Subunit Rotation of Vacuolar-type Proton Pumping ATPase:
Relative Rotation of the G as to c Subunit

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Running title: Subunit Rotation of Vacuolar ATPase.
Summary

Vacuolar-type ATPases V$_1$V$_o$ (V-ATPases) are found ubiquitously in the endomembrane organelles of eukaryotic cells. In this study, we genetically introduced a His-tag and a biotin-tag onto the c and G subunits, respectively, of *Saccharomyces cerevisiae* V-ATPase. Using this engineered enzyme, we observed directly the counter-clockwise continuous rotation of an actin filament attached to the G subunit when the enzyme was immobilized on a glass surface through the c subunit. V-ATPase generated essentially the same torque as the F-ATPase (ATP synthase). The rotation was inhibited by concanamycin and nitrate but not by azide. These results demonstrated that the V- and F-ATPase carry out common rotational catalysis.
INTRODUCTION

Vacuolar-type ATPase (V-ATPase) pumps protons into the lumens of endomembrane organelles such as vacuoles, lysosomes, endosomes and synaptic vesicles (1-4). The same enzyme is localized in the plasma membranes of specialized mammalian cells including osteoclasts and kidney intercalated cells (1-4). V-ATPase is a multisubunit complex composed of two functional sectors, $V_1$ and $V_o$: membrane peripheral $V_1$ is responsible for ATP hydrolysis and integral $V_o$ for proton translocation. In yeast, $V_1$ and $V_o$ are composed of eight ($A, B, C, D, E, F, G$, and $H$) and five ($a, c, c’, c”$, and $d$) subunits, respectively (5). The structure and function of the yeast enzyme are conserved by those of animals because yeast mutants could be complemented by the animal counterparts including the human (6), mouse (7-9) and nematode (10) ones.

The general structure of V-ATPase is similar to that of F-ATPase (ATP synthase) consisting of $F_1$ ([ ]] , [[ ]] , and [[ ]] and $F_o$ ($a, b$, and $c$) sectors. The structure, function and mechanism of F-ATPase have been studied extensively (11-15). The binding-change mechanism suggested that the three catalytic sites of F-ATPase alternate during ATP synthesis, and that the ATP releasing step requires energy (16). Protons are transported through a transmembrane pathway formed from the single $a$ subunit and a ring of multiple $c$ subunits. The F-ATPase can also hydrolyze ATP, and form an electrochemical proton gradient. We, and Junge and coworkers showed continuous rotation of a complex of [[ ]] and the $c$ ring ($c_{10-14}$) of purified F-ATPase, when the [[ ]] hexamer was immobilized (13, 17). On the contrary, the [[ ]] hexamer could rotate when the $c$ ring, either purified (18) or embedded in membranes (14), was immobilized. Finally, rotation of the $a$ subunit relative to the $c$ ring has been shown in membranes (14).

V-ATPase has five more subunits than basic F-ATPase of bacteria. Based on the results of chemical cross-linking studies (19-21) and limited sequence homology ($A$ to [[ ], $B$ to [[ ], $G$ to
b, and c to c, V - to F-ATPase), the V-ATPase subunits have been assigned to those of the F-
ATPase (1, 4). Although the structure is conserved between A and [], and B and [], there are
still significant differences between the two enzymes: G has no hydrophobic segment which b
has; and the V-ATPase c and c’ subunits are the duplicated form of F-ATPase c (22); V_o has
three hydrophobic proteolipid subunits (c, c’, and c”) (23), whereas F_o has only one (c).
Negatively stained images of bovine coated vesicle V-ATPase revealed a more complicated
structure of the secondary stalk connecting V_i and V_o (24), possibly consistent with the
presence of V-ATPase subunits of which homologues were not found in F-ATPase. V-
ATPase shows lower catalytic cooperativity (25) and a lower ratio of H⁺ transported per ATP
hydrolyzed (26, 27), when compared with F-ATPase. Thus, the two proton pumps should
have unique structures and mechanisms for their distinct physiological roles.

Although the physiological roles and structures of the two enzymes are different, it has
been proposed that V-ATPase has a similar rotary mechanism to that of F-ATPase (1). To
examine this possibility, we first focused on the V-ATPase G subunit because various lines of
evidence suggested that the G subunit located at the peripheral stalk region of the enzyme.
The G subunit exhibits homology (~24% identity) with F-ATPase b (28), which rotates
relative to the c ring. Recently, it has been reported that the G subunit may correspond to the
[] subunit of F-ATPase (20, 21). Assuming that the G subunit rotates relative to the c ring of
V-ATPase, we introduced a His-tag and a biotin-binding domain to c and G, respectively, of
the yeast enzyme. Upon ATP hydrolysis, we could observe continuous counter-clockwise
rotation of an actin filament connected to the G subunit of V-ATPase.

**EXPERIMENTAL PROCEDURES**

*Yeast Strains, Epitope Tagging and Culture Conditions—* All strains used were
derivatives of YPH499 (29) as shown in Table I. The details of the methods used for mutant
construction can be found in the Supplemental Material. Strain TH60-4C used in rotation observation carries the chromosomal genes for the c subunit with (His)₆ at the carboxyl terminus, and the G subunit fused with a biotin-tag (Lys-20 to Leu-124 of the biotin-binding domain of transcarboxylase) (30) at the carboxyl terminus. Cells were grown at 30°C in YPD (1% yeast extract, 2% peptone and 2% glucose) medium containing 50 mM succinate and 50 mM potassium phosphate adjusted to pH 5.0 with KOH (31).

Preparation of Vacuolar Vesicles and Solubilization of V-ATPase—Yeast vacuoles were obtained by the method of Takeshige et al. (32), and converted into vesicles in 10 mM MES-Tris, pH 6.9, containing 25 mM KCl and 5 mM MgCl₂. After centrifugation, the vesicles were suspended in the same buffer with 20% glycerol and stored at -80°C. For solubilization of V-ATPase, the vacuolar vesicles were suspended in buffer (10 mM Tris-HCl, pH 7.5, containing 10% glycerol, 5 mM [γ]-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride), and then centrifuged at 100,000 x g for 10 min. Zwitterionic detergent ZW 3-14 was slowly added to the vacuolar vesicle suspension (5 mg protein/ml) to give a final concentration of 14 mM. After 10 min incubation, the mixture was centrifuged at 100,000 x g for 60 min, and then the supernatant was rapidly frozen with liquid nitrogen and stored at -80°C until use.

Western Blot Analysis of the Tagged Subunits—Solubilized vacuolar proteins were denatured at 70°C for 35 min in 10 mM Tris–HCl, pH 6.8, containing 8 M urea, 5 % SDS, 5 % [γ]-mercaptoethanol, and 1 mM EDTA, and then applied to a 0 - 20 % polyacrylamide gradient gel (for detection of the His-tagged c subunit, a 15 % polyacrylamide gel was used) in the presence of SDS. After electrophoresis and blotting onto a nitrocellulose membrane, the following antibodies were used for immunological detection: anti-H⁺-ATPase 69 kDa subunit mouse monoclonal antibody clone 8B1 (1:100 dilution), obtained from Molecular Probe; mouse monoclonal anti-polyhistidine antibody clone His-1 (1:100 dilution), Sigma;
and horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution), Jackson ImmunoResearch Laboratories. Antibodies were used after dilution in TBST (100 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 0.05% Tween 20) containing 5% skim milk, except that TBST containing 5% BSA was used for the anti-polylhistidine antibody. Signals were detected with an ECL detection system (Amersham-Pharmacia). The biotin-tagged G subunit was probed with streptavidin-conjugated alkaline phosphatase (Novagen).

**Measurement of ATPase Activities and Proton Translocation**—ATPase activity and protein concentrations were determined as described previously (7). The formation of an electrochemical proton gradient in vacuolar vesicles (3 μg protein) was assayed in the buffer used for ATPase with 1 μM 9-amino-6-chloro-2-methoxyacridine (ACMA) as a fluorescence probe (excitation, 410 nm; emission, 490 nm).

**Observation of Subunit Rotation**—For rotation assay, the solubilized V-ATPase (5 μl of 3 mg/ml protein) was diluted four-fold with Buffer A (10 mM HEPES-NaOH, pH 7.2, containing 25 mM KCl, 6 mM MgCl2, and 10 mg/ml bovine serum albumin), and then introduced into a Ni2+-nitritotriacetate (Ni-NTA)-coated glass flow cell at 25°C (14). After 5 min incubation, a fluorescent (tetramethyl-rhodamine labeled) actin filament was attached to the G subunit through a biotin-tag and streptavidin (14). Immediately after introducing the reaction mixture (5 mM ATP, 0.1 mg/ml asolectin, 0.01% ZW3-14, 25 mM glucose, 1 μM biotin, 1% D-mercaptoethanol, 216 μg/ml glucose oxidase, and 36 μg/ml catalase in buffer A), a 0.6 mm² area was scanned under a Zeiss Axiovert 135 equipped with a cooled intensified CCD camera (PentaMax-512EFT, Princeton Instruments) (33).

Digital images (0.142 μm x 0.142 μm/pixel) of an actin filament were acquired at a 10 msec resolution and then subjected to centroid analysis using Winview (Roper Scientific). The rotation angle of a filament (degree) and rotational rate (revolution/sec) were calculated from the centroid of the actin filament (34). The length of the filament was taken as the
average value obtained from 10 independent images processed with Metamorph (Universal Imaging Corp.). Viscous drag was calculated as \((4\sqrt{3}) \times \mu \times L^3/[\ln(L/r)-0.447]\), where \(\mu\) is the viscosity of the medium (1.0 \times 10^{-9} \text{ pN} \cdot \text{sec} \cdot \text{nm}^2), L, the length of the actin filament (700-1100 nm), and \(r\) the radius of the filament (5 nm). Frictional torque \((N)\) was calculated as \(N = (\text{angular velocity of the filament}) \times (\text{viscous drag})\). Angular velocity is equal to \(2\mu x\) (rotational rate). Continuously rotating filaments, of which the ends were attached to V-ATPase, were used for the torque calculation.

**RESULTS**

*Expression of Epitope-Tagged Copies of the c and G Subunits in Yeast*—The system we used to examine the rotation of V-ATPase is schematically shown in Fig. 1A. We introduced sequences for the His-tag and the biotin-binding domain to the yeast chromosomal genes of the \(c\) and \(G\) subunits, respectively. The biotin-binding domain-containing proteins could be biotinylated in vivo when expressed in yeast cells (30). It has been shown that the \(c\) or \(G\) subunit with an epitope tag at the carboxyl terminus can form a functional V-ATPase (28, 35). Consistent with the previous findings, the strain expressing the V-ATPase with the His-tagged \(c\) and the biotin-binding domain fused \(G\) could grow at neutral pH similar to the wild-type, whereas a null mutant \(vma3\) (TH57-20A) could not form any colony (Fig. 1B).

The stable expression of the two epitope tagged subunits was examined by Western blot analysis. Solubilized vacuolar proteins were analyzed with anti-His-tag antibodies and streptavidin (Fig. 1C). The \(G\) subunit with the biotin-binding domain was detected at the position corresponding to molecular mass of 31 kDa, this value being larger than the predicted value (24 kDa), probably due to the high contents of positively charged residues in the engineered subunit (lysine and arginine, 16.5\% of total residues). A signal corresponding to a molecular mass of 18 kDa was detected with anti-His-tag antibody, consistent with values
for the c subunit connected with the His-tag. No signals were detected with the solubilized vacuolar vesicles from wild-type cells (TH45-2A). Expression of the A subunit was confirmed in both strains. These results indicate that a fully active V-ATPase complex with tagged subunits was present in the vacuolar membrane.

Properties of V-ATPase with Tagged Subunits—We found that the amount of the A subunit in the engineered strain (TH60-4C) was about 20% of that observed in wild-type vacuolar membranes (determined by densitometry, data not shown, and similar results were obtained with solubilized vacuolar proteins as shown in Fig. 1C), suggesting that the expression level of engineered V-ATPase was lower than that of the wild-type. The ATPase activity of the engineered V-ATPase in vacuolar vesicles was about 20% of that of the wild-type (Table II). Thus, the engineered cells expressed a smaller number of V-ATPase with wild-type activity. Initial rate of proton pumping (fluorescence quenching) was 11% of that of the wild-type, and the coupling efficiency (proton pumping activity/ATPase) was slightly lower for the engineered vesicles (Table II). These results suggest that the engineered V-ATPase does not show severe defects in turnover rate or energy coupling, although its expression level in vacuolar membranes was low.

G Subunit Rotation Relative to the c Proteolipid—We observed the rotation of an actin filament connected to the membrane-embedded F-ATPase previously (14). The method used provided an ideal system for studying rotary catalysis in membrane enzyme because it is free from any effect during solubilization and/or purification with a detergent. We examined V-ATPase rotation using vacuolar membrane fragments with or without further sonication. However, the occurrence of rotating filament is significantly low, possibly due to the absence of planar membranes in the preparation. Therefore, we examined the rotation of V-ATPase right after solubilization to avoid possible loss of any subunit(s) from the complex during purification (36, 37).
V-ATPase was introduced into a flow cell, and then immobilized on a Ni-NTA coated glass surface through the His-tag. An actin filament was attached to the G subunit through the biotin-tag and streptavidin. Upon ATP addition, we could readily observe counterclockwise rotation of the filaments (Fig. 2 A and B). However, no rotating filaments were observed when V-ATPase without the His-tag (from strain TH50-5D) or the biotin binding domain (strain TH44-5D) was introduced into the flow cell (Fig. 2C).

Inhibition of Rotation by V-ATPase-Specific Inhibitors—V-ATPase shows unique anion sensitivities: nitrate is inhibitory, but chloride has no effect (36), whereas both ions have no effect on F-ATPase. The numbers of rotating filaments were calculated in the presence of these ions: the number of rotating filaments decreased with an increase in the nitrate concentration, but chloride had no effect (Fig. 3A). ATPase activity was inhibited similarly with increasing the nitrate concentrations (Fig. 3B).

Azide and concanamycin are widely known specific inhibitors of F- and V-ATPases, respectively (34, 38). As expected, the rotation of an actin filament connected to the V-ATPase G subunit was not affected by azide (Fig. 4A), but no rotating filament was observed in the presence of concanamycin (Fig. 4B). The actin filament also stopped rotating after the addition of concanamycin (Fig. 4C). The V-ATPase rotation was inhibited possibly because the antibiotic bound to the V_o sector (25, 39, 40) or the a subunit (41).

The lags between rotational steps of each filament were variable, as shown by an expanded time scale (Fig. 5A). Thus, we selected linear segments (highlighted sections in Fig. 5A), and average rates were plotted against viscous drag applied to the filaments. Through these calculations, we estimated that V-ATPase rotation generated frictional torque of 36 ± 4 pN·nm (Fig. 5B). This value is essentially the same as the torque generated in F_oF_1 (Table III) (13, 14, 18). However, detailed analysis, using a probe other than an actin filament, is necessary to come to a definite conclusion.
DISCUSSION

In this study, we have demonstrated that V-ATPase carries out rotational catalysis. Using a genetically engineered enzyme with a His-tagged proteolipid c subunit and a biotin-binding-domain connected to the G subunit, we could observe directly the counter-clockwise rotation of an actin filament attached to the G subunit when the enzyme was immobilized on a glass surface through the His-tag. Thus, the rotary mechanism is well conserved in the two enzymes, although V-ATPase shows significant structural and functional differences from F-ATPase including: 1) a distinct subunit composition (3); 2) lower catalytic cooperativity (25); and 3) a lower ratio of H⁺ transported per ATP hydrolyzed (26, 27).

We introduced a biotin-tag to the G subunit for our rotation assay. Subunit G exhibits homology to F-ATPase subunit b (28), which forms a stator (peripheral stalk) together with subunit a, and prevents the \( \square_{3} \) hexamer from rotating when the \( \square_{F10-14} \) complex is rotating. During the study of V-ATPase rotation, Forgac and co-workers have shown that the G subunit was cross-linked to the residues located at the top portion of B subunit (20, 21). Their results suggested that subunits G and E form a peripheral stalk connecting \( V_{1} \) and \( V_{o} \) sector, and the G subunit is located near the top of \( V_{1} \) farthest away from the membrane. In F-ATPase, the peripheral stalk contains the \( \square \) subunit, which is located near the top of the entire complex (42). Thus, the G subunit may correspond to the \( \square \) subunit of \( F_{1} \) and the E subunit to the extra membrane domain of the b subunit. Although the X-ray structure of V-ATPase needs to be determined, various lines of evidence suggested that the G subunit located at the peripheral stalk of the enzyme. Therefore, it is reasonable that we could observe rotation of G relative to the c ring by introducing a probe to this subunit.

A striking difference between V-ATPase and F-ATPase is the presence of three different proteolipid subunits (Vma3p, Vma11p, and Vma16p for c, c’, and c”, respectively) within the
Vo sector compared with a single subunit c in Fo. All three proteolipids are required for functional yeast V-ATPase, since loss or mutation of any one of them results in a complete loss of V-ATPase activity (23). The specific glutamate residues in the three subunits are essential for proton translocation, similar to Asp61 of the F-ATPase subunit c (23, 43). It has been shown that each V-ATPase complex contains single copies of both Vma11p and Vma16p, and multiple copies of Vma3p (35). The rotation of an actin filament attached to the V-ATPase was similar to one attached to F-ATPase, and no effects of multiple proteolipids were observed. However, it is possible that the difference between the Vo and Fo was not observed in the rotation because an actin filament was used as a probe in the assay. The rotational rate of an actin filament observed was significantly lower than those estimated from the enzyme without a probe (36). Thus, it is of interest to examine V-ATPase rotation with different probes including gold particles (44).

Several lines of evidence indicated that V-ATPase interacts with microfilaments in mammalian cells (45-47). We have observed that the distribution of the V-ATPase in plasma membrane of osteoclasts was inhibited by cytochalasin D, which is known to depolymerize actin filaments (T. Toyomura et al., manuscript in preparation). The B1 and B2 isoforms of the B subunit of V-ATPase contain a microfilament binding site in their amino-terminal domain (46). In yeast, it has been shown that actin participates in several intracellular trafficking pathways, and Eitzen et al. (48) have found that actin, bound to the surface of purified yeast vacuoles in the absence of cytosol or cytoskeleton, regulates the last compartment-mixing stage of homotypic vacuole fusion. Thus, we can assume that the actin filament may be an anchor of the A6B3 hexamer, and the Vo c ring together with the central stalk rotates relatively in membranes.

Recently, multiple isoforms of V-ATPase subunits in higher eukaryotes were identified (7-9, 49-52). Since many of them complemented the yeast counterpart, it will be of interest
to examine their roles in the rotary mechanism using the present experimental system.

Furthermore, the rotation may be affected by mutations causing defective proton translocation (23, 43) or ATPase activity (53, 54). Our rotation assay also constitutes an efficient tool for addressing the arrangement and functions of individual subunits in V-ATPase through characterization of the rotatory mechanism with probes attached to different subunits.

Most recently, Yokoyama and coworkers showed the rotation of an isolated peripheral membrane sector of *Thermus thermophilus* H⁺-ATPase responsible for ATP synthesis (55). The bacterial enzyme has been classified into A-type ATPases found in the plasma membranes of most archea and some kinds of eubacteria (for review, see 56). A-type ATPase is related to V-ATPase but is more similar to F-ATPase (57), although the three ATPases are believed to have the same ancestor. Taken together, our present studies on V-ATPase as well as those on F-ATPase (12-14, 18) and A-ATPase (55) clearly demonstrated that these three distantly related proton-translocating ATPases carries out common rotational catalysis.
REFERENCES


ACKNOWLEDGEMENTS

We wish to thank Dr. K. Nishio for the helpful discussion and for carrying out the blind test on the rotation analysis. We are also grateful to Ms. S. Shimamura, Ms. M. Nakajima and Y. Iko for their assistance.

FOOTNOTES

Abbreviations: NTA, nitrilotriacetate; MES, 2-morpholinoethanesulfonic acid; PCR, polymerase chain reaction; SDS, sodium dodecylsulfate; V-ATPase, vacuolar H⁺-ATPase; pH, proton chemical gradient; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid.
Table I

Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
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<td>YPH499</td>
<td>MATa ura3-52 lys2-801 ade2-101 trp1::63 his3::200 leu2::1</td>
<td>(29)</td>
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<td>YW33-1C</td>
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<td>TH40</td>
<td>MATa/MATa/ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1::63</td>
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<tr>
<td></td>
<td>/his3::200/his3::200 leu2::1/leu2::1 pep4::ADE2/PEP4</td>
<td></td>
</tr>
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<td>TH44-5D</td>
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<td></td>
<td>VMA3-His-tag</td>
<td></td>
</tr>
<tr>
<td>TH45-2A</td>
<td>MATa ura3-52 lys2-801 ade2-101 trp1::63 his3::200 leu2::1 pep4::ADE2</td>
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</tr>
<tr>
<td>TH50-5D</td>
<td>MATa ura3-52 lys2-801 ade2-101 trp1::63 his3::200 leu2::1 pep4::ADE2</td>
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<td>vma3::TRP1</td>
<td>This study</td>
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<tr>
<td></td>
<td>VMA3-His-tag VMA10-biotin-tag</td>
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</table>

* The vma3::TRP1 mutation was obtained by crossing a TH40-derived strain with DV3T-B kindly provided by Dr. Ryogo Hirata.
Table II

Properties of vacuoles vesicles with the V-ATPase engineered for rotation.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Growth at pH 7.5</th>
<th>Vacuolar vesicles</th>
<th>Coupling Efficiency</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ATPase (nmol/mg-protein-min)</td>
<td>Amount of subunit A (arbitrary units)</td>
</tr>
<tr>
<td>Nonengineered</td>
<td>+++</td>
<td>495</td>
<td>100</td>
</tr>
<tr>
<td>G biotin-tag / cHis-tag</td>
<td>++</td>
<td>105</td>
<td>20</td>
</tr>
</tbody>
</table>

The function of V-ATPase in vivo was verified by monitoring cell growth at pH 7.5. Cells (TH45-2A, nonengineered, and TH60-4C, G biotin-tag / cHis-tag) were grown at 30°C on YPD medium, adjusted to pH 7.5, for three days (See Fig. 1B.). Vacuolar vesicles were isolated from TH45-2A or TH60-4C, and their ATPase activity was assayed with 0.5 mM ATP (sodium salt) at 25°C. Concanamycin A-sensitive ATPase activity in vacuolar vesicles is shown (7). To determine the amount of the A subunit, wild-type and engineered vacuoles were applied to gel electrophoresis. After immunoblotting with anti-A subunit antibodies, the relative density was estimated and is shown in arbitrary units. Vacuolar vesicles (3 μg protein / ml) were incubated in the buffer used for the ATPase assay with 1 μM ACMA for the formation of a proton gradient (ΔpH). The initial rate of ATP-dependent proton gradient (ΔpH) formation was estimated from the initial rate of fluorescence (ΔF) quenching. The ratios of the initial rate of proton gradient formation and the ATPase activity are expressed as coupling efficiency (ΔpH/ATPase). The activity of the solubilized enzyme for the rotation assay increased about two-fold.
Table III

Generation of torque from rotation in V-ATPase and F-ATPase.

<table>
<thead>
<tr>
<th>Experimental System</th>
<th>Torque generated (pN·nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>V-ATPase G biotin-tag / cHis-tag</td>
<td>36 ± 4</td>
<td>This study</td>
</tr>
<tr>
<td>F-ATPase c biotin / □His-tag</td>
<td>46 ± 13</td>
<td>(13)</td>
</tr>
<tr>
<td>F-ATPase □biotin-tag / cHis-tag</td>
<td>48 ± 17</td>
<td>(18)</td>
</tr>
<tr>
<td>F-ATPase (membrane) □biotin-tag / cHis-tag</td>
<td>42 ± 6</td>
<td>(14)</td>
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<tr>
<td>F-ATPase (membrane) a biotin-tag / cHis-tag</td>
<td>45 ± 13</td>
<td>(14)</td>
</tr>
</tbody>
</table>

Torque generated on V-ATPase rotation was determined in this study (see Fig. 5, for details).

The values for F-ATPase were calculated from the previous data.
Figure Legends

Fig. 1. Observation system for V-ATPase rotation.  A. Observation system for V-ATPase rotational catalysis. An actin filament was connected to the G subunit of V-ATPase, which was immobilized on a glass surface through a His-tag introduced into the c subunit.  B. Growth phenotype of the strain expressing the tagged subunits. Growth at pH 7.5 or 5.0 was examined for three strains: TH60-4C, strain expressing the VMA3-His-tag and VMA10-biotin-tag (for rotation); TH45-2A, wild-type; and TH57-20A, vma mutant with vma3[].  C. Expression of tagged V-ATPase subunits in strain TH60-4C. Solubilized vacuolar proteins were obtained from TH60-4C (VMA3-His-tag/VMA10-biotin-tag, lane 1) and TH45-2A (wild-type, lane 2). The vacuolar proteins (2 μg for A-subunit and biotin-tag, or 20 μg for His-tag) were subjected to gel electrophoresis, and immobilized on polyvinylidene difluoride (A subunit and biotin-tag) or nitrocellulose (His-tag) membranes. Proteins were probed with anti-A (Vma1p) V-ATPase subunit antibodies, alkaline-phosphatase (ALP) conjugated streptavidin or anti-His-tag antibodies.

Fig. 2. Rotation of an actin filament connected to the G subunit of V-ATPase.  A. Rotation of an actin filament (1.0 μm) was recorded at a 10 msec time resolution. Images are shown with arrows which schematically indicate the orientation of the filament. A video showing the rotation of an actin filament (30 frames/sec) is available (Supplemental video A).  B. Rotation of filaments of different lengths connected to the G subunit. Rotation of the filaments (0.8, 1.0 and 1.3 μm) connected to the G subunit was followed immediately after the addition of 5 mM ATP.  C. Absence of a rotating filament when V-ATPase without a His-tag or biotin-tag was used. V-ATPase without the His-tag (TH50-5D) or the biotin binding domain (TH44-5D) was introduced into a flow cell and then examined for rotation. Immediately after the addition of ATP, a 0.6 mm² area was rapidly scanned to find rotating
filaments. The experiments were repeated 12 times in each case, and the total numbers of rotating filaments are shown.

**Fig. 3. Effects of nitrate and chloride on the rotation of an actin filament connected to the V-ATPase G subunit.**  
* A. Effects of KNO₃ and KCl on the rotation of an actin filament connected to the G subunit. The rotation of an actin filament was recorded in the presence of varying concentrations of KNO₃ or KCl. KCl was omitted from the assay solution and 6 mM magnesium gluconate was used instead of MgCl₂. The number of rotating filaments were determined as described in Fig. 2C.  
* B. Effects of KNO₃ and KCl on ATPase activity. V-ATPase activities were assayed in the presence of varying concentration of KNO₃ or KCl, and the results are shown as the relative activity as to the ATPase activity without addition of KNO₃ or KCl.

**Fig. 4. Effects of azide and concanamycin A on V-ATPase rotation.**  
* A. Effect of azide on the rotation of an actin filament connected to the G subunit. The rotation of actin filaments (1.0 and 1.3 µm) was followed in the presence of 0.5 mM azide.  
* B. Rotating filaments in the presence of concanamycin A or azide. Rotating filaments (in the presence of 0.5 mM azide or 0.1 µM concanamycin A) were counted as described in the legend to Fig. 2C. The experiments were repeated 12 times, and the total numbers of rotating filaments are shown.  
* C. Effect of concanamycin A on the rotation of actin filaments (0.8 - 1.2 µm). The rotation of actin filaments was followed on the subsecond scale, and 50 µl of the assay mixture containing 0.1 µM concanamycin A was slowly introduced into the flow cell (volume, ~ 10 µl) (arrowhead), and, after standing for 30 seconds, the rotation was further recorded (red lines). As controls, reaction mixtures without the antibiotic were introduced (blue line).
Fig. 5.  **Rotational torque generated by V-ATPase.**  

*A.* Examples of rotational rate estimation. The time courses of filament (0.8 μm) rotation were expanded, and the rates were obtained from the linear segments shown by green lines.  

*B.* Effect of viscous drag on the rotational rates of actin filaments connected to the G subunit. The rotation of each filament was followed, and the average rate for 20 linear segments of the time-course was plotted against viscous drag. Colored lines represent the calculated rotational rates of filaments assuming constant torque of 30 (red), 40 (green), and 50 (blue) pN·nm. Other details and viscosity drag calculation were described in the text or previously (33).
TH57-20A (vma3) TH45-2A (Wild type) TH60-4C (Engineered)

7.5
5.0

pH

Ni-NTA-coated glass

His-tag

ADP+Pi

ATP

AB

Actin filament

Streptavidin

ADP+Pi

Biotin-tag

V1

Anti-His-tag

ALP-streptavidin

Fig. 1
Fig. 2

V-ATPase

<table>
<thead>
<tr>
<th>Biocytin-tag</th>
<th>His-tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Rotating filaments

Time (sec)

0 10 20 30

0 50 100 150 200

Rounds

0.8 µm

1.0

1.3

His-tag

Biotin-tag

+ + - +
Viscosity drag (pN·nm·sec)

Rotational rate (sec⁻¹)

Fig. 5
Supplemental Material to:

Subunit Rotation of Vacuolar-type Proton Pumping ATPase:

Relative Rotation of G as to c Subunit

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Yoh Wada$^1$, and Masamitsu Futai$^{1,2}$

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**Supplemental Methods**

**Construction of yeast mutants and plasmids**

Plasmids pTH8 and pYN5 were constructed by insertion of VMA3-\textit{His-tag} and VMA10-\textit{biotin-tag} fragments into the yeast integrating vector pRS306 (Sikorski and Hieter, 1989), respectively. The \textit{VMA3-\textit{His-tag}} fragment for pTH8 was generated by recombinant PCR with primer sets (Supplemental Table I) (TH2VMA3CSac1-F1, TH10VMA3CHIS-R, TH3VMA3CKpn1-R1 and TH9VMA3CHIS-F), and YPH499 genomic DNA as template. pYN5 was constructed as following. The \textit{VMA10} fragment containing \textit{SacII} site at its carboxyl terminus was amplified with primer sets (YN1VMA10CSac1-F, YN8VMA10CSac2-R, YN2VMA10CKpn1-R and YN7VMA10CSac2-F) and YPH499 genomic DNA. Amplified fragment was inserted into pRS306 and pYN9 was obtained. A DNA fragment coding for biotin-binding domain was amplified with a primer set (YN9TCSac2-F and YN10TCSac2-R) and Pinpoint\textsuperscript{TM} Xa-1 Vector (Promega) as template. pYN5 was generated by insertion of the fragment into \textit{SacII} site of pYN9.

A diploid strain TH40 was obtained by crossing YPH499 and YW33-1C (Table I in manuscript). YW33-1C, a null mutant of \textit{pep4} coding for a vacuolar protease, is constructed in this study. pTH8 and pYN5 were digested with HindIII and used for transformation of TH40 in order to replace the chromosomal \textit{VMA3} and \textit{VMA10} with mutant alleles, respectively. Mutations in chromosomes were introduced by two-step gene replacement (Kaiser et al., 1994). The gene replacements were confirmed by Southern analysis.

Resultant strains, TH44 (\textit{MATa/MAT\textsuperscript{\alpha}} \textit{ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1::63/trp1::63 his3::200/his3::200 leu2::1/leu2::1 \textit{pep4::ADE2/PEP4 VMA3/VMA3-\textit{His-tag}}) and TH50 (\textit{MATa/MAT\textsuperscript{\alpha}} \textit{ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1::63/trp1::63 his3::200/his3::200 leu2::1/leu2::1 \textit{pep4::ADE2/PEP4 VMA3/VMA3-\textit{His-tag}}).
VMA10/VMA10-biotin-tag) are sporulated and tetrads were dissected. Spores were grown on YPD plate (2 % glucose, 1 % yeast extract, 2 % polypeptone and 2% agar) containing 50 mM succinate and 50 mM potassium phosphate adjusted to pH 5.0 with KOH. VMA loci were investigated by PCR and haploid strains, TH44-5D, TH44-15C (MATa ura3-52 lys2-801 ade2-101 trp1-[-63]his3-[-200]leu2-[-1]VMA3-His-tag) and TH50-5D were obtained. TH60-4C was a segregant of TH60, a diploid obtained by crossing TH44-15C and TH50-5D. TH45-2A (MATa ura3-52 lys2-801 ade2-101 trp1-[-63]his3-[-200]leu2-[-1] pep4::ADE2), having nonengineered (wild type) V-ATPase, is a derivative of TH40. TH57-20A, a disruption mutant of vma3, is a segregant obtained by crossing a TH40-derived strain and DV3T-B (MATa ura3-52 lys2-801 ade2-101 trp1-[-63]his3-[-200]leu2-[-1] vma3::TRP1) which is a derivative of YPH499, kindly provided from Dr. Ryogo Hirata, RIKEN, Saitama.

References for supplemental methods
### Supplemental Table I

**Primers used for plasmid construction**

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<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>TH2VMA3CSac1-F1</td>
<td>5’-CCGAGCTCAGCTTTGTACACCGGGTTATCCATCAT-3’</td>
</tr>
<tr>
<td>TH3VMA3CKpnl-R1</td>
<td>5’-CCCGTACCCTCTTGATAGAGCTGCT-3’</td>
</tr>
<tr>
<td>TH9VMA3CHIS-F</td>
<td>5’-GTCTGTCATCATCACCACGCTCTGAAATCCATCAAAGCAG-3’</td>
</tr>
<tr>
<td>TH10VMA3CHIS-R</td>
<td>5’-GCCTTATGGTGATGATGATGACAGACAACCTTTATGATTAGCTG-3’</td>
</tr>
<tr>
<td>YN1VMA10CSac1-F</td>
<td>5’-CCGAGCTGCCACCCCTACTAAAGCTGAAAGGAA-3’</td>
</tr>
<tr>
<td>YN2VMA10CKpnl-R</td>
<td>5’-CCCGTACCCTCTTGATAGAGCGGAAAC-3’</td>
</tr>
<tr>
<td>YN7VMA10CSac2-F</td>
<td>5’-CCGTAATTTGCCATTTCTTCTTCAATCATATATATATATATATATATACC-3’</td>
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<tr>
<td>YN8VMA10CSac2-R</td>
<td>5’-TAGAAAAACTAGAATAGTAATCCCGGCAAGCCATGATATGGACCTACG-3’</td>
</tr>
<tr>
<td>YN9TCSac2-F</td>
<td>5’-CCCCGCGGAGATCTCCCGATCTTTGATGAGACC-3’</td>
</tr>
<tr>
<td>YN10TCSac2-R</td>
<td>5’-CCCCGCGGAGATCTCCCGATCTTTGATGAGACC-3’</td>
</tr>
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