Stoichiometry of the Peripheral Stalk Subunits E and G of Yeast V$_1$-ATPase Determined by Mass Spectrometry*

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Norton Kitagawa‡§, Hortense Mazon†, Albert J. R. Heck§, and Stephan Wilkens¶

From the ‡From the Department of Biochemistry and Molecular Biology, State University of New York Upstate Medical University, Syracuse, New York 13210, the §Department of Cell, Molecular and Developmental Biology, University of California, Riverside, Riverside, California 92521, and the ¶Department of Biomolecular Mass Spectrometry, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, 3584 CA Utrecht, The Netherlands

The stoichiometry of yeast V$_1$-ATPase peripheral stalk subunits E and G was determined by two independent approaches using mass spectrometry (MS). First, the subunit ratio was inferred from measuring the molecular mass of the intact V$_1$-ATPase complex and each of the individual protein components, using native electrospray ionization-MS. The major observed intact complex had a mass of 593,600 Da, with minor components displaying masses of 553,550 and 428,300 Da, respectively. Second, defined amounts of V$_1$-ATPase purified from yeast grown on $^{14}$N-containing medium were titrated with defined amounts of $^{15}$N-labeled E and G subunits as internal standards. Following protease digestion of subunit bands, $^{14}$N- and $^{15}$N-containing peptide pairs were used for quantification of subunit stoichiometry using matrix-assisted laser desorption/ionization-time of flight MS. Results from both approaches are in excellent agreement and reveal that the subunit composition of yeast V$_1$-ATPase is A$_3$B$_3$DE$_2$FG$_3$H.

Vacuolar ATPases (V-ATPases, V$_1$V$_0$-ATPases) are ATP hydrolysis-driven proton pumps found in the endomembrane system of eukaryotic organisms, where they function to acidify the interior of subcellular organelles such as lysosomes, early and late endosomes, clathrin-coated vesicles, the Golgi, the plant tonoplast, and the yeast vacuole (1–4). In higher organisms, the V-ATPase complex can also be found in the plasma membrane of polarized cells involved in acid secretion such as the ruffled membrane of bone osteoclasts or the apical membrane of renal intercalated cells. The vacuolar ATPase is a large, multisubunit complex, which can be divided into a water-soluble ATPase domain and a membrane-bound proton pore. The two domains are termed V$_1$ and V$_0$, respectively, in analogy to the F$_1$ and F$_0$ of the related F$_1$F$_0$-ATP synthase. In yeast, the V$_1$-ATPase domain contains subunits AB(C)DEFGH, whereas the membrane-bound V$_0$ is made of subunits acc$c'e'de$. Much like the F-ATP synthase, the V-ATPase is a rotary molecular motor enzyme (5, 6); ATP hydrolysis taking place on the A subunits of the A$_3$B$_3$ catalytic domain is coupled to proton translocation across the membrane domain via rotation of a central stalk made of subunits D, F, and d and a proteolipid ring (subunits c, c’, and c”). The remaining subunits C, E, G, and H are involved in forming a peripheral stator domain that provides a structural link between the catalytic domain (A$_3$B$_3$) and the membrane-bound a subunit. In the related F-ATP synthase, it is now well established that there is a single peripheral stalk, which, in the case of the bacterial enzyme, is formed by two copies of the membrane-anchored b subunits and the 6 subunit (7). The situation in the vacuolar ATPase, however, is more complicated in that there appear to be multiple peripheral stalks that connect the catalytic domain to the membrane-bound a subunit, possibly via the V-ATPase-specific H and C subunits. Using electron microscopy and single particle image analysis, we have previously shown that the C and H subunits are positioned in the interface connecting the V$_1$ and V$_0$ where they are connected to the A$_3$B$_3$ domain via elongated protein densities bound at the periphery of the B subunits (8–10). There is evidence that these elongated proteins densities are formed by the E and G subunits. First, it has been shown that these two subunits are able to form an elongated, heterodimeric complex with equimolar stoichiometry (11), and second, chemical cross-linking from cysteines on the surface of the B subunits indicates close proximity to both E and G subunits from residues distributed between the bottom of the V$_1$ and the very top (12, 13). Recently, Kane and co-workers (14) have shown that there are at least two E and two G subunits per V$_1$-ATPase complex. However, based on electron microscopic images of the intact V-ATPase and the isolated V$_1$-ATPase domain, we had speculated earlier that the number of peripheral stalks might be three as, especially in images of the intact V-ATPase, each of the three B subunits seemed to have an elongated protein density bound at its periphery (8–10).

V-ATPase activity is regulated in vivo by a reversible dissociation and reassociation mechanism, first described for the enzymes from yeast and insect (15, 16) but now also observed for the V-ATPase of animal cells (17). During dissociation, the interaction between soluble and membrane domains involving the peripheral stalks has to be broken, and as a result of that

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‡ To whom correspondence should be addressed. Tel.: 315-464-8703; Fax: 315-464-8750; E-mail: wilkenss@upstate.edu.

§ The abbreviations used are: V-ATPase, vacuolar ATPase; V$_1$, proton-pumping vacuolar ATPase; V$_0$, water soluble domain of the vacuolar proton-pumping ATPase; V$_{m}$, membrane-bound domain of the proton-pumping vacuolar ATPase; MS, mass spectrometry; MALDI, matrix assisted laser desorption ionization; TOF, time-of-flight; CV, column volumes; MBP, maltose-binding protein.
process, subunit C dissociates from the separated V1 and V0 (18). To be able to understand the structural mechanism of enzyme dissociation, knowledge of the number of peripheral stalks and the nature of their interaction with the other subunits of the stalk domain and the complex are essential. We therefore decided to determine the copy number of the E and G subunits in the yeast V1-ATPase by two different mass spectrometry approaches. First, native electrospray ionization time-of-flight mass spectrometry was used to obtain a measurement of the molecular mass of the intact V1-ATPase complex that was accurate enough so that the subunit stoichiometry could be deduced. Second, we used known amounts of isotope-labeled subunits as internal standards to directly determine the copy number of subunits E and G in yeast V1. Results from both approaches were in excellent agreement and indicated that yeast V1-ATPase complex contains three copies each of the E and G subunits. Based on this result and our earlier electron microscopy images, we propose a structural model of the complex in which three peripheral stalks, via interaction with C, H, and A subunits, connect the V1 and V0 domains in intact yeast vacuolar ATPase.

EXPERIMENTAL PROCEDURES

**Yeast V1-ATPase Purification—**V1-ATPase was purified from *Saccharomyces cerevisiae* strain SF838-5A vma10a::kanMX expressing a FLAG-tagged VMA10 in plasmid pRS315 (CEN6, LEU2) as described previously (19). Briefly, one colony of the FLAG-Vma10p yeast strain was transferred into 5 ml of liquid YPD in a fermenter (Electrolab). Yeast was grown to an OD600 of 4–5 and then transferred into 8 liters of SD leucine-dropout medium. The inoculum volume was gradually increased to 250 ml and then transferred into 8 liters of YPD in a fermenter (Electrolab). Yeast was grown to an OD600 of 8–10. Cells were harvested via low speed centrifugation. The cell pellet was resuspended in TBS (50 mM Tris, 150 mM NaCl, pH 7.4) at 1:1 w/v and frozen overnight at −20 °C. Frozen cell pellet was thawed in room temperature water, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride) and 1 mM dithiothreitol were added. Cells were lysed with 10 passes through a M110-L Microfluidizer (Microfluidics Corp.) cell disruptor. An additional 1 mM phenylmethylsulfonyl fluoride was added after the final pass. Crude lysate was centrifuged for 1 h at 250,000 × g, 4 °C, and the supernatant was passed over a 5-ml anti-FLAG M2 column (Sigma). The column was washed with 30 column volumes (CV) of TBS and eluted with 3 CV of TBS containing 100 mM NaCl, [15N]ammonium chloride (Spectra Stable Isotopes). 1 liter of culture was grown at 37 °C to an A995 = 0.6, at which point the temperature was lowered to 16 °C. After 1 h, culture was induced with 1 mM isopropyl-1-thio-β-d-galactopyranoside overnight at 16 °C. Cells were harvested by low speed centrifugation, resuspended in Column Buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7) up to a final volume of 25 ml, and frozen overnight at −20 °C. Frozen cell pellet was thawed in room temperature water and treated with 1 μg/ml lysozyme and 10 μg/ml DNase I for 30 min on ice with gentle, intermittent shaking. Cells were sonicated for three cycles of 30 s on, 30 s off at 50% power using a Virtis VirSonic 100 sonicator and then centrifuged at 15,000 × g to clarify lysate. Lysate was diluted 1:5 in Column Buffer and applied to a 20-ml amylose resin column (New England Biolabs) at −1 ml/min, washed with 30 CV of Column Buffer, and then eluted in 1.5 CV of Column Buffer containing 10 mM maltose. Fractions containing MBP fusion protein were dialyzed in DEAE binding buffer (20 mM Tris, pH 8) overnight at 4 °C. MBP fusion was then passed over a 5-ml DEAE column, washed with 10 CV of DEAE binding buffer, and then eluted with a linear gradient from 0 to 100 mM NaCl over 40 CV. Fractions containing clean fusion protein were pooled and concentrated to a volume of 1 ml using Vivaspin 20 concentrator columns with a 50-kDa molecular mass cut-off. Fusion protein was then digested using PreScission protease (GE Healthcare) and 5 mM dithiothreitol overnight at 4 °C. E subunit was separated from MBP by gel filtration (Superdex 75 HR 16/50) in TBS. E subunit-containing fractions were pooled, aliquoted, and stored in liquid N2 for storage.

**Cloning of Yeast V-ATPase Subunits E and G—**An Escherichia coli E subunit expression construct was generated as a fusion with maltose-binding protein (MBP) by subcloning the wild-type VMA4 open reading frame (minus the N-terminal Met) from *S. cerevisiae* genomic DNA into a pMAL-c2e vector (New England Biolabs) with a PreScission protease cleavage site in place of the stock enterokinase site, using the following primers: pM4, forward, 5′-GACAAGTACCCTCCGGCCTAT-TACTGTTTTCAG-3′, and pM4, reverse, 5′-GTGCCAACCTCCTAATCAAGGACGACGATGA-3′. Forward and reverse primers contained KpnI and HindIII digest sites, respectively. The resulting MBP fusion construct, pM4, was confirmed by DNA sequencing. pM4 construct was transformed into Rosetta 2 (DE3) *E. coli* cells (Novagen) and plated on LB agar containing ampicillin and chloramphenicol. An *E. coli* FLAG-tagged G subunit expression construct was generated by subcloning the FLAG-tagged VMA10 open reading frame from the previously described pRS315 construct (19) into the first multiple cloning site of a pET-Duet-1 vector (Novagen), using the following primers: pDuet1G, forward, 5′-GATAAACAT-GGACTACAAGGACGACGATGA-3′, and pDuet1G, reverse, 5′-CATATTGCGGGCGTTACAAAGCCATTGATGACTTCAG-3′. Forward and reverse primers contained NcoI and NotI digest sites, respectively. The resulting construct, pG, was confirmed by DNA sequencing. pG construct was transformed into Rosetta 2 (DE3) *E. coli* cells (Novagen) and plated on LB agar containing ampicillin and chloramphenicol.

**Protein Expression and Purification—**Single colonies of pM4-expressing cells were picked and grown overnight at 37 °C in a 25-ml inoculum of LB containing antibiotics. The entire 25 ml was used to inoculate 1 liter of M9 medium containing 1 g of [15N]ammonium chloride (Spectra Stable Isotopes). 1 liter of culture was grown at 37 °C to an A995 = 0.6, at which point the temperature was lowered to 16 °C. After 1 h, culture was induced with 1 mM isopropyl-1-thio-β-d-galactopyranoside overnight at 16 °C. Cells were harvested by low speed centrifugation, resuspended in Column Buffer (20 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7) up to a final volume of 25 ml, and frozen overnight at −20 °C. Frozen cell pellet was thawed in room temperature water and treated with 1 μg/ml lysozyme and 10 μg/ml DNase I for 30 min on ice with gentle, intermittent shaking. Cells were sonicated for three cycles of 30 s on, 30 s off at 50% power using a Virtis VirSonic 100 sonicator and then centrifuged at 15,000 × g to clarify lysate. Lysate was diluted 1:5 in Column Buffer and applied to a 20-ml amylose resin column (New England Biolabs) at −1 ml/min, washed with 30 CV of Column Buffer, and then eluted in 1.5 CV of Column Buffer containing 10 mM maltose. Fractions containing MBP fusion protein were dialyzed in DEAE binding buffer (20 mM Tris, pH 8) overnight at 4 °C. MBP fusion was then passed over a 5-ml DEAE column, washed with 10 CV of DEAE binding buffer, and then eluted with a linear gradient from 0 to 100 mM NaCl over 40 CV. Fractions containing clean fusion protein were pooled and concentrated to a volume of 1 ml using Vivaspin 20 concentrator columns with a 50-kDa molecular mass cut-off. Fusion protein was then digested using PreScission protease (GE Healthcare) and 5 mM dithiothreitol overnight at 4 °C. E subunit was separated from MBP by gel filtration (Superdex 75 HR 16/50) in TBS. E subunit-containing fractions were pooled, aliquoted, and stored in liquid N2. For purification of subunit G, single colonies of pG-expressing cells were picked and grown overnight at 37 °C in a 25-ml inoculum of LB containing antibiotics. The entire 25 ml was used to inoculate 1 liter of M9 medium containing 1 g of [15N]ammonium chloride (Spectra Stable Isotopes). 1 liter of culture was grown at 37 °C to an A995 =
0.6 and induced with 1 mM isopropyl-1-thio-β-galactopyranoside for 4 h at 37 °C. Cells lysate was prepared as above, diluted 1:5 in TBS, and applied to a 5-ml anti-FLAG M2 column at ~1 ml/min. The column was washed with 30 CV of TBS and eluted in 3 CV of TBS containing 100 μg/ml FLAG peptide. G subunit-containing fractions were pooled, concentrated to 1 ml, and subjected to gel filtration as above. Purified G subunit-containing fractions were pooled, aliquoted, and stored in liquid N2.

**Protein Concentration Determination**—Protein concentrations were routinely estimated by measuring UV absorbance in 6 M guanidine-HCl (20). For more accurate concentration determination, three samples each of V1 and individual E and G subunits were subjected to quantitative amino acid analysis (University of Texas Medical Branch).

**Electrospray Ionization Mass Spectrometry**—V1-ATPase sample was subjected to buffer exchange to 100 mM ammonium acetate, pH 6.8, by using an Ultrafree-0.5 centrifugal filter device with a cut-off of 10,000 Da (Millipore, Bedford). The sample was sprayed from solution of 2 μl containing ~0.5 μM (~0.3 mg/ml). The macromolecular mass spectrometry measurements were performed in positive ion mode using first generation modified Q-TOF 1 and LCT instruments (Micromass, Manchester, UK) (21, 22). To detect intact gas-phase ions from large protein complexes, it is generally required to cool the ions collisionally by increasing the pressure in the first vacuum stage of the mass spectrometer (23, 24). The pressures were optimized to balance preservation of noncovalent interactions and promote efficient ion desolvation in the interface region of the instrument. In this way, we were able to attain sharp ion signals enabling confident accurate mass determination, and consequently, the stoichiometry of the V1-ATPase complexes from the mass spectra. Furthermore, nanoelectrospray voltages were optimized for generation of the macromolecular protein complexes; the needle voltage was 1,500 V, and the sample cone voltage was 150 V.

**Mass Spectrometry of Individual Subunits of the V1-ATPase**—V1-ATPase was denatured by diluting in 50% acetonitrile and 0.1% formic acid at a concentration of ~0.5 μM. Mass measurements were performed in positive ion mode using an electrospray ionization time-of-flight instrument LCT (Micromass, Manchester, UK) essentially as described previously (22).

**Isotope-labeled Subunit Titration and MALDI-Mass Spectrometry**—Increasing amounts of 15N-labeled E and G subunits were mixed with a determined amount of yeast V1-ATPase, and the resulting protein mixture was separated on 12% SDS-PAGE gels. Bands containing both unlabeled and 15N-labeled E and G subunits were excised from the gels with a clean razor blade. Gel bands were diced into cubes of <1 mm3, washed three times in 400 μl of 25 mM ammonium bicarbonate, pH 8, 50% acetonitrile, shrunk in 100 μl of 100% acetonitrile, and dried for 30 min in a SpeedVac with heating. 25 μl of trypsin was added to the dried gel slices, and 25 mM ammonium bicarbonate was added to cover the swelled gel slices. After incubation overnight at 37 °C, the supernatant was transferred to a fresh Eppendorf tube, and the remaining gel slices were extracted twice with 50 μl of 50% acetonitrile, 5% trifluoroacetic acid in H2O for 15 min at room temperature. The combined extracts were dried for 1 h in a SpeedVac with heating. Dried peptide pellets were dissolved in 50% acetonitrile, 0.1% trifluoroacetic acid in H2O, purified with a ZipTip, and then pipetted onto a stainless steel MALDI probe at 1:4 and 1:10 dilutions in a saturated solution of α-cyano-4-hydroxycinnamic acid (Fluka) in 50% acetonitrile, 0.1% trifluoroacetic acid. Peptide samples were analyzed using a first generation Bruker Autoflex MALDI-TOF. Raw mass spectra were exported to CSV files using open source mMass software and processed using in-house scripts written in Ruby. Baseline was calculated as described previously (25) with some modifications. Raw spectra were divided into 40-Da windows (si), within which both median (s̄med) and fifth percentile (s̄low) signal intensity values were calculated. Noise was defined as ni = 2(s̄med − s̄low) for a given window si. The local noise envelope for a given window si was defined as s̄med ± ni. The signal trend and noise envelope were calculated by cubic spline interpolation (26) of the si data points. The noise floor, or s̄med − ni, was subtracted from the intensity value of each data point to provide a baseline correction. The noise ceiling, or s̄med + ni, was used as a threshold to accept or reject peaks. The width of each isotopic envelope was defined as the maximum width that completely contained all peaks above the noise threshold. An isotopic envelope was rejected if the envelope was perturbed by noise or overlapping signal from other peptides. Mass spectra were obtained from digests of three gels each for E + V1 and G + V1 at various ratios. 4–8 peak pair ratios from validated pairs of isotopic envelopes were averaged together for each titration point for the G and E subunit, respectively. A 1:1 peak ratio of 14N- and 15N-containing peptides was interpolated using the least squares linear regression analysis.

**RESULTS AND DISCUSSION**

**V1-ATPase Subunit Stoichiometry by Native Mass Spectrometry**—The gentle nature of electrospray ionization and the spectacular advances in mass spectrometry instrumentation enable the direct analysis of large intact macromolecular protein complexes. This field, nowadays termed macromolecular or native mass spectrometry, focuses on the structural and functional analysis of the dynamics and interactions occurring in protein complexes. For this method, the sample of interest is electrosprayed from an aqueous solution of a volatile buffer such as ammonium acetate. Desolvation of the protein assemblies in the ion source interface generates multiply charged ions of the intact complexes that can be analyzed by the mass spectrometer. Native mass spectrometry has been used to obtain accurate information about stoichiometry, stability, and dynamics of protein complexes (21, 27–32). Here, we applied macromolecular mass spectrometry to investigate the composition of V1-ATPase from the yeast *S. cerevisiae*. Before the analysis of V1-ATPase under pseudophysiological native solvent conditions, we first analyzed the complex under denaturing solvent conditions. From the resulting mass spectra, we were able to determine the accurate masses of each subunit present in the complex. The obtained subunit masses are given in Table 1, together with the predicted masses of the subunits derived from the gene sequences. The only subunit not detected by this approach was subunit D. The absence of subunit D in the mass spectra might be due to the relatively hydro-
phobic nature of the polypeptide. On the basis of the gene-predicted amino acid sequences, we concluded that most subunits lacked the N-terminal methionine residue, except subunit G, which in our purifications also contains the N-terminal FLAG tag. For subunits B and H, the masses were very close to the expected masses. For the others, we observed slightly higher experimental masses, with mass increases likely to be related to post-translational modifications, such as N-terminal acetylation (+42 Da).

Next, we investigated V1-ATPase by macromolecular mass spectrometry. Fig. 1 shows a representative native mass spectrum of yeast V1-ATPase obtained from an aqueous solution of the protein in 100 mM ammonium acetate, pH 6.8. The spectrum reveals three individual charge state distributions centered around m/z values of 10,000, 11,400, and 11,500, respectively, with the most abundant distribution around m/z 11,500. The protein mass could be easily determined by using the well resolved multiple charge states of the protein. Thus, mass determination of the ion series with the highest m/z values (around 11,500) yielded a molecular mass of 593,576 ± 3,000 Da (Table 1, complex I). When we sum the theoretical masses of each subunit (i.e. as predicted from the gene sequences) in the stoichiometry A3B3DE3FG3H, we obtain a mass of 592,454 Da, which is a very close match to the observed mass of the complex. This mass is only 0.19% higher than the measured mass for this complex. The observed deviation between the theoretical and experimental mass can be partly explained by the fact that we observed higher experimental masses for each individual subunit under denaturing conditions but also by incomplete desolvation, which may leave several water or buffer molecules attached to the protein complex (29, 33).

The most intense charge state series centered around m/z 11,400 had a mass of 553,544 ± 2,000 Da (Table 1, complex II). The closest theoretical matching mass is 552,406 Da, corresponding to a complex of A3B2DE2FG2H stoichiometry. Thus, when compared with the most abundant complex I, the complex II lacks one copy of E and one copy of G. The last ion series centered around m/z 11,500 had a mass of 428,305 ± 1,600 Da (Table 1, complex III). Here, we can unambiguously assign the mass to a subcomplex of V1-ATPase with a stoichiometry of A2B2DE2FG2H (427,195 Da). Thus, in contrast to complex I, the subunits A, B, E, and G are present only in two copies in this complex I, whereas D, F, and H are only present as a single copy. The second ions series centered around m/z 10,000, 11,400, and 11,500 correspond to the complex I (593 kDa; green circles). The charge state series centered around m/z values of 11,400 correspond to the complex II (553 kDa; blue triangles), and those centered around m/z values of 11,500 correspond to the complex III (428 kDa; red squares). The inset shows the convoluted zero charge mass spectrum revealing the presence of the three complexes of V1-ATPase exhibiting different stoichiometries. The subunit composition for complexes I, II, and III is indicated by the schematic drawings next to the charge state series. A representative spectrum of a total of three experiments obtained on two different instruments is shown.

**TABLE 1**

Comparison from theoretical and measured masses of V1-ATPase-associated proteins and the intact- and subunit-depleted V1-ATPase complexes

| Complexes | Name Composition | Experimental masses (Da) | Theoretical masses (Da) | Subunits | Mass differences |
|-----------|------------------|--------------------------|-------------------------|----------|------------------|
| I         | A3B3DE3FG3H       | 593,576 ± 3,000          | 592,454                 | A        | +496             |
| II        | A3B2DE2FG2H       | 553,544 ± 2,000          | 552,406                 | B        | +123             |
| III       | A2B2DE2FG2H       | 428,305 ± 1,600          | 427,195                 | D, F, H  |                 |

*Standard errors represent the maximum mass error obtained from the full width at half-maximum of the most intense peak for each charge state distribution.

* N-terminal Met + FLAG tag.

**FIGURE 1.** Electrospray ionization mass spectrum of yeast V1-ATPase sprayed from aqueous 100 mM ammonium acetate, pH 6.8. The most intense charge state series centered around m/z values of 11,500 correspond to the complex I (593 kDa; green circles). The charge state series centered around m/z values of 11,400 correspond to the complex II (553 kDa; blue triangles), and those centered around m/z values of 10,000 correspond to the complex III (428 kDa; red squares). The inset shows the convoluted zero charge mass spectrum revealing the presence of the three complexes of V1-ATPase exhibiting different stoichiometries. The subunit composition for complexes I, II, and III is indicated by the schematic drawings next to the charge state series. A representative spectrum of a total of three experiments obtained on two different instruments is shown.
time the copy numbers for E and G were equal. Only very minor amounts of individual E and G subunits could be seen in the low m/z range in the native mass spectra of yeast V1-ATPase, suggesting that the two subcomplexes II and III were already present in the protein solution injected for electrospray ionization-MS. It is therefore likely that the two subcomplexes already co-exist next to intact V1 in the yeast cytoplasm, but from the observed variability in the ratios of the three complexes (see above), the possibility that some loss of E and G subunits and an AB subunit pair occurred during protein purification and/or MS sample preparation cannot be ruled out at this point.

Analysis of the E and G Subunit Copy Number Using 15N-labeled Internal Standards—As an alternative to the native electrospray MS approach, we used isotope-labeled, individually purified subunits as internal standards for stoichiometry determination by peptide mass fingerprinting. Mass spectrometry is generally not considered a quantitative method due to the fact that the signal generated by specific m/z species is often not proportional to the concentration of the species in the sample analyzed. This deficiency can be overcome by including known amounts of an internal standard that can be distinguished from the peptide to be measured by isotope labels or other modifications. In this way, mass spectrometry can be used for differential quantification of protein expression but also for absolute quantification, and thus, also for stoichiometry determination in a protein complex (34–39). It is of course essential that the reference peptide is biochemically identical to the peptide to be measured to ensure that both peptides ionize and fly with the same probability. Furthermore, to obtain a statistical meaningful result, as many peptides as possible should be included in the analysis. We therefore decided to use uniformly isotope-labeled subunits as internal standards for peptide mass fingerprinting of V1-ATPase E and G subunits. To do so, V1-ATPase was purified from yeast cultures grown on 14N-containing medium, whereas individual E and G subunits were expressed in E. coli using medium containing [15N]ammonium chloride as the sole nitrogen source. The concentrations of the V1-ATPase and the individually purified E and G subunits were determined by quantitative amino acid analysis from three samples each (V1; 1.916 ± 0.27 mg/ml; E: 0.117 ± 0.006 mg/ml; G: 0.189 ± 0.012 mg/ml). For peptide mass fingerprinting, a defined amount of 14N-containing V1-ATPase was mixed with increasing amounts of 15N-labeled E or G subunit, and the resulting mixtures were separated by SDS-polyacrylamide gel electrophoresis. Fig. 2A shows SDS-PAGE for various titrations of purified subunit E (lanes 2–5), V1-ATPase alone (lane 6), and V1-ATPase titrated with the same titrations of subunit E (lanes 7–10). A similar gel for subunit G is shown in Fig. 2B. Bands containing mixed isotonically labeled populations of E and G subunits in the V1-ATPase-containing lanes were excised and trypsin-treated, and the resulting mixtures of V1-ATPase subunit peptides (14N-containing) and purified subunit peptides (15N-containing) were then subjected to MALDI-TOF mass spectrometry. Representative mass spectra for a titration of 14N-containing V1-ATPase with bacterially expressed, 15N-labeled subunit E are shown in Fig. 3. Fig. 3B shows a MALDI-TOF mass spectrum of the subunit E band excised from lane 9 of the gel shown in Fig. 2A, as indicated in Fig. 3A by the dotted line. Fig. 3, C–F, shows the 14N–15N-peptide pair at 1211.7 m/z (for the 14N- or “light” peptide) as indicated in Fig. 3B by the dotted rectangle. In Fig. 3C, the digest of the V1-ATPase subunit E band without added 15N-labeled subunit E is shown. The spectra shown in Fig. 3, D and F, were obtained from a tryptic digest of the subunit E band after adding 0.88, 1.17, and 1.76 μg of 15N-labeled E-subunit as indicated in Fig. 3B. The spectra in Fig. 3C, D, and F, were obtained from a tryptic digest of the subunit E band after adding 0.88, 1.17, and 1.76 μg of 15N-labeled E-subunit as indicated in Fig. 3B.

Determination by peptide mass finger printing. Mass spectrometry is an alternative to the native electrospray MS approach, we used isotope-labeled, individually purified subunits as internal standards for stoichiometry determination by peptide mass fingerprinting. Mass spectrometry is generally not considered a quantitative method due to the fact that the signal generated by specific m/z species is often not proportional to the concentration of the species in the sample analyzed. This deficiency can be overcome by including known amounts of an internal standard that can be distinguished from the peptide to be measured by isotope labels or other modifications. In this way, mass spectrometry can be used for differential quantification of protein expression but also for absolute quantification, and thus, also for stoichiometry determination in a protein complex (34–39). It is of course essential that the reference peptide is biochemically identical to the peptide to be measured to ensure that both peptides ionize and fly with the same probability. Furthermore, to obtain a statistical meaningful result, as many peptides as possible should be included in the analysis. We therefore decided to use uniformly isotope-labeled subunits as internal standards for peptide mass fingerprinting of V1-ATPase E and G subunits. To do so, V1-ATPase was purified from yeast cultures grown on 14N-containing medium, whereas individual E and G subunits were expressed in E. coli using medium containing [15N]ammonium chloride as the sole nitrogen source. The concentrations of the V1-ATPase and the individually purified E and G subunits were determined by quantitative amino acid analysis from three samples each (V1; 1.916 ± 0.27 mg/ml; E: 0.117 ± 0.006 mg/ml; G: 0.189 ± 0.012 mg/ml). For peptide mass fingerprinting, a defined amount of 14N-containing V1-ATPase was mixed with increasing amounts of 15N-labeled E or G subunit, and the resulting mixtures were separated by SDS-polyacrylamide gel electrophoresis. Fig. 2A shows SDS-PAGE for various titrations of purified subunit E (lanes 2–5), V1-ATPase alone (lane 6), and V1-ATPase titrated with the same titrations of subunit E (lanes 7–10). A similar gel for subunit G is shown in Fig. 2B. Bands containing mixed isotonically labeled populations of E and G subunits in the V1-ATPase-containing lanes were excised and trypsin-treated, and the resulting mixtures of V1-ATPase subunit peptides (14N-containing) and purified subunit peptides (15N-containing) were then subjected to MALDI-TOF mass spectrometry. Representative mass spectra for a titration of 14N-containing V1-ATPase with bacterially expressed, 15N-labeled subunit E are shown in Fig. 3. Fig. 3B shows a MALDI-TOF mass spectrum of the subunit E band excised from lane 9 of the gel shown in Fig. 2A, as indicated in Fig. 3A by the dotted line. Fig. 3, C–F, shows the 14N–15N-peptide pair at 1211.7 m/z (for the 14N- or “light” peptide) as indicated in Fig. 3B by the dotted rectangle. In Fig. 3C, the digest of the V1-ATPase subunit E band without added 15N-labeled subunit E is shown. The spectra shown in Fig. 3, D and F, were obtained from a tryptic digest of the subunit E band after adding 0.88, 1.17, and 1.76 μg of 15N-labeled E-subunit as indicated in Fig. 3B. The spectra in Fig. 3C, D, and F, were obtained from a tryptic digest of the subunit E band after adding 0.88, 1.17, and 1.76 μg of 15N-labeled E-subunit as indicated in Fig. 3B. As can be seen, the addition of 15N-labeled subunit E leads to the appearance of a “heavy” peptide centered on 1225.7 m/z, corresponding to a mass shift of 14 Da when compared with the light peptide at 1211.7 Da. The light (unlabeled) peptide at 1211.7 m/z corresponds to the sequence 209LLSEEALPAIR219, which contains 14 nitrogen atoms. The difference of the isotope envelopes of the light and heavy peptides can be explained by the fact that the incorporation of 15N into the bacterially expressed subunits is not 100%. However, this incompleteness of isotope labeling will not affect the overall light to heavy peptide ratio, given that the magnitude of 15N- and 14N-peptide is determined by integration of the entire envelope of the isotope distribution. Of critical importance was the correction for baseline offset and noise, which is described in detail under “Experimental Proce-
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A

B

C

control

D

0.88 µg

E

1.17 µg

F

1.76 µg
tional Procedures.” Following these corrections, the $^{14}\text{N}-^{15}\text{N}$ ratios were calculated by integration of each isotopic envelope. This procedure was then carried out for all well resolved peptide pairs. Finally, the resulting $^{15}\text{N}-^{15}\text{N}$ ratios from three independent experiments were plotted against the amounts of added $E$ and $G$ subunits (Fig. 4). This allowed us to determine the absolute amounts of subunits $E$ and $G$ present in the bands corresponding to these subunits in the $V_1$-ATPase. As can be seen from Fig. 4, $A$ and $B$, equivalence for the $^{15}\text{N}$ and $^{14}\text{N}$ peak intensities was reached by adding 1.3 and 0.66 $\mu$g of $^{15}\text{N}$-labeled $E$ and $G$ subunits to 9.6 $\mu$g of $V_1$-ATPase, respectively. The results of these calculations are shown in Table 2. The overall conclusion, as summarized in Table 2, is that the $V_1$-ATPase preparation analyzed contained each three copies of subunit $E$ and $G$, consistent with the electrospores ionization-MS data summarized above.

**Conclusion**—Given the available evidence from electron microscopy (8–10, 19), chemical cross-linking (12, 13), and protein biochemistry (11, 14), it is likely that the $E$ and $G$ subunits, as a heterodimer, bind at the periphery of the three $B$ subunits to form the peripheral stalks or stators of the $V$-ATPase enzyme. Furthermore, it has been shown that the $E$ and $G$ subunits connect the $V_1$-ATPase domain to the membrane-bound domain of the $\alpha$ subunit as well as the $V$-ATPase-specific stalk subunits $C$ and $H$ (12, 13, 40–43). Interestingly, in the related $F$-ATPase, there is a single peripheral stalk, which is known to be made of the $\delta$ and $b$ subunits (7). The question then is why there are three peripheral stalks in the vacuolar ATPase? In the $F$-ATPase, part of the $\delta$ (or OSCP) subunit occupies the dimple formed by the N-terminal domains of the $\alpha$ and $\beta$ subunits of the $F_1$, leaving room for only one peripheral stalk (44, 45). By adding extra peripheral stalks, the $V$-ATPase had to eliminate the equivalent of the N-terminal domain of $F$-ATPase $\delta$ (bacterial subunit nomenclature) because there is only space for one protein of that size on top of the $F_1$ (or $V_1$) domain. Another difference between $F$-ATPase and $V$-ATPase is that the stator subunits in $V$-ATPase have no membrane-spanning domain. The next question therefore is: how are the three stator domains of the $V$-ATPase connected to the membrane? It is known that subunits $E$ and $G$ not only interact with subunit $B$ (12, 13) but also with subunits $C$ (40, 41, 43, 46) and $H$ (42) and the N-terminal domain of subunit $a$ (41). That means that the three $E$G heterodimers connect the three $B$ subunits to the membrane-bound $a$ subunit via interaction with the $C$ and $H$ subunits, which, as we have shown earlier, are situated in the interface between the $V_1$ and $V_0$ domains (9, 10). A comparison of structural models of the bacterial $F$-ATPase and the yeast vacuolar ATPase is shown in Fig. 5. What might be the rationale behind this structural change between $F$-ATP synthase and vacuolar ATPase? As pointed out in the Introduction, the vacuolar ATPase is regulated by reversible dissociation-reassociation, a mechanism that distinguishes the $V$-ATPase from the other ATPases.

**FIGURE 4. Peak ratio analysis of $^{14}\text{N}$- and $^{15}\text{N}$-peptides from $^{15}\text{N}$-labeled subunit $E$ and $G$ titration.** Noise and baseline offset corrected peak areas of the $^{14}\text{N}$-containing, and $^{15}\text{N}$-labeled $E$ and $G$ subunit peptide peak envelopes were determined as described under “Experimental Procedures.” $^{15}\text{N}$ to $^{14}\text{N}$ peak ratios were then calculated by dividing the integrated peak envelopes for the $^{15}\text{N}$- and $^{14}\text{N}$-peptides. The normalized ratios were plotted against titrated amounts of $^{15}\text{N}$-labeled $E$ and $G$ subunit, respectively. The horizontal lines in the box symbols, from top to bottom, represent 95, 75, 50, 25, and 5 percentile, respectively. The number of individual measurements for the data points in $A$ is 7, 12, 12, 12, 12, and 12, and for $B$, it is 4, 12, 12, 8, and 12, respectively. Fitting the data without forcing the fits to go through zero lead to very similar results (not shown). The standard error(s) of the linear regression was estimated according to: $s = \sqrt{\overline{(s^2 - a + bx)^2}}(n - 2)^{-1}$, where $a = y$ intercept; $b = slope [\mug^{-1}]$; $n =$ number of data points. The estimated errors for subunits $E$ and $G$ are 10 and 9.3%, respectively.
The observation that subunit C binds neither V₁ nor V₀ after enzyme dissociation, a role for this subunit in the mechanism of reversible dissociation has been proposed (18). Based on chemical cross-linking experiments (40) and our earlier electron microscopic studies (9, 10), we speculate that in the assembled V-ATPase, subunit C binds two of the EG heterodimers, one via its foot and one via its head domain. Removal of subunit C would then result in the loss of two of the three EG-stator interactions, and the resulting loss in binding energy between V₁ and V₀ would then ultimately lead to V-ATPase disassembly. Interestingly, isolated subunit C has been crystallized in two conformations that show a difference in the relative orientations of the foot and head domain of the subunit (48). Whether only one of these two conformations is able to bind two EG heterodimers in the complex, and if so, what the mechanism might be by which subunit C is changing conformation, remain to be seen.

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FIGURE 5. Model of the subunit arrangement in the bacterial F-ATP synthase and the yeast vacuolar ATPase. A, structural model of the bacterial F-ATPase. A single peripheral stalk composed of the δ and b subunits (nomenclature of the bacterial enzyme) connects the ATPase domain to the protein channel. B, in the yeast vacuolar ATPase, three peripheral stalks, each made of a subunit EG heterodimer, are connecting the V₁ and V₀ interface. The gray circles indicate the sites of interaction between peripheral stalk subunits and ATPase and protein channel domains. For details, see “Results and Discussion.”

related F₁F₀-ATP synthase (15, 16). In the F-ATPase, each of the three stator interactions indicated in Fig. 5A by the gray circles (one between δ and F₁, one between δ and the b subunits, and one between the b subunits and subunit a) has to be able to withstand the torque generated during rotational catalysis. In the V-ATPase, on the other hand, there must be at least six interactions, three at the top of the V₁ and three in the V₁-V₀ interface (Fig. 5B). Our stoichiometry measurements indicate that the interactions of subunits EG at the top of the A₃B₃ hexamer occur with high affinity and are not broken during disassembly as all three EG heterodimers co-purify during V₁ isolation from glucose-deprived cells. Initiation of V-ATPase disassembly must therefore involve breaking some or all of the interactions of the EG heterodimers with the C, H, and a subunits in the V₁-V₀ interface. It has been shown that subunit H is not required for binding of V₁ to V₀ (47), indicating that its interaction with one of the EG heterodimers is not critical for a structural interaction between V₁ and V₀. Subunit C, on the other hand, is essential for V-ATPase assembly and based on

| No. of EG heterodimers per V₁-ATPase | Mass ratio E/V₁ | Predicted amount of ¹⁵N E subunit for 9.6 ± 1.36 µg V₁-ATPase | Ratio of 1.30 ± 0.14 µg of ¹⁵N labeled E subunit to predicted amount | Mass ratio G/V₁ | Predicted amount of ¹⁵N G subunit for 9.6 ± 1.36 µg V₁-ATPase | Ratio of 0.66 ± 0.07 µg of ¹⁵N labeled G subunit to predicted amount |
|--------------------------------------|----------------|----------------------------------------------------------|------------------------------------------------|----------------|----------------------------------------------------------|----------------------------------------------------------------|
| 1                                   | 5.14           | 0.49 ± 0.07                                              | 265 ± 48³                          | 2.68           | 0.26 ± 0.04                                              | 258 ± 40³                          |
| 2                                   | 9.54           | 0.91 ± 0.13                                              | 143 ± 26                           | 4.96           | 0.48 ± 0.07                                              | 139 ± 25                           |
| 3                                   | 13.34          | 1.28 ± 0.18                                              | 102 ± 18                           | 6.94           | 0.66 ± 0.09                                              | 99 ± 17                            |
| 4                                   | 16.66          | 1.60 ± 0.23                                              | 82 ± 15                            | 8.67           | 0.83 ± 0.12                                              | 79 ± 14                            |

* The estimated standard error of linear regression (Fig. 4) for ¹⁵N labeled E (10.00%) and for ¹⁵N labeled G (9.3%) was added to ¹⁵N labeled subunit titration values.

³ Cumulative error from protein concentration determination and linear regression. The calculated values for the copy numbers of subunits E and G are 2.9 ± 0.56 and 3 ± 0.58, respectively.
