletion mutants under conditions similar to those used for wild-type cells in Fig. 2B (5-min pulse and a 0- or 5-min chase). The results of immunoprecipitation with the 13D11 antibody are shown in Fig. 4. There is evidence of assembly of the V1 subunits in almost all of the mutant strains, and even the vma4Δ, vma5Δ, vma8Δ, vma10Δ, and even the vma1Δ mutants show some level of co-precipitation of the 100-kDa Vo subunit. The vma5Δ, vma8Δ, and vma10Δ mutants show some assembly of the V1 subunits after a 60-min pulse, so it was not surprising that some interactions could be detected at earlier times. The vma4Δ mutant did not show interactions between V1 and Vo sectors after a 60-min pulse (Fig. 3), but did appear to show some interaction at these early times of assembly. The
Early steps of assembly in mutants lacking one subunit of the yeast vacuolar H\(^+\)-ATPase. Wild-type (SF38-1D\(a\)) and a subset of the mutant strains indicated as described in Fig. 3 were converted to spheroplasts and then labeled for 5 min with Tran\(^{[35S]}\) label. An excess of methionine and cysteine was added, and the cells were either solubilized immediately (0 min chase) or chased for an additional 5 min (5 min chase). All of the samples were immunoprecipitated with the 13D11 monoclonal antibody, which recognizes the 60-kDa subunit, followed by protein A-Sepharose. Immune complexes were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography, and the positions of known V-ATPase subunits are indicated.

100-kDa subunit was present at levels in the complexes from vma43 cells comparable to wild-type after the 5-min chase, and the 17-kDa subunit was also visible at this time. These results suggest that the vma43 mutant starts to assemble the V-ATPase by a pathway similar to that seen in wild-type cells, but loses interaction between the V\(_1\) and V\(_o\) sectors at later times of assembly. In contrast, the vma32 mutant showed no evidence of early interactions between V\(_1\) and V\(_o\) sectors, but was still able to proceed with assembly of the V\(_1\) sector. These results indicate that early interactions between the 100-kDa subunit and V\(_1\) subunits are not essential for V\(_1\) assembly, and there must, therefore, be multiple potential routes for V\(_1\) sector assembly.

Assembly of the Yeast Vacuolar H\(^+\)-ATPase in sec Mutants—The pulse-chase studies described above provide an initial picture of the steps in V-ATPase assembly but do not provide any information about where in the cell the various stages of assembly might occur. Previous studies have demonstrated that three resident proteins of the ER, Vma12p, Vma21p, and Vma22p, are essential for assembly of the yeast V-ATPase (28, 32, 33) and that at least the 100-kDa V\(_o\) subunit must pass through the ER (29) and a post-Golgi, prevacuolar compartment (34) en route to the vacuole. Recent results indicate that V\(_o\) assembly occurs in the ER (49), but do not address where subunits of the V\(_1\) and V\(_o\) sectors of the V-ATPase begin to interact or the possibility of distinct free V\(_1\) and V\(_o\) subpopulations raised by the results described above. We attempted to obtain a preliminary correlation of assembly and transport of the V-ATPase to the vacuole by examining the extent of assembly of the V-ATPase in several yeast sec mutants blocked at various steps in transport through the secretory pathway (35, 36). The yeast sec mutants are all temperature-sensitive mutants that allow normal transport through the secretory pathway at the permissive temperature (usually 25 °C) but exhibit an arrest in transport at elevated temperature. Epistasis studies of these mutants and their subsequent molecular characterization indicate that they arrest transport at different stages in the secretory pathway (35, 36). sec1-1 is a late-acting sec mutant that arrests vesicle fusion with the plasma membrane but does not affect the transport of soluble or membrane proteins to the vacuole (36–38). sec12-4 is an early acting sec mutant that prevents exit of newly synthesized secreted and vacuolar proteins from the ER (35, 38). sec18-1 acts at multiple places in the secretory pathway but exhibits an early block (similar to that seen in sec12-4 cells) in exit of newly synthesized secreted and vacuolar proteins from the ER (35, 36). As shown in Fig. 5A, the sec1-1 mutant assembled both fully assembled V-ATPase complexes, immunoprecipitated by the 8B1 and 13D11 antibodies, and free V\(_o\) complexes, immunoprecipitated by the 10D7 antibody, at both the permissive and nonpermissive temperatures. These complexes were comparable to those formed in wild-type cells (Fig. 5A). sec18-1 mutant cells also assembled an apparently wild-type V-ATPase when incubated at 25 °C for 30 min (Fig. 5B). However, labeling at the nonpermissive temperature for 30 min resulted in accumulation of complexes that contained the 100-kDa subunit and V\(_1\) subunits but no 17-kDa subunit. There was relatively little difference in the V\(_o\) complexes formed at
the permissive or nonpermissive temperature. Very similar results were observed in a sec12-4 mutant (data not shown).

Some of the sec mutants have been shown to be reversible; transport intermediates formed at elevated temperature can be chased through to their normal location when the mutants are returned to the permissive temperature (35). In order to distinguish whether the complexes formed at elevated temperature in the sec18-1 mutants were true assembly intermediates or “dead-end” complexes resulting from a compromised secretory pathway, we determined whether these complexes could go on to assemble with the 17-kDa subunit during a subsequent chase period at 25 °C. As shown in Fig. 5B, the V1 subunit-containing complexes formed at elevated temperature in sec18-1 mutants can combine with the labeled 17-kDa subunit during a subsequent 30-min chase at 25 °C but do not assemble with the 17-kDa subunit if the cells are chased at 37 °C. A similar pattern was seen in the sec12-4 mutant (data not shown).

Both the arrest of transport from the ER and the reversal of the arrest with the return to the permissive temperature were confirmed by immunoprecipitating the soluble vacuolar protein carboxypeptidase Y from the same cells shown in Fig. 5. A and B. CPY exhibits easily distinguishable forms, characteristic of organelle-specific posttranslational modifications, as it transits from the ER (p1 form) to the Golgi apparatus (p2 form) to the vacuole (mature form) (38, 47). As expected, sec1 mutant cells exhibit all three forms of CPY, representing newly synthesized CPY at various stages in transport, at both the permissive and nonpermissive temperatures (Fig. 5C). sec18 mutant cells exhibit all three forms after a 30-min labeling at the permissive temperature but accumulate the p1 form, indicating an ER block, at the nonpermissive temperature. Significantly, the accumulated p1 form can be converted to the p2 and m forms during a chase at the permissive, but not the nonpermissive, temperature. The results suggest that during the 30–60-min incubation at elevated temperature, we stabilized a genuine assembly intermediate in the sec18 mutant, similar to intermediate complexes seen at very early (0–2 min) chase times in wild-type cells. Furthermore, this intermediate appears to be competent for full assembly of the V-ATPase when the sec block is released.

There is a potential question of whether wild-type complexes lacking the labeled 17-kDa subunit at early time points (Figs. 1 and 2B) are true assembly intermediates or merely complexes containing unlabeled 17-kDa subunit. The results with the sec mutants argue that these complexes are true intermediates that become competent for assembly with the 17-kDa subunit at a well-defined stage of transport. These results do not support an exchange of the 17-kDa subunit from the V0 complexes formed at elevated temperature to the V1V0 complexes but instead suggest that full assembly of the free V0 sectors can occur before these early sec blocks.

**DISCUSSION**

**Multiple Assembly Pathways for the Yeast Vacuolar H+-ATPase**—The methods used here have allowed us to identify new features of the assembly of the yeast V-ATPase. Their greatest strength is the ability to follow the kinetics of biosynthesis in vivo. A number of potential assembly intermediates present at steady state have been identified and demonstrated to form interactions necessary for assembly in vitro, but these experiments do not directly address the early steps of biosynthesis. We have temporally resolved the incorporation of two V1 and one V0 subunit into complexes and achieved a picture of both V1V0 and V0 sector assembly in vivo. Based on the results of these experiments, in combination with previous experiments, we propose that assembly of the yeast vacuolar H+-ATPase, and probably other V-type ATPases, may proceed through at least two pathways, shown schematically in Fig. 6.

The central feature of the model in Fig. 6 is that cells have the capacity to assemble the vacuolar H+-ATPase either by forming early interactions between V1 and V0 subunits and then adding to both sectors (concerted V1V0 assembly) or by combining preassembled V1 and V0 sectors, possibly with addition of one or more subunits that are not initially present in either sector (independent assembly of V1 and V0).

The results presented here argue that in wild-type cells, in which all of the subunits of the enzyme are present, biosynthetic assembly of the V1V0 complex occurs predominantly by the concerted pathway, with early interactions between V1 subunits and at least the 100-kDa subunit of the V0 sector, followed by a gradual addition of the 17- and 36-kDa subunits to the V0 sector. Labeled 17- and 36-kDa subunits are incorporated at earlier times into the free V0 complexes (Fig. 2B), suggesting that the free V0 complexes are being formed shortly after synthesis of the individual subunits. It is possible that the 17- and 36-kDa subunits are incorporated at later times in assembly of V1V0 complexes or that an unlabeled pool of the 17- and 36-kDa subunits is preferentially recruited to V1V0 complexes with the newly synthesized 100-kDa subunit. However, the second possibility was eliminated by the experiments shown in Fig. 5, which strongly support the concerted model of assembly. The one piece of data that initially appears to be inconsistent with this model is the failure of the 100-kDa subunit to bind to the V1 subunits at early times in mcmA Δ mutant. It has been shown, however, that loss of one V0 subunit destabilizes the other V0 subunits (25, 41) and could potentially generate other long-term changes in the cell, for example, loss of an assembly factor that affects the behavior of the 100-kDa subunit in assembly.

We also have evidence of assembly of a pool of free V0 sectors, consistent with the independent assembly pathway in Fig. 6, but we have no evidence that these sectors go on to form fully assembled V1V0 complexes in wild-type cells. As described above, we saw a pool of free V0 sectors that can be immunoprecipitated by the 10D7 antibodies and that is not chased into intact V-ATPase complexes with increasing time. Although we cannot readily address whether there are free V0 sectors being formed simultaneously by the methods used here, there is ample evidence of a pool of free V1 sectors in the cytosol of
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wild-type yeast cells (18, 19, 26). The assembly pathway of these free \( V_1 \) sectors has been examined in detail by Tomashek et al. (18). In certain deletion mutants, independent assembly of \( V_1 \) and \( V_o \) sectors appears to become the predominant assembly pathway. Depending on the subunit that is missing, cells can proceed to assemble either \( V_1 \) or \( V_o \) sectors with no evidence of early interaction between subunits of the two sectors. Under certain circumstances, for example, in the \( uma4\Delta \) mutant, the initial steps of concerted \( V_1V_o \) assembly appear to occur, but the complexes formed are then lost, apparently because a subunit missing in the mutant is essential for stabilization of the complexes.

Although we did not see intact V-ATPase complexes forming by the independent assembly pathway in our studies, there is substantial evidence that the free \( V_1 \) and \( V_o \) sectors contain the structural information necessary for assembly. Using two different \textit{in vitro} reconstitution systems, \( V_o \) sectors assembled at the vacuole in \( uma4\Delta \) cells were shown to be competent for structural interaction with \( V_1 \) sectors (17, 18) and activation of ATPase activity in \( uma4\Delta \) cells (19, 20). In certain deletion mutants, independent assembly of \( V_1 \) and \( V_o \) sectors appears to become the predominant assembly pathway (18). In certain deletion mutants, independent assembly of \( V_1 \) and \( V_o \) sectors occurs during exit from the ER or in a subsequent compartment in wild-type cells. In the \textit{sec12-4} and \textit{sec18-1} mutants at the nonpermissive temperature, the assembly intermediate that accumulated after 30 min resembled the complexes present in wild-type cells at the 2–5-min chase times (Fig. 1). Based on this evidence, we would suggest that attachment of the 100-kDa subunit to \( V_1 \) subunits in the concerted pathway takes place before the \textit{sec18-1} block but that acquisition of the 17-kDa subunit takes place later in transport to the vacuole.

In the course of these experiments, we have also biochemically identified a new protein, with a molecular mass of approximately 19 kDa, that exhibits properties expected of an assembly factor (specifically, transient appearance at early stages of both \( V_1 \) and \( V_o \) sector assembly). We do not yet know the identity of this protein. Polyclonal antibodies against Vma12p, a previously identified assembly factor (28), did not immunoprecipitate the 19-kDa protein. The 19-kDa protein seems to disappear as the 17-kDa protein appears in the \( V_V_1 \) and \( V_o \) complexes, but it is not an unprocessed form of the 17-kDa subunit because it does not undergo a molecular mass shift when Vma3p is epitope-tagged (data not shown). It is still possible that the 19-kDa protein represents one of the other proteolipid subunits (Vma11p or Vma16p) (45), but the best evidence available suggests that both of these protein are part of the final V-ATPase complex. Further experiments will be necessary to identify this protein.

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