Reconstitution in Vitro of V₁ Complex of Thermus thermophilus V-ATPase Revealed That ATP Binding to the A Subunit Is Crucial for V₁ Formation*

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Vaccular-type H⁺-ATPase (V-ATPase or V-type ATPase) is a multisubunit complex comprised of a water-soluble V₁ complex, responsible for ATP hydrolysis, and a membrane-embedded V₀ complex, responsible for proton translocation. The V₁ complex of Thermus thermophilus V-ATPase has the subunit composition of A₃B₃DF, in which the A and B subunits form a hexameric ring structure. A central stalk composed of the D and F subunits penetrates the ring. In this study, we investigated the pathway for assembly of the V₁ complex by reconstituting the V₁ complex from the monomeric A and B subunits and DF subcomplex in vitro. Assembly of these components into the V₁ complex required binding of ATP to the A subunit, although hydrolysis of ATP is not necessary. In the absence of the DF subcomplex, the A and B monomers assembled into A₁B₁ and A₃B₃ subcomplexes in an ATP binding-dependent manner, suggesting that ATP binding-dependent interaction between the A and B subunits is a crucial step of assembly into V₁ complex. Kinetic analysis of assembly of the A and B monomers into the A₁B₃ heterodimer using fluorescence resonance energy transfer indicated that the A subunit binds ATP prior to binding the B subunit. Kinetics of binding of a fluorescent ADP analog, N-methylanthraniloyl ADP (mant-ADP), to the monomeric A subunit also supported the rapid nucleotide binding to the A subunit.

Acidification of intracellular acidic compartments of eukaryotic cell, such as lysosomes, endosomes, or synaptic vesicles is important for various cellular processes, including receptor-mediated endocytosis and secondary transport, and is accomplished by ATP-driven proton translocation of the vacuolar-type H⁺-ATPases (V-ATPases), which is localized in the membranes of these compartments (1–3). V-ATPases are also found in the plasma membrane of some specialized cells, such as macrophages (4), renal intercalated cells (5), or osteoclasts (6), and transport proton from the cytosol to the extracellular space. It is known that the V-ATPases in the plasma membrane is also responsible for various cellular functions such as acidification of extracellular space or homeostasis of pH in the cytosol (1–3). V-ATPases are large protein complexes consisting of two major parts, a peripheral V₁ complex and a membrane-embedded V₀ complex. V₁ and V₀ are responsible for ATP hydrolysis and proton translocation, respectively. Proton translocation at V₀ is accompanied by rotation of the membrane-spanning c-ring, which is driven by ATP hydrolysis at V₁ (7–10). In addition to the role in proton translocation, it was recently suggested that V₀ complex is directly involved in membrane fusion events (11, 12). Homologues of eukaryotic V-ATPases have also been found in the plasma membranes of some bacteria (10, 13, 14). Although the bacterial V-ATPases have a simpler subunit composition and function as an ATP synthase or Na⁺/H⁺ antiporter rather than a proton pump, all subunits have significant homology with eukaryotic counterparts (10, 15).

It is known that most of the V-ATPase subunits are essential for formation of a functional V₁/V₀ complex in vivo (1). When any of the genes encoding V-ATPase subunits, except H subunit, are deleted, formation of V₁/V₀, and/or V₁,V₀ complexes is inhibited. Assembly of V-ATPase subunits also requires other proteins or compounds. For example in yeast, it is known that Vma12p/Vma21p/Vma22p proteins, which are not final components of the V-ATPase, aid the assembly of V₀ subunits in the endoplasmic reticulum (16, 17), and that a protein complex, RAVE, which binds to the free cytosolic V₁ complex, plays a role in association of V₁ and V₀ (18, 19). In addition, it has been suggested that both under in vitro (20) and in vivo (21) conditions, binding of ATP to the A subunit is important for assembly. Another important feature of assembly of V-ATPase components is that proton translocation by V-ATPase is regulated by reversible association/dissociation of V₁ and V₀ complexes (22). Proton translocation activity of the V-ATPase is completely abolished by the dissociation of the V₁ complex from the V₀ complex.

Here, we report in vitro reconstitution of the V₁ complex of Thermus thermophilus from its components. The V₁ complex of T. thermophilus V-ATPase is composed of 8 subunits with a subunit composition of A₃B₃DF (15). The A and B subunits form a hexameric A₃B₃ ring, in which A and B are...
arranged alternately, and the D and F subunits constitute a central rotor shaft, which penetrates into the cavity of the A$_3$B$_3$ ring. We investigated the role of ATP in V$_1$ formation in detail, and found that ATP binding to the A subunit is a crucial step for assembly of the V$_1$ components into the complex. Furthermore, potential intermediate subcomplexes were identified. We also describe a model for the assembly of the V$_1$ complex.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—ATP, ADP, and bovine serum albumin were products of Sigma. AMP-PNP and restriction endonucleases were purchased from Roche. N-Methylanthraniloyl ADP (mant-ADP) was purchased from Invitrogen. Cy$_3$-maleimide and Cy$_5$-maleimide were from GE Healthcare. Other reagents were from Wako Pure Chemicals (Osaka, Japan), unless otherwise stated.

**Expression and Purification of Proteins**—Expression of the T. thermophilus V$_1$ complex (A$_3$B$_3$DF) in *Escherichia coli* containing a His$_{6N}$ tag at the N terminus of the A subunit was performed as described previously (23). The *E. coli* cells were suspended in 0.1 M sodium phosphate, pH 8.0, 20 mM imidazole-HCl, 0.3 M NaCl and disrupted by sonication, followed by heat treatment at 65 °C for 30 min. After removal of denatured *E. coli* proteins by centrifugation at 19,000 × g for 60 min, the supernatant was applied to a nickel-nitrilotriacetic acid Superflow column (Qiagen), which was then washed thoroughly with 0.1 M sodium phosphate, pH 8.0, 0.2 M imidazole-HCl, 0.3 M NaCl. The buffer was switched to 20 mM Tris-HCl, pH 8.0, containing 1 mM EDTA by ultrafiltration (AmiconUltra, Millipore) and the solution was applied to a UNO-Q column (Bio-Rad). Proteins were eluted with a linear gradient of NaCl, from 0 to 400 mM. V$_1$ and the A subunit were eluted in different fractions and were stored at 4 °C. The B subunit of *T. thermophilus* was expressed in *Escherichia coli* strain BL21(DE3)-CodonPlus-RP (Stratagene) harboring a plasmid pHis-B, in which genes for the B subunits were inserted between the EcoRI and SalI sites of pUC18. A His$_{6F}$ tag was fused to the C terminus of the F subunit. The *E. coli* cells were suspended in 0.1 M sodium phosphate, pH 8.0, 20 mM imidazole-HCl, 0.3 M NaCl and disrupted by sonication, followed by centrifugation at 27,000 × g for 90 min. The supernatant was applied to a nickel-nitrilotriacetic acid Superflow column, which was then washed thoroughly with 0.1 M sodium phosphate, pH 8.0, 60 mM imidazole-HCl, 0.3 M NaCl, 0.05% dodecyl maltoside and eluted with 0.1 M sodium phosphate, pH 8.0, 0.2 M imidazole-HCl, 0.3 M NaCl, 0.05% dodecyl maltoside. Fractions containing DF were pooled and stored at 4 °C. Protein concentration of the V$_1$ complex, the A and B subunits, were determined from UV absorbance calibrated by quantitative amino acid analysis using molar extinction coefficient of 360,000, 82,000, and 42,000 M$^{-1}$ cm$^{-1}$, respectively. The protein concentration of the A$_3$B$_3$ subcomplex was determined by the same method assuming that it has the same extinction coefficient as V$_1$. Protein concentrations of the DF subcomplex were estimated with SDS-PAGE by comparing density of D subunit with that of known concentrations of V$_1$.

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The A$_3$B$_3$ subcomplex was then equilibrated with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl. The flow rate was 0.5 ml/min. Molecular weight standards were purchased from Sigma.

**Fluorescent Labeling of Proteins**—To label the A and B subunits with a fluorescent dye, Glu$_{114}$ or Glu$_{374}$ of the A subunit and Thr$_{107}$ or Lys$_{304}$ of the B subunit were replaced with cysteine. All intrinsic cysteine residues were changed to serine or alanine (A-Cys$_{28}$, A-Cys$_{508}$, and B-Cys$_{268}$ to Ser, and A-Cys$_{255}$ to Ala). The A and B subunits, each containing the single introduced cysteine, were purified, and then incubated with 10 mM dithiothreitol for 30 min to reduce the sulfhydryl group of cysteine. After removal of dithiothreitol by gel permeation chromatography equilibrated with 20 mM MOPS-NaOH, pH 7.0, 150 mM NaCl, proteins were incubated with 5-fold molar excess of Cy$_3$-maleimide (A subunit) or Cy$_5$-maleimide (B subunit) at room temperature for 2 h. Non-reacted fluorescent dye was removed with NAP-10 (GE Healthcare) and Superdex HR200. The labeling ratios of dye to protein were 0.85, 0.89, 0.69, and 0.72, respectively, for A$_{E114C-Cy3}$, A$_{E374C-Cy3}$, B$_{T107C-Cy5}$, and B$_{K304C-Cy5}$ as estimated by spectrophotometry. The molar extinction coefficients used were 150,000 M$^{-1}$ cm$^{-1}$ at 550 nm (Cy$_3$) and 250,000 M$^{-1}$ cm$^{-1}$ at 650 nm (Cy$_5$).

**FRET Measurement between A and B Subunits**—FRET from the donor (Cy$_3$) in the A subunit to the acceptor (Cy$_5$) in the B subunit was monitored with a fluorescence spectrometer (FP-6500, JASCO). To acquire fluorescent spectra, the donor was selectively excited with light at 532 nm (bandwidth 10 nm) and the fluorescence intensity from 550 to 700 nm was scanned (bandwidth 3 nm). For time course measurements, the donor was excited with light at 532 nm (bandwidth 5 nm) and the fluorescence intensity was measured at 565 nm (bandwidth 5 nm). All measurements were performed in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mg/ml bovine serum albumin at 37 °C.

**ATPase Assay**—ATPase activity was measured at 25 °C, as previously described (24). Briefly, protein was added to a solution comprised of 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 2 mM MgCl$_2$, MgATP, and an ATP regenerating system (0.1 mg/ml pyruvate kinase, 0.1 mg/ml lactate dehydrogenase, 2 mM phospho-enolpyruvate, and 0.2 mg/ml NADH), and the decrease in absorbance at 340 nm was monitored with a spectrometer (V-550, JASCO).
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FIGURE 1. Reconstitution of \( T. \) thermophilus \( V_1 \) complex. A, isolation of \( V_1 \) components. Isolated \( V_1 \) components were analyzed by SDS-PAGE; \( V_1 \) complex (lanes 1 and 3), \( A_B \) subcomplex (lane 2), \( A \) subunit (lane 3), \( B \) subunit (lane 4), and DF subcomplex (lane 6). \( B \) reconstitution of \( V_1 \) from \( A \) and \( B \) subunits and DF subcomplex. The \( A \) and \( B \) subunits (13.3 mM) were mixed with DF subcomplex (2.1 mM). The mixtures were incubated without nucleotide (lane 3) or with 1 mM MgATP (lanes 1, 2, and 4), or with MgADP (lane 5) in 50 mM Tris–HCl, pH 8.0, 150 mM NaCl (buffer A, total volume of 15 μl) for 1 h at 37 °C, then applied to 7.5% Native PAGE, followed by staining with Coomassie Brilliant Blue. Lane 6, \( V_1 \) complex. C, reconstitution of \( V_1 \) using nucleotide analog or mutant \( A \) subunit. The mixtures of 13.3 mM B subunit and 2.1 mM DF subcomplex were incubated with 13.3 mM each of the wild-type \( A \) (lanes 1–3), \( A_{K234A/T235A} \) (lane 4), or \( A_{K277A} \) (lane 5) with the indicated nucleotides in buffer A (total volume of 15 μl) for 1 h at 37 °C. The mixtures were applied to 7.5% Native PAGE, followed by staining with Coomassie Brilliant Blue. Lane 6, \( V_1 \) complex. D, formation of subcomplexes of \( V_1 \). The mixtures of 10 μM each of \( A \) and \( B \) subunit were incubated with 0 (lane 1), 0.8 (lane 2), 1.6 (lane 3), 2.4 (lane 4), and 3.2 μM (lane 5) of DF subcomplex in the presence of 1 mM MgATP, followed by analysis on Native PAGE. Lane 6, \( V_1 \) complex. E, subunit composition of subcomplexes of \( V_1 \). Each band of Native PAGE shown in \( D \) was excised, followed by separation with SDS-PAGE. Lane 1, \( V_1 \); lane 2, the band indicated by the asterisk in \( D \); lane 3, \( A_B \); lane 4, \( F \) reconstitution of \( V_1 \) from \( A_B \) and DF subcomplexes. The mixtures of 0.4 μM \( A_B \) and 1.1 μM DF subcomplexes were incubated with (lane 4) or without (lane 1–3) 1 mM MgATP at 37 °C for 1 h, followed by analysis on Native PAGE. The gels were stained with Coomassie Brilliant Blue.

RESULTS

Requirement of ATP for Reconstitution of the \( V_1 \) Complex in Vitro—The subunit composition of the \( V_1 \) complex of \( T. \) thermophilus is \( A_B \)DF. It is an ATP-driven rotary motor, in which \( D \) and \( F \) subunits rotate relative to \( A_B \), hexamer (7, 9). There are three catalytic sites in one \( V_1 \) complex. Most of the residues that participate in catalysis are located in the \( A \) subunit (2). To analyze the assembly process of \( V_1 \) subunits, the \( A \) and \( B \) subunits, and DF subcomplex of \( T. \) thermophilus were expressed in \( E. \) coli and purified to near homogeneity (Fig. 1A). They were mixed together in the absence and presence of ATP and submitted to native polyacrylamide gel electrophoresis (Native PAGE) to determine whether the \( V_1 \) complex and/or small subcomplex is formed. In the absence of ATP, no \( V_1 \) formation was observed (Fig. 1B, lane 3). In contrast, \( A \), \( B \), and DF were assembled into the \( V_1 \) complex in the presence of ATP (Fig. 1B, lane 4). This is consistent with the previous observation that ATP is required for the in vitro reconstitution of yeast \( V_1 \) complex from yeast cell extract (20). A small band that was observed just below the \( V_1 \) complex is the \( A_B \) subcomplex (Fig. 1, B and C), which we describe later. To determine whether both ATP binding and hydrolysis are required or only ATP binding is necessary for \( V_1 \) formation, we carried out reconstitution experiments using mutant \( A \) subunits and an ATP analog. As shown in Fig. 1C, the use of a non-hydrolyzable ATP analog, AMP-PNP, instead of ATP supported assembly of subunits into the \( V_1 \) complex. We also assessed whether the \( A_{E257A} \) mutant could be incorporated into the \( V_1 \) complex. Glu in the \( A \) subunit is equivalent to Glu in the \( \beta \) subunit of \( Bacillus \) PS3 F1-ATPase, which has been suggested to be an essential residue for hydrolysis, but not for binding ATP (25, 26). When \( A_{E257A} \) was mixed with the \( B \) subunit, the DF subcomplex, and ATP, the \( V_1 \) complex was successfully reconstituted (Fig. 1C, lane 4). These results clearly suggest that hydrolysis of ATP is not necessary for \( V_1 \) formation. Moreover, \( A \), \( B \), and DF assembled into the \( V_1 \) complex when incubated with ADP (Fig. 1B, lane 5). Next, we assessed assembly using the \( A_{K234A/T235A} \) mutant (hereafter \( A_{KTA} \) mutant), in which the conserved Lys and Thr within the Walker motif A (GXXGXXGT) of the \( A \) subunit were replaced with alanine. It has been reported that the AAA + protein ClpB with this mutation in the Walker motif has greatly reduced capacity to bind nucleotide (27). Indeed, the \( A_{KTA} \) mutant lost the ability to bind the fluorescent nucleotide, mant-ADP as described below. As expected, no assembly was observed when the \( A_{KTA} \) mutant was examined (Fig. 1C, lane 5). These results indicate that binding of nucleotide to the catalytic sites is necessary for assembly of the subunits into the \( V_1 \) complex, and that hydrolysis of ATP is not necessary.

Nucleotide-dependent Formation of Subcomplexes—Because \( T. \) thermophilus \( V_1 \) is an assembly of 8 subunits, there should be several intermediate subcomplexes when \( V_1 \) subunits assemble to form the complex. Existence of intermediates in assembly is consistent with the observation that yeast strains lacking one or more genes encoding \( V_1 \) subunits accumulate various subcomplexes (28, 29). During reconstitution of the \( V_1 \) complex, we observed two bands on Native PAGE (Fig. 1D), which migrated slightly faster than the \( V_1 \) complex. This suggested formation of two large subcomplexes during the assembly process. One subcomplex migrated slower than the \( V_1 \) complex on Native PAGE even in the absence of DF and contained only \( A \) and \( B \) subunits (Fig. 1, B, lane 1; D, lane 1; E, lane 3). Thus, this subcomplex should be the \( A_B \) subcomplex (also see below). The amount of

\(^4\) All nucleotides used in this study were a 1:1 mixture with MgCl₂. For example, 1 mM ATP means 1 mM ATP and 1 mM MgCl₂.
The A$_3$B$_3$ subcomplex assembled from the A and B subunits decreased as the amount of DF subcomplex increased (Fig. 1D). On the other hand, another subcomplex was observed when the amount of DF increased (Fig. 1, D, lane 4 and 5), and contained A, B, and DF (Fig. 1E, lane 2). Because this subcomplex contained 77% of the B subunit compared with V$_1$, its subunit composition is thought to be A$_2$B$_2$DF.

Next, we purified the A$_3$B$_3$ subcomplex using gel permeation chromatography, and examined whether it can accommodate the DF subcomplex to form the V$_1$ complex. Interestingly, the A$_3$B$_3$ and DF subcomplexes assembled into the V$_1$ complex even in the absence of ATP (Fig. 1F, lane 3). This suggests that the A$_3$B$_3$ subcomplex might be a precursor of the V$_1$ complex, and that ATP, which is obligatory for V$_1$ formation, is not used for the assembly step in which the DF subcomplex is incorporated into the cavity of the A$_3$B$_3$ subcomplex. Although A$_3$B$_3$ and DF assembled into V$_1$ complex in the presence of ATP, formation of the A$_2$B$_2$DF subcomplex was also observed under these conditions (Fig. 1F, lane 4). The A$_2$B$_2$DF subcomplex might assemble from the DF and A$_1$B$_1$ heterodimer, as the A$_3$B$_3$ disassembles in the presence of ATP (see below). It is also likely that A$_2$B$_2$DF is a direct precursor of the V$_1$ complex. However, because of the instability of this subcomplex, this could not be demonstrated.

We next investigated the assembly of the A and B subunits in the absence of DF in detail. When the A and B subunits were incubated with ATP, formation of the A$_3$B$_3$ subcomplex was observed as shown in Fig. 2A. The A$_3$B$_3$ subcomplexes were also formed when AMP-PNP or AE257A was used, suggesting that hydrolysis of ATP is unnecessary (Fig. 2B, lanes 2 and 3), although the amount of A$_3$B$_3$ formed was decreased significantly compared with the case where wild-type A subunit and ATP were used. The decrease in the amount of A$_3$B$_3$ observed might suggest that A$_3$B$_3$ is stabilized by hydrolysis of bound ATP. We also analyzed the assembly of the A and B subunits using gel-permeation chromatography. As shown in Fig. 2C, only one peak with an estimated molecular mass of 62 kDa was observed in the absence of ATP. This peak should be a mixture of the monomeric A and B subunits, which have calculated molecular masses of 64 and 53 kDa, respectively. In contrast, additional 309- and 122-kDa peaks were observed in the presence of ATP (Fig. 2C), indicating the formation of the A$_1$B$_1$ heterodimer in addition to the A$_3$B$_3$ subcomplex. Both peaks were also observed when AE257A mutant was used instead of the wild-type A subunit (Fig. 2C), or when AMP-PNP was used instead of ATP (data not shown). The A$_1$B$_1$ heterodimer did not clearly resolve from the B subunit by Native PAGE (Fig. 2B, asterisks). On the other hand, when AKTAA mutant was used neither A$_3$B$_3$ nor A$_1$B$_1$ was observed (Fig. 2C). This indicates that binding of ATP to the A subunit induces or stabilizes interaction between A and B subunits and subsequent formation of A$_1$B$_1$, which would then assemble into the A$_3$B$_3$ complex. Given that the V$_1$ complex did not assemble when ATP was absent or AE257A was used, the formation of the A$_1$B$_1$ heterodimer induced by ATP binding is most likely the primary step of V$_1$ complex formation.
The amount of A$_3$B$_3$ formed was dependent on the concentration of monomeric subunits (Fig. 2D). The A and B subunits must be at or above the critical concentration, which seems to be a few micromolar, for assembly into A$_3$B$_3$.

**Nucleotide-dependent Disassembly of A$_3$B$_3$ Subcomplex**—In the absence of nucleotides, the purified A$_3$B$_3$ subcomplex was stable at 4 °C for a few days (data not shown), and appeared as a single band on Native PAGE (Fig. 3A, lane 1) and eluted as a single peak in gel-permeation chromatography (Fig. 3B). However, when A$_3$B$_3$ was incubated with ATP, a band representing the A$_3$B$_3$ subcomplex disappeared and a new band appeared on Native PAGE (Fig. 3A, lane 2). Gel-permeation chromatography also showed disappearance of A$_3$B$_3$ and emergence of a 115-kDa complex (Fig. 3B). This indicates that ATP induced disassembly of the A$_3$B$_3$ subcomplex into A$_3$B heterodimer. Disassembly of A$_3$B$_3$ was also observed when incubated with AMP-PNP or ADP (Fig. 3, A, lanes 3 and 4, and B), except that a trace amount of A$_3$B$_3$ was still present after treatment with ADP. Although A$_3$B$_3$ was completely disassembled by ATP under the conditions shown in the figure, a substantial amount of A$_3$B$_3$ remained after treatment with ATP when the concentration of A$_3$B$_3$ was much higher (data not shown). This apparent resistance of A$_3$B$_3$ to nucleotides might be caused by reassembly of A$_3$B$_3$ to form A$_3$B$_3$. Because the amount of assembled A$_3$B$_3$ is highly dependent on the concentration of the components (Fig. 2D), it does not appear that reassembly occurs at low concentrations. It is not clear why dissociation of A$_3$B$_3$ complex by addition of ATP yielded only trace amounts of the monomers (Fig. 3B), whereas addition of ATP to the monomeric A and B subunits gave a substantial amount of monomers (Fig. 2C). One possible reason for this inconsistency is that the isolated subunits used for assembly experiments contain inactive species that are partially denatured and cannot assemble. In contrast to the A$_3$B$_3$ subcomplex, nucleotide-induced disassembly was not observed for V$_1$ complex (data not shown). The presence of the central stalk must prevent nucleotide-induced conformational changes that lead to disassembly of the A$_3$B$_3$ ring.

**Asymmetric Binding of the A Subunit with B Subunits**—In the V$_1$ complex, one A subunit interacts with two B subunits, and vice versa, because the A and B subunits are arranged alternately (Fig. 4A). One of the two interfaces between A and B subunits includes a catalytic site, whereas the other does not. Thus, two A$_1$B$_1$ species might exist, i.e. A-B and B-A. To determine whether A$_1$B$_1$ assembled from the A and B monomers contains one or two A$_1$B$_1$ complexes, we tried to estimate the relative distance between two positions within the A$_1$B$_1$ subcomplex using FRET. FRET is a powerful tool for estimating distances within the complex and for investigating dynamics of various biological processes, including protein interaction and ligand binding (30). Glu$^{114}$ or Glu$^{374}$ of the A subunit, and Thr$^{107}$ or Lys$^{304}$ of the B subunit were replaced by cysteine, and the intrinsic cysteine residues were replaced by substitution. Glu$^{114}$ of the A subunit and Lys$^{304}$ of the B subunit are close to the catalytic site, whereas Glu$^{374}$ of the A subunit and Thr$^{107}$ of the B subunit are distant from the catalytic site (Fig. 4A). These unique cysteine residues were selectively labeled by Cy3 (A subunit) or Cy5 (B subunit) dyes. If the distance between Cy3 (donor) and Cy5 (acceptor) dyes are close enough, decrease in the donor fluorescent intensity and increase in the acceptor fluorescent intensity associated with interaction of the Cy3-labeled A and the Cy5-labeled B subunits was expected. First, we examined whether FRET is observed between the Cy3-labeled A and the Cy5-labeled B subunits. Donor intensity from the Cy3-labeled A subunit did not change upon addition of Cy5-labeled B subunit to a solution containing Cy3-labeled A subunit. However, when ATP was added to the mixture of Cy3-labeled A and Cy5-labeled B subunits, the donor intensity...
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The fluorescence change noted above must reflect formation of the A₂B₂ heterodimer, not the A₃B₃, under the conditions of this experiment. Next, we measured the fluorescence spectrum of a mixture of the Cy3-labeled A subunit and the Cy5-labeled B subunit at 0 and 60 min after addition of ATP. Fig. 4C shows four sets (A₁₁₄C-Cy₃ and B₁₀₇C-Cy₅, A₁₁₄C-Cy₃ and B₃₀₄C-Cy₅, A₃₇₄C-Cy₃ and B₁₀₇C-Cy₅, and A₃₇₄C-Cy₃ and B₃₀₄C-Cy₅) of fluorescence spectra. The highest FRET induced by ATP was seen between A₁₁₄C-Cy₃ and B₃₀₄C-Cy₅, whereas the lowest FRET was between A₃₇₄C-Cy₃ and B₁₀₇C-Cy₅. If the A subunits interact with the B subunit via a non-catalytic interface, higher FRET between A₃₇₄C-Cy₃ and B₁₀₇C-Cy₅ should be observed. Thus, this result strongly suggests that the A subunit interacts with the B subunit via the interface containing the catalytic site in the assembled A₁B₁.

Binding of the A Subunit with ATP Precedes Binding with the B Subunit—We next asked how A, B, and ATP assemble into the A₁B₁ heterodimer. Kinetic analyses of real-time donor fluorescent changes associated with dimerization of A₁₁₄C-Cy₃ and B₃₀₄C-Cy₅ were carried out. In this experiment, concentrations of the B subunit and ATP varied and were kept much higher than that of the A subunit, which was fixed at 4 nM to maintain the changes in the concentration of free ATP and the monomeric B subunit at negligible levels. Fig. 5A represents a typical time course of donor fluorescence change, which was well fitted with a single exponential equation, indicating that there is only one rate-limiting step in interaction of the A and B subunits. Thus, there are five possible models for A₁B₁ formation: the A subunit interacts: 1) simultaneously with the B subunit and ATP to form A₁B₁; 2) very rapidly with the B subunit first, then slowly with ATP to stabilize the complex; 3) slowly with the B subunit first, then very rapidly with ATP; 4) very rapidly with ATP first, then slowly with the B subunit; or 5) slowly with ATP first, then very rapidly with the B subunit. However, the gradual decrease in fluorescence observed clearly rules out model 2.

The apparent rate constants increased linearly with B subunit concentration (Fig. 5, B and C), consistent with models 1, 3, and 4. On the other hand, the apparent rate constants increased with ATP concentration, but were saturated at high ATP concentrations (Fig. 5, D and E). This result clearly rules out models 1, 3, and 5, because models 1 and 5 predict that apparent rate constants increase linearly with ATP concentration, and model 3 predicts that apparent rate constants decrease with ATP concentration. In contrast, B subunit concentration and ATP concentration dependence of the apparent rate constants were well fitted with the equation derived from model 4 (Fig. 5, C and E). In conclusion, the A subunit and ATP are in rapid equilibrium with A₁B₁ heterodimer, not the A₃B₃, under the conditions of this experiment.
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FIGURE 5. Kinetic analysis of FRET between A and B subunits. Donor fluorescent intensity at 565 nm of the mixture containing AE114C-Cy3 and BK304C-Cy5 was monitored at 37°C. At 300 nM, AE114C-Cy3 was fixed at 4 nM in this experiment. A, estimation of apparent rate constants of donor fluorescent change. Typical fluorescent change after addition of ATP is shown. An apparent rate constant of the fluorescent decrease was estimated by fitting donor fluorescent intensity (gray line) with a single exponential function (red line). ATP was added at time 0, and data between 0 and 20 s were excluded from analysis. B, donor fluorescent change at various concentrations of B subunit. Donor fluorescent intensity was monitored at a fixed ATP concentration (1 mM) and at various B subunit concentrations (red, 50 nM; orange, 100 nM; light green, 150 nM; blue, 200 nM; purple, 250 nM). C, apparent rate constants at various concentrations of B subunit. Apparent rate constants estimated from measurements in B were plotted against concentration of B subunit. From the y intercept of the fits with a linear segments (black line) $k_{on} = 2.0 \times 10^{-3} \text{s}^{-1}$ was deduced. D, donor fluorescent change at various concentrations of ATP. Donor fluorescent intensity was monitored at fixed B subunit (200 nM) and at various ATP concentrations (red, 0.1 mM; orange, 0.2 mM; light green, 0.4 mM; green, 0.6 mM; blue, 1.0 mM; purple, 2.0 mM). E, apparent rate constants at various concentrations of ATP. Apparent rate constants estimated from measurements in D were plotted against concentration of ATP. Fits with an equation, $k_{app} = k_{on} + [B \text{ subunit}] [ATP] / (k^{TRP}_{off} + [ATP])$, are shown as the black line, where $k^{TRP}_{off} = 1.7 \times 10^{7} \text{M}^{-1} \text{s}^{-1}$, $k_{on} = 2.0 \times 10^{-3} \text{s}^{-1}$ and $k^{TRP}_{off} = 0.28 \text{ms}$ (see text for details).

A subunit (Fig. 6B). These data clearly indicate that the monomeric A subunit can bind nucleotide. On the contrary, a similar fluorescence increase was not observed with the AKTAA mutant (Fig. 6A, bottom left panel), indicating that affinity for nucleotide was significantly decreased by the mutation. Fluorescence increase was not observed with the B subunit (Fig. 6A, bottom right panel). This observation is not consistent with previous reports that the isolated B subunit can bind nucleotide (32). The monomeric B subunit might have low affinity for mant-ADP that might not be detected using this method. We next monitored real-time changes in emission from the acceptor molecule upon addition of the A subunit. As shown in Fig. 6C, the acceptor fluorescence change was completed within 1 min at

unit was added to the mant-ADP solution, fluorescence around 435 nm, which is emitted from mant-ADP, was increased (Fig. 6A, top left panel). Excess free ATP suppressed the increase in fluorescence (Fig. 6A, top right panel). In addition, the extent of the fluorescence increase was dependent on the amount of the
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37 °C after adding the A subunit. This is much faster than formation of the A3B3 subcomplex observed using FRET (Fig. 5), and supports our conclusion that binding of ATP to the A subunit precedes binding of the B subunit.

Analysis of Continuous ATP Hydrolysis of A3B3 Subcomplex—We have previously demonstrated that the A3B3D subcomplex is the minimum unit for rotation and that the F subunit is not absolutely required for either ATP hydrolysis or assembly and disassembly (23). However, it is still unclear whether the D subunit is required for ATP hydrolysis. The ATPase activity of the A3B3 subcomplex, which assembled from wild-type A and B subunits and ATP, was measured by a coupling assay using an ATP regeneration system, but there was no detectable ATPase activity (data not shown). The A3B3 subcomplex, however, contained about 1.5 mol of ADP per mol of A3B3, suggesting that A3B3 can hydrolyze ATP but is inhibited by bound ADP. The inhibitory effect of ADP on the V1 complex was shown earlier (24). The V1 complex with the A-S232A/A-T235S mutations (we call it TSSA mutations) is much less susceptible to ADP inhibition than wild-type (7). We then measured ATPase activity of the A3B3(TSSA) subcomplex, although A3B3(TSSA) is slightly different from wild-type A3B3 in that A3B3(TSSA) disassembles in the presence of ATP into monomeric A and B subunits (data not shown), whereas wild-type subunits into A1B1. Fig. 7D shows the time-dependent change of the rate of hydrolysis of 40 μM ATP by A3B3(TSSA) indicating that the ATPase activity decelerated exponentially during hydrolysis and reached a steady-state rate within a few minutes. This time course suggests that the initial rapid hydrolysis is catalyzed by the A3B3(TSSA) free from degradation components, and that A3B3(TSSA) gradually disassembled, causing the observed decrease in activity. Finally, the steady-state hydrolysis is catalyzed by the A3B3(TSSA) where disassembly and reassembly is in equilibrium. The apparent rate constants for conversion from the active to the inactive form \( k_{a-i} \) and from the inactive to the active form \( k_{i-a} \) at initial A3B3(TSSA) concentrations of 59 nM, which were estimated from the rate of deceleration \( k_{a-i} + k_{i-a} \) and the ratio of the steady state activity to initial activity \( k_{a-i}/(k_{a-i} + k_{i-a}) \), are shown in Fig. 7D and E. The \( k_{a-i} \) and \( k_{i-a} \) values most likely correspond to disassembly and reassembly rates, respectively. According to this assumption, disassembly of the A3B3 and reassembly into the A3B3 takes \( \approx 57 \) and \( \approx 1200 \) s, respectively, at 40 μM ATP. In addition, \( k_{a-i} \) and \( k_{i-a} \) increased with the concentration of ATP (Fig. 7E), further supporting the fact that ATP is critical for both the assembly and disassembly.
DISCUSSION

Although assembly of V-ATPase subunits has been extensively investigated both in vivo (16, 17, 33) and in vitro (20, 34 – 37), how subunits assemble to form the \( V_1 \) complex has been ambiguous. In this study, we investigated the assembly pathway for the \( V_1 \) complex by reconstituting the \( T. \) thermophilus \( V_1 \) complex from its components in vitro. One of the most important findings of this study is that the binding of nucleotide to the catalytic site of the A subunit is essential for interaction of the A and B subunits to form the \( V_1 \) complex. This is consistent with previous reports that ATP is required for assembly of yeast \( V_1 \) in vitro (20) and that mutations in the Walker A motif of the A subunit leads to defective formation of a functional V-ATPase in vivo (21). Furthermore, based on both the kinetics of FRET change between A and B subunits and the mant-ADP binding assay, we found that binding of nucleotide to the monomeric A subunit precedes assembly of the A and B subunits. It was also suggested that only one of the two interfaces is involved in the nucleotide-induced interaction of the A subunit with the B subunit. There are at least two possible models for how nucleotide binding to the A subunit induces interaction between the A and B subunits. One possible model is that binding of the nucleotide changes the conformation of the A subunit into one that interacts with the B subunit. This is based on several lines of evidence that indicate that the catalytic \( \beta \) subunit of F\( _1 \)-ATPase, which has some similarity to the A subunit of V-ATPase, undergoes a large conformational change upon binding of ATP (38 – 40). Another possible model is that bound nucleotide acts as “glue” that pastes the A and B subunits together. This idea is based on the fact that ATP is bound to the catalytic site that is formed at the interface between the A and B subunits in A\( _2 \)B\( _1 \) heterodimer. In the reconstitution of \( V_1 \) complex, we also detected A\( _2 \)B\( _1 \), A\( _1 \)B\( _2 \), and A\( _2 \)B\( _1 \)DF subcomplexes. It is very likely that these subcomplexes are intermediates in the assembly process. Other possible subcomplexes, such as A\( _1 \)B\( _2 \) or A\( _1 \)B\( _2 \)DF, might exist, but could not be detected by our methods. Based on the results of this study, a model for the assembly process for \( T. \) thermophilus \( V_1 \) from its components is shown in Fig. 8. At the first step of assembly, the A subunit binds ATP, followed by binding of the B subunit, to form the A\( _2 \)B\( _1 \) subcomplex. There might be at least two pathways from the A\( _2 \)B\( _1 \) to the \( V_1 \) complex. One is that two A\( _2 \)B\( _1 \) and one DF assemble into A\( _2 \)B\( _2 \)DF, followed by assembly into the \( V_1 \) complex by binding another A\( _1 \)B\( _2 \) complex. The other is that A\( _1 \)B\( _2 \) first trimerizes into the A\( _2 \)B\( _2 \) subcomplex, which then binds the DF dimer to form the \( V_1 \) complex. Although we showed that nucleotide is required for assembly of A and B to A\( _2 \)B\( _1 \) but not for assembly of A\( _2 \)B\( _1 \) and DF to \( V_1 \), it is not clear whether or not other assembly steps require nucleotide. Of course, it should be taken into account that the assembly in vivo is more complex. For example, it has been shown for yeast V-ATPase that some populations of \( V_1 \) subunits are incorporated into the membrane-embedded \( V_o \) fraction before whole \( V_1 \) complex is assembled (33). This means \( V_o \) is sometimes assembled on the \( V_1 \) complex, and that \( V_o \) complex might affect assembly of \( V_1 \) subunits. However, assembly properties derived from in vitro reconstitution experiments would also be important for assembly in vivo.

V-ATPase belongs to a group of Walker-type ATPases, which shares a conserved motif, GXXXGK(T/S), called the Walker motif. It is known that some of Walker-type ATPases have a ring-shaped oligomeric structure like the \( V_1 \) complex, and interestingly, require nucleotide for subunit oligomerization. These include E. coli F\( _1 \)-ATPase (41), circadian clock protein KaiC (42, 43), and AAA+$\$+ chaperone ClpB (27). Nucleotide-dependent oligomerization in these complexes might proceed through the mechanism similar to that of V-ATPase.

Interestingly, the A\( _2 \)B\( _3 \) subcomplex disassembles in the presence of nucleotide. How does nucleotide induce disassembly of A\( _2 \)B\( _3 \)? It seems unlikely that ATP binding to the catalytic site causes disassembly because the A\( _2 \)B\( _3 \) with the TSSA mutation shows continuous ATPase activity. It might be possible that the A\( _2 \)B\( _3 \) subcomplex is “programmed” to disassemble after it hydrolyzes a certain number of ATP molecules. If so, the A\( _2 \)B\( _3 \) would hydrolyze a certain number of ATP molecules, on average, independent of ATP concentration. However, this mechanism is ruled out because the average number of ATPs hydrolyzed per single A\( _2 \)B\( _3 \) subunit is not constant, and also because the ATPase activity is not continuous (Fig. 7F) and because wild-type A\( _2 \)B\( _3 \), which does not show ATPase activity probably due to ADP inhibition, also disassembles by ATP. It has been suggested that a low affinity non-catalytic ATP binding site is formed on the B subunit in the \( V_1 \) complex (44 – 46) and also on the isolated B subunit (32). Thus, another possibility is that ATP binding to this low affinity site weakens the interaction between A and B subunits. This model well explains why the number of ATP hydrolyzed by the A\( _2 \)B\( _3 \) subcomplex before inactivation is large and varies with [ATP] (Fig. 7F). Why T. thermophilus V-ATPase has a system of ATP-dependent disassembly that seems quite wasteful is not clear. One possible explanation is that this system prevents the accumulation of a non-productive dead-end complex, A\( _2 \)B\( _3 \)-\( V_o \), which is formed when A\( _2 \)B\( _3 \) is
mixed with the V_o complex in vitro. Accumulation of A_3B_3-V_o will waste V_o complex, resulting in the reduction of the amount of functional V-ATPase complex. Because the A_3B_3 moiety in A_3B_3-V_o also is disassembled by nucleotide in vitro, the amount of A_3B_3 and A_3B_3-V_o must be kept at a very low level in vivo.

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