The F Subunit of *Thermus thermophilus* V$_1$-ATPase Promotes ATPase Activity but Is Not Necessary for Rotation*

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V$_1$-ATPase from the thermophilic bacterium *Thermus thermophilus* is a molecular rotary motor with a subunit composition of A$_3$B$_3$DF, and its central rotor is composed of the D and F subunits. To determine the role of the F subunit, we generated an A$_3$B$_3$D subcomplex and compared it with A$_3$B$_3$DF. The ATP hydrolyzing activity of A$_3$B$_3$D ($V_{\max} = 20$ s$^{-1}$) was lower than that of A$_3$B$_3$DF ($V_{\max} = 31$ s$^{-1}$) and was more susceptible to MgADP inhibition during ATP hydrolysis. A$_3$B$_3$D was able to bind the F subunit to form A$_3$B$_3$DF. The C-terminally truncated F$_{\text{ens}}$ subunit was also bound to A$_3$B$_3$D, but the F$_{\text{ens}}$ subunit was not, indicating the importance of residues 69–84 of the F subunit for association with A$_3$B$_3$D. The ATPase activity of A$_3$B$_3$DF$_{\text{ens}}$ was intermediate between that of A$_3$B$_3$D and A$_3$B$_3$DF. A single molecule experiment showed the rotation of the D subunit in A$_3$B$_3$D, implying that the F subunit is a dispensable component for rotation itself. Thus, the F subunit binds peripherally to the D subunit, but promotes V$_1$-ATPase catalysis.

The pH within many intracellular compartments, such as the Golgi apparatus, endosomes, and lysosomes, is regulated by a family of H$^+$-pumping ATPases known as the vacuolar H$^+$-ATPases (V-ATPases). V-ATPases exist in the membranes of these organelles of all eukaryotic cells and in the plasma membranes of some specific eukaryotic cells and are involved in a variety of physiological processes. V-ATPases are multisubunit enzymes arranged as a peripheral V$_1$ complex that is responsible for MgATP hydrolysis and that is attached to a membrane-embedded V$_0$ complex containing a proton pore. Based on the functional and structural similarity between V-ATPases and F-ATPases, it was assumed that V-ATPases use a rotary mechanism similar to that used by F-ATPases. Recent studies have shown that this is indeed the case (3, 4).

A family of V-ATPases also exists in the plasma membranes of some bacteria (5–7). One example is V-ATPase from the thermophilic bacterium *Thermus thermophilus* (5, 8–10). The *T. thermophilus* V-ATPase is capable of both ATP-driven proton translocation and proton-driven ATP synthesis *in vitro* and functions as an ATP synthase *in vivo* (4, 11). The subunit structure of this V-ATPase is simpler than that of its eukaryotic counterpart. It is composed of nine subunits, A, B, D, F, C, E, G, I, and L (10), with several lines of evidence indicating that the D, F, C, and L subunits form a central rotor and that the I, E, and G subunits constitute a stator apparatus with an A$_3$B$_3$ hexamer (3, 4, 12, 13).

The V$_1$ complex from *T. thermophilus* is ATPase-active and is thus known as V$_1$-ATPase. The V$_1$-ATPase is composed of four subunits with a presumed subunit composition of A$_3$B$_3$DF. Unlike the F$_1$-ATPases, the structure and enzymatic properties of V$_1$-ATPase are not well characterized. The central rotor in bacterial F$_1$-ATPase is composed of two subunits, $\gamma$ and $\epsilon$ (14). The high resolution structure of F$_1$-ATPase shows a hexameric arrangement of alternating $\alpha$ and $\beta$ subunits that surround a highly $\alpha$-helical $\gamma$ subunit, forming the minimum rotary unit, $\alpha_3\beta_2\gamma$ (15). The F$_1$-ATPase $\epsilon$ subunit functions as an endogenous regulator of ATPase activity (16–19). The central rotor of V$_1$-ATPase is also composed of two different subunits, D and F (3). Electron microscopic and cross-linking experiments suggest that the D subunit is located in the central cavity of the A$_3$B$_3$ hexamer and thus is a functional homolog of the $\gamma$ subunit of F$_1$-ATPase (10, 20). Site-directed and random mutagenesis studies of the yeast D subunit also suggest an analogy between the V$_1$-ATPase D subunit and the F$_1$-ATPase $\gamma$ subunit (21). In contrast, the function of the F subunit is, to date, still largely unknown.

To elucidate the function of the F subunit, two different V$_1$ complexes (A$_3$B$_3$DF and A$_3$B$_3$D) were generated and characterized. The results reveal a novel role for the F subunit in V$_1$-ATPase catalysis.

EXPERIMENTAL PROCEDURES

**Chemicals—**Pyrobest™ DNA polymerase and a DNA ligation kit were purchased from Takara (Kyoto, Japan). ATP and ADP were from Sigma. Restriction endonucleases, pyruvate kinase, lactate dehydrogenase, and NADH were obtained from Boehringer. Oligonucleotides were from Hokkaido System Science (Sapporo, Japan). All other reagents were purchased from Wako Pure Chemical Industries Inc. (Osaka, Japan) unless otherwise noted.

**Plasmids—**DNA fragments containing genes coding for the F, A, B, and D subunits were amplified from *T. thermophilus* chromosomal DNA by PCR using oligonucleotide primers 5'-CCAGGGTCATCCGGGGAAGAGGTTGATGTTGCCTCGTGAAG-3' and 5'-AGGAAGAGGGCGGTTGACGCGC-3'. After digestion with EcoRI and HindIII, the amplified fragment was ligated between the EcoRI and HindIII sites of pUC18 to obtain pG1-FABD. A His$_6$ tag was introduced at the N terminus of the A subunit by PCR-based mutagenesis to obtain pG3-FABD. Plasmid pG3-FABD was used for expression of wild-type V$_1$-ATPase. Plasmid pG1-DBA, which was used for expression of the A$_3$B$_3$D subcomplex, was constructed as follows. The gene for the D

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subunit was amplified from *T. thermophilus* chromosomal DNA by PCR using oligonucleotide primers 5'-GGCGCTGGGGCGC-3' and 5'-CTCTTAGAGGCTTAAAAGGCCGGCGCCCGCATCTCCACC-3'. The gene for the B subunit was amplified using oligonucleotide primers 5'-AGGCCACCGCGTTAGAGGTTATAAAGGCCGGCCGGAGGATCTTCGTAAG-3' and 5'-GCTCTAGCTAGTGCTATATGTAAGGGGCTTGAAG-3'. The amplified D, B, and A fragment genes were digested with EcoRI and BamHI sites of pET-17b (Novagen). The N terminus of the F subunit expressed by pET-F and pG3-FABD was modified by the addition of three amino acid residues (Met-Val-Pro) for efficient expression.

Expression and Purification of F Subunit and the A/3B3D Subcomplex—*Escherichia coli* BL21-Codon Plus-RP cells (Stratagene) harboring pG3-FABD or pG1-DBA were cultured for 20 h at 37 °C in Super broth (32 g/liter Tryptone, 20 g/liter yeast extract, and 5 g/liter sodium chloride) containing 100 μM ampicillin, 20 μg/ml chloramphenicol, and 0.6 M ammonium sulfate 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 solution was applied to a nickel-nitriiotriacetic acid; NTA, nickel-nitrilotriacetic acid; MOPS-KOH, 3-[N-morpholino]propanesulfonic acid; and 0.5% (w/v) bovine serum albumin. MgADP inhibition was determined using the BCA protein assay kit (Pierce) with bovine serum albumin as a standard. ATPase activity was measured at 25 °C in the presence of an ATP-regenerating system as described (11). Briefly, the reaction was initiated by the addition of enzyme solution (which was kept on ice for at least 30 min) to 2 ml of assay mixture consisting of 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 2 mM MgCl2, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 0.1 mg/ml pyruvate kinase, 0.1 mg/ml lactate dehydrogenase, and a range of MgATP concentrations. The rate of ATP hydrolysis was monitored continuously as the rate of oxidation of NADH, which was determined by the absorbance decrease at 340 nm. The initial ATP hydrolysis rates for both A3B3DF and A3B3D did not fit a simple Michaelis-Menten model, but did fit a typical substrate inhibition model (Scheme 1). Kinetic parameters and error estimates were determined from least-squares fits to the following equation:}

\[
E_0^* \cdot [MgATP]/V_{max}/K_s + [MgATP] + [MgATP]^2/2K_s, \text{ where } [E_0^*] \text{ is the enzyme concentration in the assay mixture.}
\]

The amount of nucleotide binding to V1-ATPase and the A3B3D subcomplex was determined as described previously (11).

**Rotation Experiments**—The His8-tagged wild-type A3B3DF and A3B3D subcomplexes were analyzed by SDS-PAGE. Lane 1, molecular mass markers; lane 2, A3B3DF; lane 3, A3B3D.

![FIG. 1. Purified A3B3DF and A3B3D subcomplexes. Purified His8-tagged wild-type A3B3DF and A3B3D subcomplexes were analyzed by SDS-PAGE. Lane 1, molecular mass markers; lane 2, A3B3DF; lane 3, A3B3D.](Image)

1 The abbreviations used are: NTA, nickel-nitriiotriacetic acid; MOPS, 4-morpholinesulfonpholic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
and 3 mM phosphoenolpyruvate). Rotation of the beads was observed at 25 °C with an Olympus IX70 bright-field microscope (magnification \( \times 1000 \)). Images of A3B3DF and A3B3D were recorded with a CCD camera at frame rates of 125 and 30 s\(^{-1} \), respectively. Analysis of rotation was performed as described previously (24).

RESULTS

The Kinetics of the A\(_{3}B_{3}D\) Subcomplex Differ from Those of A\(_{3}B_{3}DF\)—Wild-type A\(_{3}B_{3}DF\) (V\(_{1}\)-ATPase) and A\(_{3}B_{3}D\) from \( T. \) thermophilus were expressed in \( E. \) coli and purified to homogeneity (Fig. 1). After purification, the wild-type A\(_{3}B_{3}DF\) and A\(_{3}B_{3}D\) subcomplexes exhibited almost no ATPase activity due to tightly bound MgADP, which inactivates V\(_{1}\) (11). To reactivate the enzymes, the bound nucleotide was removed by phosphate/EDTA treatment as described under “Experimental Procedures.” One mol of the final A\(_{3}B_{3}DF\) and A\(_{3}B_{3}D\) subcomplexes used in this work contained 0.33 and 0.30 mol of ADP, respectively. Fig. 2 shows the time course of MgATP hydrolysis catalyzed by A\(_{3}B_{3}DF\) and A\(_{3}B_{3}D\) with the ATP-regenerating system. After the addition of A\(_{3}B_{3}DF\) or A\(_{3}B_{3}D\) to the assay mixture, an apparent short initial lag was observed. As observed previously (11), the initial lag phase was rapidly followed by a second phase of a rapid rate of hydrolysis, which decelerated slowly. Although the overall profiles of ATP hydrolysis of A\(_{3}B_{3}DF\) and A\(_{3}B_{3}D\) were similar, the ATPase activity of A\(_{3}B_{3}D\) was apparently lower than that of A\(_{3}B_{3}DF\) (Fig. 2a). To compare the steady-state kinetic parameters of A\(_{3}B_{3}DF\) and A\(_{3}B_{3}D\), the rate of rapid ATP hydrolysis after the initial lag phase was measured at various concentrations of MgATP. [S] – \( v \) plots for both A\(_{3}B_{3}DF\) and A\(_{3}B_{3}D\) exhibited typical kinetics for substrate inhibition rather than simple Michaelis-Menten kinetics (Fig. 2b). The \( V_{\text{max}} \), \( K_{m} \), and \( K_{i} \) values for A\(_{3}B_{3}DF\) were calculated to be 31 s\(^{-1} \), 0.26 mM, and 24 mM, respectively (Table I). The \( V_{\text{max}} \) and \( K_{m} \) values were consistent with previously reported values (34 s\(^{-1} \) and 0.24 mM, respectively) (11). In contrast, the \( V_{\text{max}} \) of A\(_{3}B_{3}D\) was significantly lower than that of A\(_{3}B_{3}DF\) (Table I). In addition, the \( K_{i} \) values were increased in A\(_{3}B_{3}D\) (Table I). These results suggest that the F subunit promotes V\(_{1}\) activity by increasing catalytic turnover and affinity for MgATP.

The A\(_{3}B_{3}D\) Subcomplex Is More Susceptible to Inhibition at Higher MgATP Concentrations—As described previously (11), the ATPase activity of A\(_{3}B_{3}DF\) decelerated during ATP hydrolysis due to MgADP inhibition (Fig. 2a). A similar inactivation has also been observed for CaATP hydrolysis of yeast V\(_{1}\) (25). It has been suggested that the active form of F\(_{1}\)-ATPase is converted to the MgADP-inhibited form (which is completely inactive) during MgATP hydrolysis. At the same time, the inhibited form is also converted to the active form (26). We assumed that this kind of interconversion also occurred in V\(_{1}\)-ATPase during MgATP hydrolysis. Based on this scheme, the rate constants for A\(_{3}B_{3}DF\) deceleration at 0.05, 0.2, and 2 mM MgATP were estimated to be 0.0009, 0.0020, and 0.0024 s\(^{-1} \), respectively (Table I). The estimated rate constant for deceleration of A\(_{3}B_{3}D\) ATPase activity at 2 mM MgATP was significantly higher than that for A\(_{3}B_{3}DF\) (Table I). In contrast, the rate constants at 0.05 and 0.2 mM MgATP were very similar to those for A\(_{3}B_{3}DF\) (Table I).

![Fig. 2. MgATP hydrolysis catalyzed by A\(_{3}B_{3}D\) and A\(_{3}B_{3}DF\).](image)

**Table I**

| Protein     | \( V_{\text{max}} \) \( \text{s}^{-1} \) | \( K_{m} \) \( \text{mM} \) | \( K_{i} \) \( \text{mM} \) | Inhibition rate\(^{a} \) \( \text{s}^{-1} \) |
|-------------|-----------------|-----------------|-----------------|-----------------|
| A\(_{3}B_{3}DF\) | 30.9 ± 0.8       | 0.26 ± 0.02     | 24.2 ± 3.5      | 2.4 \( \times \) 10\(^{-3} \) (2 mM) |
| A\(_{3}B_{3}D\) | 20.2 ± 0.4       | 0.49 ± 0.03     | 36.7 ± 5.4      | 6.9 \( \times \) 10\(^{-3} \) (2 mM) |
| A\(_{3}B_{3}D\) + F | 29.7 ± 0.7       | 0.24 ± 0.02     | 28.7 ± 4.3      | 3.7 \( \times \) 10\(^{-3} \) (2 mM) |
| A\(_{3}B_{3}D\) + F\(_{\text{S185-188}}\) | 23.7 ± 0.7       | 0.41 ± 0.03     | 34.5 ± 6.4      | 2.3 \( \times \) 10\(^{-3} \) (2 mM) |

\(^{a}\) Rate constants for deceleration of ATPase activity at specific MgATP concentrations (in parentheses).
Role of the F Subunit in V₁-ATPase

The F Subunit Is Not Essential for Rotary Movements of V₁-ATPase—The central rotor of V₁-ATPase from T. thermophilus is composed of two subunits, D and F, which rotate relative to the A₈B₈ hexamer (3). However, to date, it has been unclear if the F subunit is an indispensable component for rotation of V₁-ATPase. To answer this question, we attempted to observe rotation of the D subunit in A₈B₈ using a single molecule technique. All intrinsic Cys residues were replaced with Ser except for Cys²⁵⁵ of the A subunit, as it was found that the A(C255S) mutation led to a decrease in ATPase activity (Table II). Other substitutions did not significantly affect ATPase activity (Table II). The D subunit was specifically labeled with biotin (data not shown), and A₈B₈D or A₈B₈DF was immobilized on an Ni²⁺-NTA-coated glass surface via a His₈ tag. Streptavidin-coated beads (0.56 μm) attached to the D subunit were observed under a bright-field microscope after infusion of buffer containing 4 mM MgATP into the flow cell. Beads attached to A₈B₈DF were observed to rotate at ~6.1 rotation/s (Fig. 4c), which is much higher than the previously reported value of ~2.6 rotation/s (3). When A₈B₈D was used, bead rotation was also observed. Like A₈B₈DF, the direction of the rotation was always counterclockwise when viewed from the membrane side (Vₒ side). Bead rotation was also observed for A₈B₈D, although at a lower rate of ~1.8 rotation/s (Fig. 5), which is much higher than the previously reported value of ~1.8 rotation/s (3). When A₈B₈D was used, bead rotation was also observed. Like A₈B₈DF, the direction of the rotation was always counterclockwise when viewed from the membrane side (Vₒ side). Bead rotation was also observed for A₈B₈D, although at a lower rate of ~1.8 rotation/s (Fig. 5).

These results indicate that the F subunit is not an essential subunit for rotary catalysis of V₁-ATPase and that A₈B₈D is the minimum ATP-driven rotary unit of V-ATPase rather than A₈B₈DF. These results also suggest that the F subunit binds peripherally to the D subunit rather than being located within the central cavity of the A₈B₈ hexamer.

The A₈B₈ D Subcomplex Binds to the Isolated F Subunit—A₈B₈D was mixed with a 10-fold molar excess of the purified F subunit, followed by gel filtration chromatography to remove the free F subunit (Fig. 3a). As shown in Fig. 3 (b and c, lanes 5), the F subunit eluted together with the A₈B₈ D subcomplex from the gel filtration column, clearly demonstrating that A₈B₈D was able to bind to the isolated F subunit. To assay which regions of the F subunit are important for binding to A₈B₈D, we constructed six expression plasmids with deletion mutants of the F subunit (F₃₁–₈, F₃₁–₁₃, F₃₁–₂₄, F₃₁–₄₄). Among them, only F₃₁–₁₃ and F₃₁–₂₄ were expressed in E. coli. MALDI-TOF mass spectrometry showed that the F₃₁–₁₃ and F₃₁–₂₄ mutants as well as the intact F subunit were expressed as designed (data not shown). However, four mutants with deletions of N-terminal residues (F₃₁–₈, F₃₁–₁₃, F₃₁–₂₄, and F₃₁–₄₄) were not expressed in E. coli (data not shown). This suggests that the N-terminal region of the F subunit is important for folding and/or expression. Interactions between the A₈B₈ D subcomplex and F₃₁–₁₃ and F₃₁–₂₄ were also assayed by reconstitution. As shown in Fig. 3 (b and c, lanes 6 and 7), F₃₁–₁₃ bound to A₈B₈D, whereas F₃₁–₁₃ did not. These results indicate that the C-terminal 22 residues of the F subunit are involved in binding to A₈B₈D with residues 69–84 being essential.

Steady-state Kinetics and MgADP Inhibition of Reconstituted V₁-ATPases—The ATP hydrolyzing activity of the A₈B₈ D subcomplex was measured in the presence of a 20-fold molar excess of the isolated F subunit or F₃₁–₁₃ and F₃₁–₂₄. Fig. 4a shows the time course of the ATPase assay for A₈B₈D in the presence of the intact F subunit or F₃₁–₁₃ at 2 mM MgATP. MgATP hydrolysis by the A₈B₈ D subcomplex was significantly enhanced by the addition of either the F subunit or F₃₁–₁₃. The isolated F subunit did not show ATPase activity (data not shown). The addition of the F subunit to A₈B₈D resulted in a significant increase in Vₘₐₓ (20 s⁻¹) and a decrease in Kₘ (0.24 mM) and Kᵢ (29 mM) (Fig. 4b and Table I). These values are comparable with those for A₈B₈DF, suggesting that the observed differences between the A₈B₈ D and A₈B₈ D subcomplexes are due principally to the F subunit. However, the inhibition rate for A₈B₈D bound to the F subunit at 2 mM MgATP was slightly higher than that for A₈B₈DF (Table I). The reason for this higher inhibition rate is not clear at present. Binding of F₃₁–₁₃ to A₈B₈D also increased Vₘₐₓ (24 s⁻¹) and decreased Kₘ (0.41 mM), but the effect was less than that seen for the intact F subunit (Fig. 4b and Table I). These results suggest that the C-terminal 22 residues of the F subunit may play a role in catalytic events.

The reasons for the F₃₁–₁₃ mutants as different from F₃₁–₂₄ being essential.

The central cavity of the A₈B₈ hexamer.
The F subunit is a component of the V$_1$ complex of V$_0$V$_1$-ATPase, forming the central rotor of V$_1$-ATPase together with the D subunit (3). However, little is known about its function. To investigate the function of the F subunit, the A$_3$B$_3$DF (V$_1$-ATPase) and A$_3$B$_3$D subcomplexes of _T. thermophilus_ V-ATPase were prepared and characterized. The ATPase activity of A$_3$B$_3$D was significantly lower than that of A$_3$B$_3$DF. Both the $V_{\text{max}}$ and affinity for MgATP in A$_3$B$_3$D were lower compared with those in A$_3$B$_3$DF. In addition, the MgADP inhibition rate for A$_3$B$_3$D at the highest MgATP concentration was $\approx$3 times greater than that for A$_3$B$_3$DF. The reconstituted V$_1$-ATPase from A$_3$B$_3$D and the isolated F subunit exhibited the same ATPase activity kinetic parameters as V$_1$-ATPase. These results indicate that the F subunit functions as an intrinsic activator of the ATP hydrolysis reaction of V$_1$-ATPase. It is not clear at present, however, if the F subunit affects V$_1$-ATPase catalysis by directly interacting with the catalytic A subunit or by changing the conformation of the D subunit. The ATP hydrolysis reaction catalyzed by V$_1$-ATPase is thought to proceed via the following steps: 1) ATP binding to V$_1$-ATPase, 2) hydrolysis of ATP to ADP and P$_i$, and 3) release of ADP and P$_i$. The F subunit should affect Steps 2 and/or 3 because the rate of MgATP hydrolysis by A$_3$B$_3$DF is $\approx$1.5-fold higher than that of A$_3$B$_3$D under $V_{\text{max}}$ conditions in which Step 1 is no longer rate-limiting. Moreover, it seems that the F subunit also participates in Step 1 by changing the affinity of the A subunit for ATP because the $K_m$ for A$_3$B$_3$D is $\approx$2-fold higher than that for A$_3$B$_3$DF. The result of the single molecule assay that the rotation speed of A$_3$B$_3$D was lower than that of A$_3$B$_3$DF indicates that binding of the F subunit to A$_3$B$_3$D also affects rotary catalysis in the A$_3$B$_3$D rotary unit. However, the rotation speed in the single molecule experiment was significantly lower than that estimated from the ATP hydrolysis rate in the bulk phase assay (assuming that V$_1$ hydrolyzes 3 molecules of ATP/rotation) for both complexes. This indicates that the rotation speed of the bead-attached enzyme depends largely on both the torque generated by ATP hydrolysis and the frictional load due to attached beads in this experiment. Thus, the lower rotation rate for A$_3$B$_3$D compared with A$_3$B$_3$DF cannot be solely accounted for by the lower ATP hydrolysis rate for A$_3$B$_3$D in the bulk phase assay. One possible reason for the lower rotation speed of A$_3$B$_3$D is that the torque is decreased in the absence of the F subunit.

The F subunit of V-ATPase resembles the $\epsilon$ subunit of bacterial F$_o$F$_1$-ATPase in the sense that both F and $\epsilon$ subunits bind to a central shaft subunit that forms the central rotor of V$_1$ and F$_1$, respectively. In addition, the F and $\epsilon$ subunits have similar molecular masses (12–14 and 13–15 kDa, respectively). However, there are significant differences between the two subunits. Unlike the F subunit, the $\epsilon$ subunit acts as an endogenous inhibitor of ATP hydrolysis by both F$_1$- and F$_o$F$_1$-ATPases (16–19). Recent studies indicate that the $\epsilon$ subunit regulates ATP hydrolysis/synthesis of F-ATPase through a drastic conformational rearrangement of the C-terminal $\alpha$-helical domain (27–29). Moreover, it has been predicted that the structure of

**DISCUSSION**

**TABLE II**

| Protein                  | Turnover at 4 mM MgATP |
|-------------------------|------------------------|
| A(S232A/T235S)          | 52                     |
| A(C282S/S232A/T235S)    | 46                     |
| A(S232A/T235S/C507S)    | 11                     |
| A(S232A/T235S/C507S/B(C264S) | 56                 |
| A(S232A/T235S/C507S/B(C264S) | 49                 |
| A(C282S/S232A/T235S/C507S/B(C264S) | 54                 |

**ATPase activity of Cys-substituted V$_1$ mutants**

**FIG. 4** MgATP hydrolysis catalyzed by A$_3$B$_3$D bound to the F subunit and F. a, time course of ATP hydrolysis catalyzed by A$_3$B$_3$D and A$_3$B$_3$DF at 25°C and at 2 mM MgATP. The reaction was started by the addition of 20 μl of enzyme solution containing 2 μM A$_3$B$_3$D and 40 μM F subunit or F$_{\text{LAE-106}}$ (arrowhead) to 2 ml of assay mixture (see “Experimental Procedures” for details). b, $v$ vs. $[S]$ plot of MgATP hydrolysis. The rate of rapid MgATP hydrolysis after the initial lag phase (see “Results” for details) was measured at various MgATP concentrations. O, A$_3$B$_3$D + F; ●, A$_3$B$_3$D + F$_{\text{LAE-106}}$. The solid lines show fitting functions with $V_{\text{max}}$ = 29.7 s$^{-1}$, $K_m$ = 0.24 mM, and $K_i$ = 28.7 mM (A$_3$B$_3$D + F) and with $V_{\text{max}}$ = 23.7 s$^{-1}$, $K_m$ = 0.41 mM, and $K_i$ = 34.5 mM (A$_3$B$_3$D + F$_{\text{LAE-106}}$). The inset shows a magnification of the low [MgATP] range. mAU, absorbance milliunits.

**FIG. 5** Time course of rotation of beads attached to the D subunit. Shown is the bead rotation in A$_3$B$_3$DF (a) and A$_3$B$_3$D (b) at 4 mM MgATP.
the yeast F subunit has an αβ-fold and lacks a long C-terminal α-helix (30), although the ε subunit consists of an N-terminal β-sandwich and a C-terminal α-helical domain (14, 31). Taken together, these data strongly suggest that the F subunit of V-ATPase is not a functional homolog of the ε subunit of bacterial F-ATPase.

We have recently demonstrated that the central stalk region of V-ATPase is quite different from that of F-ATPase (13). In F-ATPase, the central rotor of the F$_c$ complex directly interacts with the proteolipid ring (32). In contrast, it seems that the central rotor of V$_1$ interacts directly with the C subunit (d subunit in eukaryotic V-ATPase), not with the proteolipid ring (12, 13). Cross-linking experiments based on the crystal structure of the C subunit from T. thermophila have shown that the F subunit is in close proximity to the C subunit (13). Experiments on the yeast enzyme also suggest that the F subunit is located at the interface between V$_0$ and V$_1$ (22). The distinct central stalk architecture at the interface between V$_0$ and V$_1$ of V-ATPase could be relevant to the unique reversible dissociation/association of V$_1$ and V$_0$ (1), and the F subunit might be one of the candidates for a regulator of the dissociation/association of V$_0$ and V$_1$. Although the F subunit is a very small subunit, it might be a multifunctional subunit involved in assembly, catalysis, and regulation.

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