The V₀V₁-ATPase of Thermus thermophilus catalyzes ATP synthesis coupled with proton translocation. It consists of an ATPase-active V₁ part (ABDF) and a proton channel V₀ part (CLEG), but the arrangement of each subunit is still largely unknown. Here we found that acid treatment of V₀V₁-ATPase induced its dissociation into two subcomplexes, one with subunit composition ABDFC and the other with EGI. Exposure of the isolated V₀ to acid or 8 M urea also produced two subcomplexes, ABDFCL and the other with EGI. Thus, the C subunit (homologue of d subunit, yeast Vma6p) associates with the L subunit ring tightly, and I (homologue of 100-kDa subunit, yeast Vma10p) associates with the L subunit ring. Based on these observations and our recent demonstration that D, F, and L subunits rotate relative to A₃B₃ (Imamura, H., Nakano, M., Noji, H., Muneyuki, E., Ohkuma, S., Yoshida, M., and Yokoyama, K. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 2312–2315; Yokoyama, K., Nakano, M., Imamura, H., Yoshida, M., and Tamakoshi, M. (2003) J. Biol. Chem. 278, 24255–24258), we propose that C, D, F, and L subunits constitute the central rotor shaft and A, B, E, G, and I subunits comprise the surrounding stator apparatus in the V₀V₁-ATPase.

V₀V₁-ATPases (V-ATPases) are the ATPase/ATP synthase superfamily that catalyzes the exchange of the energy between proton translocation across membranes and the energy of ATP hydrolysis/synthesis (1–3). They are widely distributed in different types of eukaryotic cells and some bacteria (2, 4). In eukaryotic cells, V₀V₁-ATPases exist in both intracellular compartments and plasma membranes, and are responsible for the acidification of intracellular compartments, renal acidification, bone resorption, and tumor metastasis (2). On the other hand, most prokaryotic V₀V₁-ATPases produce ATP using the energy of a transmembrane proton electrochemical gradient that is generated by a respiratory chain (4, 5).

The overall structure of V₀V₁-ATPases is similar to that of F₀F₁-ATPases (α₁β₁γ₁δ₁ε₁δ₁γ₁c₁0–₁₄), which are responsible for ATP synthesis in mitochondria, chloroplast, and plasma membranes of eubacteria (3, 6). Both are composed of two functional domains, the peripheral catalytic V₁ or F₁ and a membrane embedded ion translocating domain called V₀ or F₀.

The structure and subunit arrangements of F₀F₁-ATPases are well characterized. The x-ray structure of F₁ revealed a hexamer of alternating α and β surrounding a central cavity containing a highly α-helical γ subunit (7). The γ and ε subunit constitute a central shaft, which directly contacted with the c subunit ring in F₀ (8). The b subunit has a hydrophobic N-terminal domain anchored in the membrane, and a hydrophilic C-terminal domain forms an elongated peripheral stalk that interacts with the F₁ moiety as a stator (9). The a subunit in F₀, which consists of a stator part together with b subunit, is situated peripherally to the c subunit ring and plays a crucial role in the proton translocation (3, 10, 11).

Like the F₀F₁-ATPases, the peripheral V₁ part contains a catalytic core, which is composed of three copies each of A and B subunits. The A subunit contains a catalytic site, and the A and B subunits are arranged alternately, forming a hexameric cylinder. The D subunit, which fills the central cavity of the A₃B₃ cylinder, makes up a central shaft with the F₁ subunit (12, 13).

The V₀ moiety contains at least two kinds of hydrophobic proteins, proteolipid subunits and 100-kDa subunit. The V₀V₁-ATPases from yeast contain three members of the proteolipid family, which are predicted to contain at least four transmembrane helices, and they constitute a hetero-oligomer (14, 15). The 100-kDa subunit has a bipartite structure containing a hydrophilic N-terminal domain and a hydrophobic C-terminal domain containing multiple transmembrane helices (16, 17). Although no significant sequence homology was found between the 100-kDa subunit and F₀-γ subunit, several lines of evidence have suggested that the 100-kDa subunit might be a functional equivalent to F₁-γ subunit (18–20). The d subunit (yeast VMA6 products) has been reported as a member of V₀ part (21), although it is a hydrophilic protein.

Based on the functional and structural similarity between V₀V₁-ATPases and F₀F₁-ATPases, it has been assumed that V₀V₁-ATPases would use a similar rotary mechanism as the F₀F₁-ATPases (3, 6, 22). The central shaft composed of γ and ε subunits in F₁ are directly associated with the c subunit ring in F₀ (8). Thus, the rotation of the central shaft in turn drives the rotation of the c subunit ring. This movement of the c subunit ring relative to F₀-α subunit, which is kept fixed to the α₁β₁ hexamer by a peripheral stalk, is thought to be directly responsible for proton translocation (3, 6). Recently, we visualized the rotation of single molecules of V₁-ATPase, establishing that V₀V₁-ATPases functions through a rotary mechanism (12). As with the F₀F₁-ATPase, V₁ and V₀ are connected by both central

Subunit Arrangement in V-ATPase from Thermus thermophilus*

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The structure and subunit arrangements of F₀F₁-ATPases are well characterized. The x-ray structure of F₁ revealed a hexamer of alternating α and β surrounding a central cavity containing a highly α-helical γ subunit (7). The γ and ε subunit constitute a central shaft, which directly contacted with the c subunit ring in F₀ (8). The b subunit has a hydrophobic N-terminal domain anchored in the membrane, and a hydrophilic C-terminal domain forms an elongated peripheral stalk that interacts with the F₁ moiety as a stator (9). The a subunit in F₀, which consists of a stator part together with b subunit, is situated peripherally to the c subunit ring and plays a crucial role in the proton translocation (3, 10, 11).

Like the F₀F₁-ATPases, the peripheral V₁ part contains a catalytic core, which is composed of three copies each of A and B subunits. The A subunit contains a catalytic site, and the A and B subunits are arranged alternately, forming a hexameric cylinder. The D subunit, which fills the central cavity of the A₃B₃ cylinder, makes up a central shaft with the F₁ subunit (12, 13).

The V₀ moiety contains at least two kinds of hydrophobic proteins, proteolipid subunits and 100-kDa subunit. The V₀V₁-ATPases from yeast contain three members of the proteolipid family, which are predicted to contain at least four transmembrane helices, and they constitute a hetero-oligomer (14, 15). The 100-kDa subunit has a bipartite structure containing a hydrophilic N-terminal domain and a hydrophobic C-terminal domain containing multiple transmembrane helices (16, 17). Although no significant sequence homology was found between the 100-kDa subunit and F₀-γ subunit, several lines of evidence have suggested that the 100-kDa subunit might be a functional equivalent to F₁-γ subunit (18–20). The d subunit (yeast VMA6 products) has been reported as a member of V₀ part (21), although it is a hydrophilic protein.

Based on the functional and structural similarity between V₀V₁-ATPases and F₀F₁-ATPases, it has been assumed that V₀V₁-ATPases would use a similar rotary mechanism as the F₀F₁-ATPases (3, 6, 22). The central shaft composed of γ and ε subunits in F₁ are directly associated with the c subunit ring in F₀ (8). Thus, the rotation of the central shaft in turn drives the rotation of the c subunit ring. This movement of the c subunit ring relative to F₀-α subunit, which is kept fixed to the α₁β₁ hexamer by a peripheral stalk, is thought to be directly responsible for proton translocation (3, 6). Recently, we visualized the rotation of single molecules of V₁-ATPase, establishing that V₀V₁-ATPases functions through a rotary mechanism (12). As with the F₀F₁-ATPase, V₁ and V₀ are connected by both central
and peripheral stalks (2), although the subunit composition of these stalks has not been established.

We have previously identified $V_0V_1$-ATPase in a thermophilic eubacterium, *Thermus thermophilus* (23, 24). The $V_0V_1$-ATPase is capable of both ATP-driven proton translocation and proton-driven ATP synthesis and functions as ATP synthase *in vivo* (5, 23). The *T. thermophilus* has a simple subunit structure, composed of nine different subunits, A, B, C, D, F, G, I, and L, with molecular sizes of 64, 54, 25, 12, 36, 21, 13, 17, and 2 kDa, respectively (Table I). Although the molecular size of some subunits of *T. thermophilus* is smaller than that of eukaryotic counterparts, each subunit of *T. thermophilus* shows a sequence similarity to its eukaryotic counterpart (see Table I). For instance, the L subunit shows an overall sequence similarity to eukaryotic V$_c$-a subunit (100-kDa subunit). Although the molecular size of the L subunit is ~50% of the eukaryotic V$_c$-a subunit (16-kDa proteolipid subunit), the L subunit shows an obvious sequence similarity to the V$_c$-a subunit.

The hydrophilic V$_i$ part of *T. thermophilus*, which is ATPase active and hence called V$_i$-ATPase, is made up of four subunits with a stoichiometry of A$_4$B$_4$D$_4$F$_4$ (23). The G, E, and C subunits are also hydrophilic, but they are not contained in the V$_i$ (23, 24).

Here we report isolation of several subcomplexes of $V_0V_1$-ATPases of *T. thermophilus*, and we propose subunit arrangement as well as rotor/stator identification in the complex.

**MATERIALS AND METHODS**

**Construction of a His$_6$-$V_0V_1$-ATPase, V$_0$ Expressing *T. thermophilus* Strain—** A mutant *T. thermophilus* strain, AH8, in which the atp$_A$ gene was replaced with a modified atp$_A$ gene encoding a His$_6$-tagged A subunit, was constructed as follows. At first, an atp$_A$-his$_6$ gene was constructed; the cloned atp$_A$ operon was subjected to PCR mutagenesis. The mutation primer was 5’-AAT GGA GGG ACG ATC CAA CAC CAT CGC GGG GTG ATC CAG AAG ATC (25) and the pyrE gene, which carries the pyrE gene cassette, was constructed; the XbaI-EcoRI fragment containing the euB gene of pT8euB (25) was cloned in pUC119, and then the NdeI-EcoRI fragment was replaced with the NdeI-EcoRI fragment containing the pyrE gene of pIVN (26). The sequence corresponding to a 1550-bp region, which is upstream of the atp$_A$ gene and includes the termination codon of the atpF gene, was amplified with primers InteA5/5/Sph (5’-GGGACATGGAGGTGGTGAGAATCGGCGCC-3’), and InteA5/3/Sal (5’-GGTTCAGCTACAGCTTGATGCTCAAAACCGATGTC-3’), followed by SphI and SalI digestion. The sequence corresponding to a 1570-bp region containing most parts of the atp$_A$-his$_6$ gene with its Shine-Dalgarno sequence was amplified with primers InteA5/5/EcoS (5’-GATCTTACGATGGAGGACATGGAGATCGGCGCC-3’), and InteA5/3/EcoS (5’-GAATTCCTCCCTTTAGGGACATGGACGCTAA-3’), followed by EcoRV and EcoRI digestion. These two fragments were cloned in the SphI-SalI and EcoRV-EcoRI sites of pUTpyrE, respectively. The resulting plasmid pyrE strain, *T. thermophilus* TTY1 (26), was genetically transformed as described previously in order to insert the pyrE gene as a selective marker and includes the termination codon of the atp$_F$ gene on the chromosome with the modified atp$_A$ gene encoding the His$_6$-tagged A subunit (25). Transformants were selected on a minimum-medium plate without uracil. Chromosomal DNA was prepared from a transformed strain, AH8, and integration of the pyrE gene into the site between the atp$_F$ gene and the atp$_A$ gene was confirmed by Southern blot analysis (data not shown).

**Isolation of V$_i$, V$_0$, and V$_{0i}$—** The recombinant *T. thermophilus* was grown as described previously (24). The cells (200 g) harvested at log phase growth were suspended in 400 ml of 50 mM Tris-Cl (pH 8.0), containing 5 mM MgCl$_2$, and disrupted by sonication. The membranes were precipitated by centrifugation at 100,000 g for 20 min and washed with the same buffer twice. The washed membranes were suspended in 20 mM sodium imidazole (pH 8.0), 0.1 M NaCl, and 10% Triton X-100 (w/v), and then the suspension was sonicated. The debris and insoluble materials were removed by centrifugation at 100,000 x g for 60 min, and the supernatant was applied onto a Ni-NTA superflow column (Qiagen, 3 x 10 cm) equilibrated with 20 mM sodium imidazole (pH 8.0), 0.1 M NaCl, 0.1% Triton X-100. The column was washed with 200 ml of the same buffer. The protein was eluted with a linear imidazole gradient (20–100 mM). The fractions containing the V$_i$-ATPases were analyzed with PAGE in the presence of SDS-PAGE, and then were combined and dialyzed against 20 mM Tris-Cl (pH 8.0), 0.1 mM EDTA, and 0.05% Triton X-100 for 3 h. The dialyzed solution was applied to a Resource Q column (6 ml, Amersham Biosciences) equilibrated with 20 mM Tris-Cl (pH 8.0), 0.1 mM EDTA, and 0.05% Triton X-100. The proteins were eluted with a linear NaCl gradient (0–0.5 M). The purity of each fraction was analyzed by SDS-PAGE and PAGE in the presence of alkyl ether sulfate (Softy 12, LION corp., AES-2-PAGE, Ref. 24). Each fraction containing V$_i$-ATPases, V$_o$, and V$_{0i}$ was combined and stored at 4°C until use.

Preparation of CL and EGG Subcomplexes from V$_0$—The V$_0$ fraction (purity, >90%) was dialyzed overnight against acetate buffer, containing 0.1 M sodium acetate (pH 4.0), 0.1 M EDTA, 5 mM DTT, 0.05% Triton X-100, or urea buffer, containing 8 M urea, 10 mM Tris-Cl (pH 8.0), 0.1 mM EDTA, 5 mM DTT, 0.05% Triton X-100. The dialyzed solution was concentrated with ultrafiltration using Centricon (Millipore Corp.). The concentrated solution was subjected to FPLC with a Superdex HR-200 column (Amerham Biosciences) equilibrated with 50 mM Tris-Cl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, 0.05% Triton X-100. The proteins were eluted with

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1 The recent versions of the nucleotide sequences, and protein sequences of each subunit have been submitted to GenBank™. The X subunit was termed as C subunit in that version and in this paper.

2 The abbreviations used are: AES, alkyl ether sulfate (Softy 12, LION); DCCD, dicyclohexylcarbodiimide; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; FPLC, fast protein liquid chromatography; Ni-NTA, nickel-nitrilotriacetic acid; DTT, dithiothreitol.
the same buffer, and each fraction was analyzed by AES-PAGE. Each subcomplex was subjected to re-chromatography by FPLC with the Superdex HR-200. The fractions containing each subcomplex were combined and stored at 4°C until use.

Preparation of V_{1-CL} Subcomplex—The V_{0V1}-ATPase was dialyzed overnight against acetate buffer containing 0.1 M sodium acetate (pH 4.0), 0.1 mM EDTA, 5 mM DTT, 0.05% Triton X-100. The dialyzed solution was concentrated by ultrafiltration and then subjected to FPLC with the Superdex HR-200 equilibrated with 50 mM Tris-Cl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA, and 0.05% Triton X-100. The fractions were analyzed by AES- and SDS-PAGE. The fractions containing the V_{1-CL} complex were combined and stored at 4°C until use.

Reconstitution of V_{0} into Liposomes and Measurement of Proton Permeability of the Liposomes—Proteoliposomes containing V_{0} were reconstituted according to the procedure by Richard et al. (27). Reconstitution was performed at 25°C in 25 mM potassium phosphate buffer (pH 7.3) and 500 mM K_{2}SO_{4}. Unilamellar liposomes were prepared by reverse phase evaporation using phosphatidylcholine (type II, Sigma) and resuspended at a lipid concentration of 4 mg/ml. Triton X-100 was added to a final concentration of 8 mg/ml. Then 10^{5}/H\text{H}_{9262} \text{lo fV}_{0} solution (5 mg of protein/ml) was added to 850/\text{H}_{9262} \text{l of the liposome solution.}\text{N-Octyl-
glucopyranoside was added to a final concentration of 20 m M, and the mixture was incubated for 5 min. Then pyranine (excitation, 450 nm; emission, 510 nm) were added to the mixture at a final concentration of 0.2 \text{M. The detergent was removed by four successive additions of 80 mg/ml washed Bio-Beads SM-2 (Bio-Rad). Two milliliters of 25 mM potassium phosphate buffer (pH 7.3) and 100 mM Na}_{2}SO_{4} were added to 200 \mu l of liposome solutions and incubated for 10 min at 25°C. Valinomycin was added to the mixture at a final concentration of 20 \mu M, and then carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) was added to the mixture at a final concentration of 0.1 \mu M.

Others—Chemicals used were reagent-grade and purchased from Sigma or Wako Pure Chemicals. Protein concentrations were determined by BCA protein assay (Pierce), and bovine serum albumin was used as the standard. PAGE in the presence of SDS or AES was carried out as described previously (24). The proteins were stained with Coomassie Brilliant Blue.

RESULTS

Purification of His-tagged V_{0V1}-ATPase—To obtain a large amount of highly purified V_{0V1}-ATPase from T. thermophilus, His\text{tag was introduced at the N-terminal of atpA with a shuttle integration vector system (25, 26, 28). The His-tagged V_{0V1}-ATPase in the membranes was solubilized with Triton X-100 and purified with a Ni-NTA-agarose column. The AES-PAGE analysis revealed that V_{0V1}-ATPase was the major component in the eluted fractions (Fig. 1a). Typically, ~30 mg of V_{0V1}-ATPase was obtained from 200 g of the recombinant cells.

![Fig. 2. Purified V_{0V1}-ATPase, V_{1}, and V_{0}. a, left, 6% AES-PAGE; right, 18% SDS-PAGE. Complexes were visualized by Coomassie Brilliant Blue R staining. Lane 1, 20 \mu g of V_{0V1}-ATPase. Lane 2, 20 \mu g of V_{1}. Lane 3, 20 \mu g of V_{0}. b, elution profiles of V_{0V1}-ATPase, V_{1}, and V_{0} from gel permeation high pressure liquid chromatography. Purification of each complex and the conditions of gel permeation FPLC were described under “Materials and Methods.”](http://www.jbc.org/)
bands on the AES gel electrophoresis (Fig. 2a, left). Fig. 2b shows elution profiles of the \( V_{0}V_{1} \)-ATPase, \( V_{1} \), and \( V_{0} \). The molecular size of \( V_{0} \) was estimated to be 350 kDa. SDS-PAGE analysis revealed that the \( V_{0} \) was composed of I, L, E, G, and C (Fig. 2a, right, lane 3).

The \( V_{0} \) was reconstituted into liposome in order to examine proton channel activity. As shown in Fig. 3, the lumens of \( V_{0} \) liposome were rapidly acidified in response to a membrane potential imposed by \( K^{+} \) diffusion mediated by valinomycin. Further incorporation of protons was induced by the addition of an uncoupler, FCCP. The prior treatment of the \( V_{0} \) liposomes with dicyclohexylcarbodiimide (DCCD) resulted in loss of proton translocation. No rapid acidification was observed for simple liposomes without \( V_{0} \). The results indicate that the isolated \( V_{0} \) is a functional DCCD-sensitive proton channel.

**IEG and CL Subcomplexes from \( V_{0} \)—**The \( V_{0} \) fraction was exposed to acidic buffer (pH 4.0) and then applied to gel permeation chromatography. Two new peaks appeared with estimated molecular mass of \( \sim 250 \) and \( \sim 130 \) kDa (Fig. 4a). Each fraction showed a single band in the AES gel (Fig. 4b, upper panel). The SDS-PAGE analysis revealed that the 250-kDa complex was composed of subunits I, E, and G, and the 130-kDa complex was composed of subunits C and L (Fig. 4b, lower panel). These complexes were also obtained from \( V_{0} \) by treatment with 6 M urea, suggesting that hydrophilic E and G subunits are associated tightly with hydrophobic I subunit and hydrophilic C subunit with the L subunit ring.

**\( V_{1} \)-CL Subcomplex—**The \( V_{0}V_{1} \)-ATPase was exposed to the low pH acetate buffer and applied to gel permeation chromatography. As shown in Fig. 4c, following this separation, a new peak appeared after the peaks corresponding to \( V_{0}V_{1} \)-ATPase and the IEG subcomplex. AES-PAGE and SDS-PAGE analysis revealed that the complex in the new peak was composed of C, L, A, B, D, and F (Fig. 4b, lower panel). The E, G, and I were not present in the complex (Fig. 4a, b, and c). This result indicates that the LC subcomplex binds to the central shaft composed of D and F subunits.

**DISCUSSION**

The precise arrangement of the subunits in the \( V_{0}V_{1} \)-ATPase remains an important, unclarified issue. In particular, the structure and subunit composition of both central and peripheral stalk have yet to be clarified. In an attempt to obtain insight into the subunit arrangement and function, we have studied the \( T. thermophilus \) \( V_{0}V_{1} \)-ATPase, which has a much simpler subunit composition compared with eukaryotic counterpart (Table I). The \( V_{0}V_{1} \)-ATPase of \( T. thermophilus \) partially dissociated into \( V_{0} \) and \( V_{1} \) during the ion exchange column chromatography, and they were easily isolated. The \( V_{1} \) part of \( T. thermophilus \) is made up of four different subunits with a stoichiometry of \( A_{3}B_{3}D_{1}F_{1} \). The D subunit had been the most probable candidate of rotor subunit in \( V_{1} \) portion (2). Cross-linking studies have suggested that the D subunit was adjacent to B subunit at central cavity region of \( A_{3}B_{3} \) hexamer, and the F subunit was associated with the D subunit (29, 30). In contrast, studies on the V-ATPase from \( M. sexta \) suggested that subunit E, rather than subunit D, was the rotor subunit (31). Electron microscopic study of Na\(^{+}\)-pumping \( V_{0}V_{1} \)-ATPase from \( C. bartonii \) also suggested that the E subunit was the...
Table I

Comparison between V₀V₁-ATPase subunits in T. thermophilus, Saccharomyces cerevisiae, and Homo sapiens.

| Yeast V-ATPase | Identities between Tth V and yeast V | T. thermophilus V-ATPase | Identities between Tth V and human V | Human V-ATPase |
|----------------|------------------------------------|-------------------------|------------------------------------|----------------|
| A (70 kDa)     | 50                                 | A (64 kDa)              | 50                                 | A (68 kDa)     |
| B (58 kDa)     | 55                                 | B (54 kDa)              | 55                                 | B (57 kDa)     |
| D (28 kDa)     | 28                                 | D (25 kDa)              | 28                                 | D (28 kDa)     |
| F (13 kDa)     | 21                                 | F (12 kDa)              | 21                                 | F (13 kDa)     |
| d (40 kDa)     | 17                                 | C (36 kDa)              | 16                                 | d (40 kDa)     |
| E (26 kDa)     | 16                                 | E (21 kDa)              | 20                                 | E (26 kDa)     |
| G (13 kDa)     | 22                                 | G (13 kDa)              | 13                                 | G (14 kDa)     |
| a (96 kDa)     | 16                                 | I (72 kDa)              | 16                                 | a (96 kDa)     |
| c (16 kDa)     | 37                                 | L (8 kDa)               | 37                                 | c (16 kDa)     |
| c' (17 kDa)    |                                   |                         |                                   | c' (17 kDa)    |
| c'' (23 kDa)   |                                   |                         |                                   | c'' (23 kDa)   |
| H (54 kDa)     |                                   |                         |                                   | H (55 kDa)     |
| C (44 kDa)     |                                   |                         |                                   | C (44 kDa)     |

Fig. 5. Structural model of V₀V₁-ATPase and subcomplexes. Model shows most probable subunit arrangement in V₀V₁-ATPase of T. thermophilus. The central stalk is postulated to include C, D, and F subunits, whereas the peripheral stalk includes E and G subunits. The V₀V₁-ATPase (holoenzyme) partially dissociated into V₀ (proton channel) and V₁ (ATP-driven motor) during ion exchange column chromatography. The V₀ dissociated into CL subcomplex (V₀ rotor) and IEG subcomplex (stator) by the low pH treatment. The low pH treatment of holoenzyme also induced dissociation of the stator subcomplex from the holoenzyme (left side).

Rotor and Stator Subunits in the V-ATPase

The V₀ moiety of T. thermophilus, which shows proton channel activity, is composed of five different subunits, two typical membrane proteins, subunits I and L, and three hydrophilic subunits, E, G, and C (Fig. 2c). In the rotary mechanism, each subunit should be classified as part of the rotor or the stator part. Subunit I (72 kDa) shows an apparent sequence similarity to yeast V₁-ATPase, which interacts with the proteolipid ring and also plays a critical role in proton translocation (18). Thus, the I subunit is thought to be functional homologue of F₁γ, and to constitute the stator part with other subunits. The L subunit is a member of a highly conserved family of hydrophobic subunits, often termed as proteolipid due to their solubility in organic solvents (15). The proteolipid subunit, both in F₀F₁-ATPase and V₀V₁-ATPase, forms a ring structure and has an essential carboxyl residue involved in proton translocation (2, 8). We have demonstrated recently [33] the rotation of L subunit ring relative to A₃B₃ hexamer, indicating that the L subunit is part of the rotor region along with the D and F subunits.

To address the question of the localization of C, E, and G subunits in V₀V₁-ATPase, V₀V₁-ATPase or V₀ was exposed to low pH buffer or with 8 M urea to dissociate them into subcomplexes. The V₀ part can be divided into two complexes, one is composed of subunits E, G, and I, and the other is composed of subunits L and C. The E subunit was predicted to be a highly hydrophilic α-helical protein and one of candidates of F₁γ subunit homologue (31, 32). Our results are consistent with the cross-linking studies by Arata et al. (29, 30) and strongly suggest that the subunit E is a stator subunit, rather than a rotor subunit. The G subunit of T. thermophilus shows significant similarity (−20% identity, overall sequence) to the F₁γ subunit homologue (31, 32). Our results are consistent with the cross-linking studies by Arata et al. (29, 30) and strongly suggest that the subunit E is a stator subunit, rather than a rotor subunit. The G subunit of T. thermophilus shows significant similarity (−20% identity, overall sequence) to the F₁γ subunit homologue (31, 32). Our results are consistent with the cross-linking studies by Arata et al. (29, 30) and strongly suggest that the subunit E is a stator subunit, rather than a rotor subunit. The G subunit of T. thermophilus shows significant similarity (−20% identity, overall sequence) to the F₁γ subunit homologue (31, 32). Our results are consistent with the cross-linking studies by Arata et al. (29, 30) and strongly suggest that the subunit E is a stator subunit, rather than a rotor subunit. The G subunit of T. thermophilus shows significant similarity (−20% identity, overall sequence) to the F₁γ subunit homologue (31, 32). Our results are consistent with the cross-linking studies by Arata et al. (29, 30) and strongly suggest that the subunit E is a stator subunit, rather than a rotor subunit. The G subunit of T. thermophilus shows significant similarity (−20% identity, overall sequence) to the F₁γ subunit homologue (31, 32). Our results are consistent with the cross-linking studies by Arata et al. (29, 30) and strongly suggest that the subunit E is a stator subunit, rather than a rotor subunit.
the V₁ and V₀ domain (1, 2). For instance, the assembly state of the yeast V-ATPase is post-translationally regulated by glucose in vivo (36, 37). The V₅V₇-ATPase of M. sexta also shows a similar type of regulation (4, 38). In contrast, the V₅V₇-ATPase of T. thermophilus functions as an ATP synthase, and no reversible dissociation has been observed for this enzyme. The lower affinity of both E and G subunits to the membrane domain in eukaryotes may be important in the reversible dissociation of the V₁ and V₀ domain.

Subunit C, a homologue of Vma6p (or the d subunit) assigned to be the V₇ part in yeast V₅V₇-ATPase (21), was also a member of the V₅ complex of T. thermophilus. The CL subcomplex was stable against the treatment with 8 M urea, suggesting that the C subunit tightly binds to the L subunit ring. Interestingly, the IEG subcomplex is easily removed from V₅V₇-ATPase by low pH treatment, leaving an ATPase active V₁-CL subcomplex (illustrated in Fig. 5). Based on the electron microscopy studies of subcomplexes with different subunit composition, Chab et al. (32) suggested that the C subunit of C. fervidus V₅V₇-ATPase is a component of the central stalk. The V₁ complex from Methanosarcina mazei was made up from five different subunits, A–D and F (39), and each subunit shows an apparent sequence homology to counterpart of T. thermophilus (32) suggested that the C subunit of E. coli is a component of the central stalk. The V₁ complex consists of three parts, the V₁, which acts as an ATP-driven motor, the V₀ rotor part composed of subunits C and L, and the stator part composed of subunits I, E, and G (Fig. 5). These subcomplexes would also be useful for future structural studies.

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Subunit Arrangement in V-ATPase from *Thermus thermophilus*

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