ATP Hydrolysis and Synthesis of a Rotary Motor V-ATPase from Thermus thermophilus

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Vacuolar-type H⁺-ATPase (V-ATPase) catalyzes ATP synthesis and hydrolysis coupled with proton translocation across membranes via a rotary motor mechanism. Here we report biochemical and biophysical catalytic properties of V-ATPase from Thermus thermophilus. ATP hydrolysis of V-ATPase was severely inhibited by entrapment of Mg-ADP in the catalytic site. In contrast, the enzyme was very active for ATP synthesis (2.5 s⁻¹) with the Kₘ values for ADP and phosphate being 47 ± 0.5 and 460 ± 30 μM, respectively. Single molecule observation showed V-ATPase rotated in a 120° stepwise manner, and analysis of dwelling time allowed the binding rate constant kₑ for ATP to be estimated (~1.1 × 10⁶ M⁻¹ s⁻¹), which was much lower than the kₑ on (kₑ on = Vₑ max/Kₘ) for ADP (1.4 × 10⁷ M⁻¹ s⁻¹). The slower kₑ off than kₑ on and strong Mg-ADP inhibition may contribute to prevent wasteful consumption of ATP under in vivo conditions when the proton motive force collapses.

Vacuolar-type H⁺-ATPases (V-ATPases) are found in a wide range of organisms. V-ATPase in eukaryotes functions as an ATP hydrolysis-driven proton pump that carries out acidification of cellular compartments, such as lysosomes, and extracellular fluid in the case of renal acidification, bone resorption, and tumor metastasis (1). A family of V-ATPases is also found in archaea and some eubacteria (the prokaryotic V-ATPase family) (2–7). A major role of the V-ATPase in prokaryotes is to produce ATP, a function performed by FₒF₁ in eukaryotes and most eubacteria. V-ATPase and FₒF₁, function by a rotary ATP synthase/ATPase mechanism (1). The hydrophilic domain of both V-ATPase and FₒF₁ (called V₁ and F₁, respectively) is responsible for ATP synthesis/hydrolysis and is connected via the central rotor and peripheral stator stalks to the transmembrane domain (V₉ and F₉, respectively), which functions as an ion channel (1, 8). Although composition and arrangement of subunits differ considerably between V-ATPase and FₒF₁, they seem to share a common rotary catalysis mechanism, catalyzing the interconversion of the energy from proton translocation across membranes and the energy of ATP synthesis/hydrolysis through rotation of the central rotor subunits (8). It is thought that rotary catalysis is basically reversible (8, 9). When the transmembrane electrochemical gradient of protons (proton motive force (pmf)) is of sufficient strength, pmf drives rotation of a central rotor shaft to synthesize ATP. In contrast, when pmf is weak, the enzymes become an ATP-driven proton pump that rotates in the opposite direction driven by the energy released by ATP hydrolysis. Indeed, it has been shown that yeast V-ATPase, which functions as a proton pump in vivo, is able to catalyze ATP synthesis when exposed to an electrochemical gradient in vitro (10). In addition the F₁ portion of FₒF₁ can synthesize ATP when the rotor shaft is forced to rotate in a direction opposite that of ATP hydrolysis (11, 12). It is well known that the ATP hydrolysis reaction catalyzed by both V-ATPase and FₒF₁ is highly regulated by a number of different mechanisms to prevent wasteful ATP consumption (1, 13). One such mechanism is Mg-ADP inhibition, whereby Mg-ADP binds into the catalytic site of the V₁ and F₁ domains and thus inhibits ATP hydrolysis (14–17). Some enzymes appear to be inhibited irreversibly by these mechanisms (18, 19).

V-ATPase from the thermophilic bacterium, Thermus thermophilus, has been extensively investigated by biochemical and biophysical methods. Subunit rotation coupled to ATP hydrolysis of the V₁ portion has been visualized using a single mole-
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cule detection technique (20–22), and recent work has succeeded in resolving each step in the ATP hydrolysis reaction of the V₁ domain (23). In these previous studies, a mutant enzyme (the S232A/T235S double substitution in subunit A, the TSSA mutation) was used to investigate the suppression of Mg-ADP inhibition. However, rotation analysis of the wild type enzyme has yet to be described. Moreover, the effects of the V₀ domain on rotary catalysis by the V₁ domain, including effects on stepwise rotation, dwell time, torque, etc., remain unclear. Previous work has shown that the V-ATPase is highly active as an ATP synthase with an H⁺/ATP ratio of 4.0 ± 0.1 (24). However, the kinetic parameters (Kₘ and Vₘₐₓ) of the ATP synthesis reaction by the V-ATPase have not been reported yet. In addition, the effect of the TSSA mutation on ATP synthesis has not been investigated. It is clear that a detailed quantitative analysis of ATP synthesis/hydrolysis by the holo-enzyme (i.e. V-ATPase) is required for a deeper understanding of this remarkable rotary catalysis mechanism.

In this study, we visualized rotation steps, which correspond to each catalytic reaction, of wild type V-ATPase from a thermophilic eubacterium, *T. thermophilus*, at a single molecule level. We also quantitatively analyzed ATP synthesis using enzyme reconstituted into liposomes. It was possible to determine several kinetic parameters of V-ATPase for both ATP synthesis and hydrolysis reactions. These parameters explain why *T. thermophilus* V-ATPase has a higher preference for ATP synthesis than ATP hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Preparation of Avi Tag or His Tag Introduced V₁**—The Avi Tag sequence (GLNDIFEAQKIEWHE) was introduced at the C terminus of the V₀-c subunits using an integration vector system (26, 27). The recombinant *T. thermophilus* strain was grown at 70 °C for ~24 h under strong aeration in a medium containing 8 g of polypeptone, 4 g of yeast extract, and 2 g of NaCl/l. The harvested cells (30 g) were suspended in 200 ml of 50 mM Tris-HCl (pH 8.0) and disrupted by sonication. The membranes were precipitated by centrifugation at 96,000 × g for 30 min and the supernatant was applied to a nickel-nitritotriacetic acid (Ni²⁺-NTA) Superflow column (Qiagen), which was then washed thoroughly and eluted with 20 mM sodium phosphate (pH 8.0), 200 mM imidazole-HCl, 100 mM NaCl, and 0.05% (w/v) octaethylene glycol monododecyl ether. The fractions containing proteins were collected and dialyzed against 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.05% (w/v) octaethylene glycol monododecyl ether for 10 h at 4 °C. The dialyzed solution was applied to a Resource Q column (Amersham Biosciences). Proteins were eluted with a linear NaCl gradient, from 0 to 500 mM. The V₀ and V-ATPase were eluted in different fractions and applied to a Superdex HR200 column and eluted with 20 mM MOPS-NaOH (pH 7.0), 150 mM NaCl, and 0.05% (w/v) n-dodecyl β-d-maltoside (DDM) (buffer B). To obtain the reconstituted V-ATPase, the purified V₁ and V₀ were mixed (V₁/V₀ molar ratio was >3) for 1 h at room temperature, and unreacted V₁ was removed by a Superdex HR200 column equilibrated with buffer B. The reconstituted V-ATPase was concentrated with an Amicon Ultra filtration unit and stored at 4 °C until use.

**Protein Concentration, Bound Nucleotide, and ATP Hydrolysis Assays**—The protein concentration of V₁ was determined from UV absorbance calibrated by quantitative amino acid analysis; 1 µM gives 0.36 A at 280 nm. The protein concentrations of V₀ and V-ATPase were determined by the BCA assay kit (Pierce) using known concentrations of V₁ as standards.

Nucleotides tightly bound to wild type V₁ were removed as described previously (25). The enzyme solution was dialyzed against 100 mM sodium phosphate (pH 8.0) and 10 mM EDTA (buffer C) and heated at 65 °C for 10 min, followed by cooling on ice for 30 min; this process was repeated five times. The enzyme solution was then applied to a PD-10 column (Amersham Biosciences) equilibrated with buffer C. After repeating this application procedure several times, the enzyme solution was applied to a Superdex 200HR column and eluted with 20 mM MOPS-NaOH (pH 7.0) and 150 mM NaCl and concentrated with an Amicon Ultra filter unit. The enzyme was stored at 4 °C before use. The amount of nucleotide bound to the enzymes was measured using an ODS-80TS column (TOSOH) eluted with 100 mM sodium phosphate (pH 6.9), containing 4 mM EDTA, monitoring absorbance at 260 nm.

The ATP hydrolysis activities of the Avi-tagged reconstituted V-ATPase and the Avi-tagged V₁ were measured as follows; the reactions were initiated by the addition of V₁ or V-ATPase solutions into 1.6 ml of assay mixture consisting of 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 2 mM MgCl₂, 0.05% DDM, 0.1 mg of sodium octyl sulfate, and 10 mM ATP. The ATP hydrolysis activities were measured by the increase in the amount of ADP produced and the ADP hydrolysis activities were measured as the decrease in the amount of ATP consumed.
(w/v) DDM, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 50 µg/ml pyruvate kinase, 50 µg/ml lactate dehydrogenase, and a range of concentrations of Mg-ATP. The rate of ATP hydrolysis was monitored continuously as the rate of oxidation of the NADH, determined by the absorbance decrease at 340 nm. N, N’-Dicyclohexylcarbodiimide (DCCD) sensitivity of ATP hydrolysis activity was measured after a 1-h preincubation of 100 µM DCCD with V1 or V-ATPase. These experiments were carried out at 25 °C.

Single Molecular Analysis for ATP Hydrolysis—Streptavidin-coated beads and nickel-nitrotriacetic acid (Ni2+-NTA)-coated coverslips were prepared as described before (28, 29). A flow cell (5–10 µl) was made of two coverslips (bottom, 24 × 36 mm², and top, 24 × 24 mm²) separated by two spacers of 50-µm thickness. The glass surface of the bottom coverslip was coated with Ni2+-NTA. Buffer D (50 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.05% (w/v) DDM) containing 1 mg/ml bovine serum albumin was first applied to the flow cell and incubated for 5 min to block nonspecific binding of the enzyme. The biotinylated V1 or V-ATPase (1–10 nm) in buffer D containing 1 mg/ml bovine serum albumin was then applied to the flow cell and incubated for 5 min. Unbound V1 or V-ATPase was washed out with 20 µl of buffer D and 40 µl of 20 mM potassium phosphate (pH 8.0) containing 0.05% (w/v) DDM (buffer E). Then 0.1% (w/v) streptavidin-coated beads in buffer E were applied to the flow cell and incubated for 10 min. Unbound beads were removed by washing with 40 µl of buffer E and 40 µl of buffer D. Finally, observation of rotation was initiated after infusion of 80 µl of buffer D supplemented with a range of concentrations of Mg-ATP, 2 mM MgCl₂, 1 mM phosphoenolpyruvate, and 50 µg/ml pyruvate kinase. Rotation of the bead was recorded with a charge-coupled device camera (300-RCX, Dage-MTI, Michigan City, IN) at 30 frames/s (fps) using a phase-constant microscope (IX70, Olympus) with ×100 objective lens (N.A., 1.30, Olympus). For rapid recording, we acquired images of the rotating bead with a dark field microscope (IX70, Olympus) equipped with a mercury lamp and with a complementary metal oxide semiconductor camera (Hi-Dcam, NAC Image Technology, Tokyo) at 1,000 fps. Custom software (created by Ryohi Yasuda, Kengo Adachi, and Library) was used for analyses of the bead movements and dwelling times of steps. Time-averaged rotation speed was calculated over five consecutive revolutions. All experiments were carried out at 23–25 °C. For single molecular experiments, the Avi-tagged WT-V1 isolated from E. coli cell cultures was used for a single molecular experiment. To assess the effects of the TSSA mutation (20, 21), bulk phase ATP hydrolysis activity of the recombinant WT-V1 was measured. The recombinant V1 was referred to as WT-V1 hereafter. The ATP hydrolysis activity of the isolated WT-V1 was very low (~1.9 s⁻¹) because the enzyme contains 1.21 ± 0.05 (mean ± S.D.) mol of inhibitory ADP per 1 mol of enzyme on average. To measure the ATP hydrolysis activity of the wild type enzymes, the bound nucleotides were removed by successive phosphate/EDTA treatments as described previously (25). The WT-V1 used for measurement of ATP hydrolysis activity contained 0.38 ± 0.01 (mean ± S.D.) mol of ADP per 1 mol of enzyme after five times treatments. The ATP hydrolysis activity of WT-V1 was dependent on the amount of bound ADP (see supplemental Fig. 1), indicating that the WT-V1 is inactivated by entrapment of one ADP molecule per one enzyme molecule. The WT-V-ATPase was reconstituted from the activated V1 and V0, which was isolated from the membrane fraction of T. thermophilus cells. The ATP hydrolysis activity of the WT-V1 and WT-V-ATPase was measured at various concentrations of ATP in the presence of 0.05% of DDM. The raw data for ATP hydrolysis by the enzymes are shown in Fig. 1A. After the reaction was initiated by the addition of WT-V1, an apparent deceleration of the ATP hydrolysis rate was observed with the ATP hydrolysis activity mainly lost within 15 min. This result is similar to the ATP hydrolysis profiles by the WT-V1 in the absence of detergent (25). ATP hydrolysis by WT-V-ATPase was also observed, but the calculated inactivation rate of (3.6 ± 0.3) × 10⁻³ s⁻¹ (mean ± S.E.) was lower than that of WT-V1 of (7.9 ± 0.1) × 10⁻³ s⁻¹ (mean ± S.E.). After incubation of the activated enzymes with Mg-ADP, the ATP hydrolysis activities were completely abolished (Fig. 1A). The re-inactivated enzymes contained 1.21 ± 0.08 (mean ± S.D.) mol of ADP per 1 mol of enzyme.

Fig. 1B shows [S]−v plots for both the wild type V-ATPase and V1. The rate of ATP hydrolysis of WT-V1 obeyed simple Michaelis-Menten kinetics. The Vmax and Km values for WT-V1 were calculated to be 39.9 ± 0.3 s⁻¹ and 205 ± 7 µM (mean ± S.D.) mol of ADP per 1 mol of enzyme.

RESULTS

ATP Hydrolysis by Wild Type Enzymes—In this study, the Avi-tagged WT-V1 isolated from E. coli cell cultures was used for a single molecular experiment. To assess the effects of the TSSA mutation (20, 21), bulk phase ATP hydrolysis activity of the recombinant WT-V1 was measured. The recombinant V1 was referred to as WT-V1 hereafter. The ATP hydrolysis activity of the isolated WT-V1 was very low (~1.9 s⁻¹) because the enzyme contains 1.21 ± 0.05 (mean ± S.D.) mol of inhibitory ADP per 1 mol of enzyme on average. To measure the ATP hydrolysis activity of the wild type enzymes, the bound nucleotides were removed by successive phosphate/EDTA treatments as described previously (25). The WT-V1 used for measurement of ATP hydrolysis activity contained 0.38 ± 0.01 (mean ± S.D.) mol of ADP per 1 mol of enzyme after five times treatments. The ATP hydrolysis activity of WT-V1 was dependent on the amount of bound ADP (see supplemental Fig. 1), indicating that the WT-V1 is inactivated by entrapment of one ADP molecule per one enzyme molecule. The WT-V-ATPase was reconstituted from the activated V1 and V0, which was isolated from the membrane fraction of T. thermophilus cells. The ATP hydrolysis activity of the WT-V1 and WT-V-ATPase was measured at various concentrations of ATP in the presence of 0.05% of DDM. The raw data for ATP hydrolysis by the enzymes are shown in Fig. 1A. After the reaction was initiated by the addition of WT-V1, an apparent deceleration of the ATP hydrolysis rate was observed with the ATP hydrolysis activity mainly lost within 15 min. This result is similar to the ATP hydrolysis profiles by the WT-V1 in the absence of detergent (25). ATP hydrolysis by WT-V-ATPase was also observed, but the calculated inactivation rate of (3.6 ± 0.3) × 10⁻³ s⁻¹ (mean ± S.E.) was lower than that of WT-V1 of (7.9 ± 0.1) × 10⁻³ s⁻¹ (mean ± S.E.). After incubation of the activated enzymes with Mg-ADP, the ATP hydrolysis activities were completely abolished (Fig. 1A). The re-inactivated enzymes contained 1.21 ± 0.08 (mean ± S.D.) mol of ADP per 1 mol of enzyme.

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S.E.), respectively. The ATP hydrolysis activity of WT-V-ATPase showed similar kinetics to WT-V1 with the $V_{\text{max}}$ and $K_m$ values calculated to be $41.4 \pm 0.3$ s$^{-1}$ and $134 \pm 3$ μM (mean ± S.E.) from the [S]-v plot, respectively. These results indicate that the enzymatic properties of V-ATPase are similar to those of V1, suggesting that the catalytic properties of V1 are not affected by association of the V0 domain.

Analysis of the TSSA mutants (S232A/T235S double mutation in the A subunit) revealed almost continuous ATP hydrolysis for both TSSA-V1 and TSSA-V-ATPase in contrast to the wild type enzymes. This indicates that the mutations suppress Mg-ADP inhibition (20) (Fig. 1C). The TSSA mutant of V1 has been used for previous single molecule experiments because of this characteristic. The bound nucleotide in the TSSA enzymes was almost all removed by a single phosphate/EDTA treatment with 1 mol of the TSSA-V1, used in these experiments containing <0.03 mol of ADP. As shown in Fig. 1D, the rate of ATP hydrolysis obeyed simple Michaelis-Menten kinetics. The $V_{\text{max}}$ and $K_m$ values for the TSSA-V1 were calculated to be 55.8 ± 0.3 s$^{-1}$ and $587 \pm 21$ μM (mean ± S.E.), respectively, consistent with previous results (23). Unlike the wild type enzyme, the $V_{\text{max}}$ value for the TSSA-V-ATPase was 2-fold lower than that of TSSA-V1, 30.6 ± 0.3 s$^{-1}$ (mean ± S.E.). In contrast, the $K_m$ value for the TSSA-V-ATPase was almost identical to that of the TSSA-V1, $510 \pm 11$ μM (mean ± S.E.). The kinetic parameters for ATP hydrolysis by either V1 or V-ATPase are summarized in Table 1.

**Rotation Assay of Wild Type Enzymes**—Single molecule analysis is a powerful technique in the determination of the enzymatic properties of molecular motors as it eliminates artifacts through contamination with denatured or inactivated enzyme molecules. Although our group and others have reported direct observation of subunit rotation of V-ATPase, the rotational steps corresponding to a single ATP hydrolysis reaction could not be observed (21, 22). Observation of stepwise rotation of V-ATPase will provide mechanistic insights into the rotary catalysis and allow an assessment of the effect of V0 on the enzymatic properties of V1. In this study, we used the rotation analysis system of V-ATPase as depicted in Fig. 2A. V-ATPase was immobilized onto a Ni$^{2+}$-NTA-coating glass surface via His$_6$ tags introduced into the C termini of the V$_{0\alpha\gamma}$ subunits. This method differs from those previously described for immobilizing the protein molecules (21). As shown in Fig. 2B, highly purified V$_0$ from *T. thermophilus* membranes and recombinant V1 expressed in *E. coli* were used for the reconstitution of V-ATPase. Specific biotinylation of the A subunits in V-ATPase was confirmed by Western blotting, as shown in Fig. 2C. Fig. 2D shows the sensitivity of V-ATPase to inactivation by N,N'-dicyclohexylcarbodiimide (DCCD), a specific inhibitor that modifies a critical carboxylate in the proteolipid subunit. DCCD has been generally used as a marker to show that F$_0$F$_1$ is intact (30); if proton translocation and ATP hydrolysis are uncoupled, ATP hydrolysis activity is no longer sensitive to DCCD inhibition. The reconstituted V-ATPase was almost completely inactivated by preincubation with DCCD; the residual activity was nearly 10% after 60 min of incubation at room temperature. The ATP hydrolysis catalyzed by WT-V-ATPase, WT-V1, TSSA-V-ATPase, and TSSA-V1 were measured using the [S]-v plot of ATP hydrolysis rate catalyzed by WT-V-ATPase (open circles) and WT-V1 (filled circles) at various ATP concentrations. Error bars represent S.D. The solid lines show fit with the Michaelis-Menten equation, $V_{\text{max}} = 41.4 \pm 0.3$ s$^{-1}$, $K_m = 134 \pm 3$ μM (WT-V-ATPase), and $V_{\text{max}} = 39.9 \pm 0.3$ s$^{-1}$, $K_m = 205 \pm 7$ μM (WT-V1) (mean ± S.E.). C, time course of ATP hydrolysis catalyzed by TSSA-V-ATPase and TSSA-V1, at 4 min ATP. Upper, activated enzymes; lower, ADP-inhibited enzymes. D, [S]-v plot of ATP hydrolysis rate catalyzed by TSSA-V-ATPase (open circles) and TSSA-V1 (filled circles) at various ATP concentrations. Error bars represent S.D. The solid lines show fit with the Michaelis-Menten equation, $V_{\text{max}} = 30.6 \pm 0.3$ s$^{-1}$, $K_m = 587 \pm 21$ μM (TSSA-V-ATPase), and $V_{\text{max}} = 55.8 \pm 0.3$ s$^{-1}$, $K_m = 510 \pm 11$ μM (TSSA-V1) (mean ± S.E.).

**TABLE 1**

| Protein             | $V_{\text{max}}$ s$^{-1}$ | $K_m$ μM | $k_{\text{cat}}$ for ATP μs$^{-1}$ | $K_m \times k_{\text{cat}}$ μM s$^{-1}$ |
|---------------------|---------------------------|----------|-----------------------------------|--------------------------------------|
| WT-V-ATPase         | 41.4 ± 0.3                | 134 ± 3  | (1.03 ± 0.04) × 10$^6$ (1 μM)     | 143                                  |
| WT-V1               | 39.9 ± 0.3                | 205 ± 7  | (1.11 ± 0.01) × 10$^6$ (2 μM)     | 271                                  |
| TSSA-V-ATPase       | 30.6 ± 0.3                | 587 ± 21 | (1.38 ± 0.02) × 10$^6$ (0.5 μM)   | 34.7                                  |
| TSSA-V1             | 55.8 ± 0.3                | 510 ± 11 | (6.21 ± 0.23) × 10$^7$ (10 μM)    | 87.7                                  |

This indicates that the mutations suppress Mg-ADP inhibition (20) (Fig. 1C). The TSSA mutant of V1 has been used for previous single molecule experiments because of this characteristic. The bound nucleotide in the TSSA enzymes was almost all removed by a single phosphate/EDTA treatment with 1 mol of the TSSA-V1, used in these experiments containing <0.03 mol of ADP. As shown in Fig. 1D, the rate of ATP hydrolysis obeyed simple Michaelis-Menten kinetics. The $V_{\text{max}}$ and $K_m$ values for the TSSA-V1 were calculated to be 55.8 ± 0.3 s$^{-1}$ and $587 \pm 21$ μM (mean ± S.E.), respectively, consistent with previous results (23). Unlike the wild type enzyme, the $V_{\text{max}}$ value for the TSSA-V-ATPase was 2-fold lower than that of TSSA-V1, 30.6 ± 0.3 s$^{-1}$ (mean ± S.E.). In contrast, the $K_m$ value for the TSSA-V-ATPase was almost identical to that of the TSSA-V1, $510 \pm 11$ μM (mean ± S.E.). The kinetic parameters for ATP hydrolysis by either V1 or V-ATPase are summarized in Table 1.
temperature. In contrast, the isolated V$_1$ showed only a slight drop in activity under the same conditions. These results indicate that the engineered V-ATPase used for the rotation assay was fully functional.

The wild-type enzymes have a strong propensity to lapse into the Mg-ADP inhibited form during catalytic turnover; the rate of inactivation of enzyme is dependent on the ATP concentration in the assay buffer. At 4 mM ATP, almost half the WT-V-ATPase molecules are inhibited within 5 min of addition of ATP, and nearly all molecules cease ATP hydrolysis within 15 min (see Fig. 1A). The inhibition rate is too fast to allow observation of the rotating molecules under ATP-saturated conditions. Below 2 $\mu$M ATP, however, it was possible to observe the rotating beads. A bead attached to the A subunit of WT-V-ATPase rotated stepwise, pausing every 120° (Fig. 3, A and B), like V$_1$. The dwell time between successive 120° steps in WT-V-ATPase at low ATP concentrations corresponds to the time that WT-V-ATPase is waiting for binding of ATP, because binding of ATP is the rate-limiting step under these conditions. Based on histogram analysis (Fig. 3, C and D), $k_{on}$ for ATP of WT-V-ATPase was estimated to be $(1.03 \pm 0.04) \times 10^6$ M$^{-1}$ s$^{-1}$ and $(1.11 \pm 0.01) \times 10^6$ M$^{-1}$ s$^{-1}$ (mean ± S.E.) at both 1 and 2 $\mu$M ATP, respectively.

Rotation of activated WT-V$_1$ was also investigated in the presence of 0.05% DDM. To visualize the rotation of the D subunit, the enzymes were immobilized on a Ni$_2$-NTA-coated glass surface by a His$_6$ tag introduced into the A subunit, and a duplex of streptavidin-coated beads was attached to the biotin-labeled D subunit. To determine the $k_{on}$ value for ATP to WT-V$_1$ in the presence of detergent, we observed stepwise rotation of WT-V$_1$ at low ATP concentration. Stepwise rotational beads were found at both 0.5 and 2 $\mu$M ATP (Fig. 3, E and F). Based on the histogram analysis for dwell times (Fig. 3, G and H), the $k_{on}$ value of WT-V$_1$ for ATP was estimated at 0.5 and 2 $\mu$M ATP to be $(1.38 \pm 0.02) \times 10^6$ M$^{-1}$ s$^{-1}$ and $(1.26 \pm 0.01) \times 10^6$ M$^{-1}$ s$^{-1}$ (mean ± S.E.), respectively. The $k_{on}$ value of WT-V$_1$ is nearly equal to that of WT-V-ATPase. The $V_{max}$ of WT-V$_1$ for ATP hydrolysis is roughly estimated to be $\approx 270$ s$^{-1}$ as a product of $k_{on}$ of $1.3 \times 10^6$ M$^{-1}$ s$^{-1}$ (single molecular analysis) and a $K_m$ of 205 $\mu$M (bulk-phase analysis). These kinetic parameters estimated by single molecule assay are summarized in Table 1.

**Single Molecule Analysis of TSSA Mutants**—Rotation of single molecules of the TSSA-V-ATPase was also investigated. The rotational speed was basically proportional to the ATP concentration between 10 and 100 $\mu$M (Fig. 4A). At 4 mM ATP, a rotational rate of 9 revolutions/s was observed. One-third of the bulk-phase ATP hydrolysis rate of the TSSA-V-ATPase was roughly equal to the rotation rate below 500 $\mu$M ATP, suggesting that three ATPs were consumed per rotation. Below 50 $\mu$M ATP, the bead attached to the A subunit rotated stepwise, pausing every 120° (Fig. 4, B and C), like WT-V-ATPase. The dwell time between successive 120° steps in the TSSA-V-ATPase at low ATP concentrations corresponds to the time that the TSSA-V-ATPase is waiting for binding of ATP. Fig. 4, D and E, show histograms of the dwell times at 10 and 20 $\mu$M ATP, respectively. By fitting the dwell times with a single exponential equation, $k_{on}$ for ATP to the TSSA-V-ATPase was estimated at 10 and 20 $\mu$M ATP to be $(6.21 \pm 0.23) \times 10^6$ M$^{-1}$ s$^{-1}$ and $(5.62 \pm 0.11) \times 10^6$ M$^{-1}$ s$^{-1}$ (mean ± S.E.), respectively. These values for the TSSA-V-ATPase are about 20-fold lower than that of WT-V-ATPase, suggesting that the TSSA mutant V-ATPase has reduced affinity for ATP.

The rotation of the TSSA-V$_1$ was also investigated in the presence of detergent. Similar to the TSSA-V-ATPase, the mutant V$_1$ exhibited an ATP hydrolysis rate comparable with the rotation rate of the enzyme concentrations of ATP of less than 100 $\mu$M (see supplemental Fig. 2A). The TSSA-V$_1$ showed...
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![Figure 3](Image)

**FIGURE 3.** A-D, ATP-driven rotation of WT-V-ATPase. Rotation was visualized under a microscope by attaching a duplex of 220 nm beads to the A subunit. A, stepwise rotation of the V-ATPase at 1 μM ATP recorded at 30 fps. B, stepwise rotation at 2 μM ATP recorded at 30 fps. Insets, A and B, centroid of the rotating bead. C, histogram of dwell time between successive steps at 1 μM ATP (n = 1500, 13 molecules) fitted with a single exponential equation: \( k_{\text{on}} = (1.03 \pm 0.04) \times 10^6 \text{M}^{-1} \text{s}^{-1} \) (mean ± S.E.). D, histogram of dwell time between successive steps at 2 μM ATP (n = 1598, 9 molecules) fitted with a single exponential equation: \( k_{\text{on}} = (1.11 \pm 0.01) \times 10^6 \text{M}^{-1} \text{s}^{-1} \) (mean ± S.E.). E-H, ATP-driven rotation of WT-V1. Rotation was visualized under a microscope by attaching a duplex of 220 nm beads to the D subunit. E, stepwise rotation of the D subunit at 0.5 μM ATP recorded at 30 fps. F, stepwise rotation of the D subunit at 2 μM ATP recorded at 30 fps. Insets, E and F, the centroid of the rotating bead. G, histogram of dwell time between successive steps at 0.5 μM ATP (n = 440, 5 molecules) fitted with a single exponential equation: \( k_{\text{on}} = (1.38 \pm 0.02) \times 10^6 \text{M}^{-1} \text{s}^{-1} \) (mean ± S.E.). H, histogram of dwell time between successive steps at 2 μM ATP (n = 919, 5 molecules) fitted with a single exponential equation: \( k_{\text{on}} = (1.26 \pm 0.01) \times 10^6 \text{M}^{-1} \text{s}^{-1} \) (mean ± S.E.).

![Figure 4](Image)

**FIGURE 4.** ATP-driven rotation of TSSA-V-ATPase. Rotation was visualized under a microscope by attaching a duplex of 220 nm bead to the A subunit. A, ATP dependence of rotation rate and bulk-phase ATP hydrolysis rate. Time-averaged rotation rates of the A subunit of single molecule TSSA-V-ATPase (open squares) and one-third of bulk-phase ATP hydrolysis rates (filled circles) are plotted against ATP concentration. Error bars represent S.D. Using the ATP concentration dependence of the rates observed with the Michaelis-Menten equation, bead rotation (dotted line) occurred with a \( V_{\text{max}} \) of 6.9 ± 0.4 Hz and a \( K_m \) of 288 ± 60 μM, and bulk-phase ATP hydrolysis (solid line) occurred with a \( V_{\text{max}} \) of 10.2 ± 0.1 s⁻¹ and a \( K_m \) of 587 ± 21 μM (mean ± S.E.). B, stepwise rotation of the TSSA-V-ATPase at 10 μM ATP recorded at 30 fps. C, stepwise rotation at 20 μM ATP recorded at 30 fps. Insets, B and C, the centroid of the rotating bead. D, histogram of dwell time between successive steps at 10 μM ATP (n = 578, 13 molecules) fitted with a single exponential equation: \( k_{\text{on}} = (6.21 \pm 0.23) \times 10^6 \text{M}^{-1} \text{s}^{-1} \) (mean ± S.E.). E, histogram of dwell time between successive steps at 20 μM ATP (n = 1162, 14 molecules) fitted with a single exponential equation: \( k_{\text{on}} = (5.62 \pm 0.11) \times 10^6 \text{M}^{-1} \text{s}^{-1} \) (mean ± S.E.).

stepwise rotation below 10 μM of ATP concentration (see supplemental Fig. 2, B and C). The \( k_{\text{on}} \) value for ATP of the TSSA-V1 was estimated at 4 and 10 μM ATP to be (1.93 ± 0.03) × 10^6 M⁻¹ s⁻¹ and (1.51 ± 0.02) × 10^6 M⁻¹ s⁻¹ (mean ± S.E.), respectively (see supplemental Fig. 2, D and E). These values are comparable with previous results in the absence of detergent (23).

Kinetic Analysis of ATP Synthesis by V-ATPase—The physiological role of the V-ATPase of *T. thermophiles* is ATP synthesis (14). To determine kinetic parameters for the ATP synthesis reaction, the isolated WT- and TSSA-V-ATPase were reconstituted into proteoliposomes using a freeze-thaw and sonication method. The proteoliposomes were energized by a
transmembrane pH difference (acid-base transition: pH$_{\text{out}}$ = 8.4, pH$_{\text{in}}$ = 4.9) and a K$^+$/valinomycin diffusion potential ([K$^+$_out] = 100 mM, [K$^+$_in] = 1 mM). The generated pmf is estimated to be 295 mV from the Nernst equation. The ATP synthesized by the enzymes was measured by a luciferin/luciferase system as described under “Experimental Procedures.” A, [S]-v plot of ATP synthesis rate catalyzed by WT-V-ATPase (open circles, WT) and TSSA-V-ATPase (open circles, TSSA) at various ADP concentrations in the presence of 10 mM sodium phosphate. The solid lines show fit with the Michaelis-Menten equation. $V_{\text{max}}$ = 67.4 ± 1.5 s$^{-1}$, $K_m$(ADP) = 4.7 ± 0.5 μM (WT-V-ATPase), and $V_{\text{max}}$ = 14.9 ± 0.5 s$^{-1}$, $K_m$(ADP) = 17.1 ± 3.1 μM (TSSA-V-ATPase) (mean ± S.E.). B, [S]-v plot of ATP synthesis rate catalyzed by WT-V-ATPase (filled circles, WT) and TSSA-V-ATPase (open circles, TSSA) at various phosphate concentrations in the presence of 1.1 mM ADP. The solid lines show fit with the Michaelis-Menten equation. $V_{\text{max}}$ = 73.2 ± 1.3 s$^{-1}$, $K_m$(Pi) = 0.46 ± 0.03 mM (WT-V-ATPase) and $V_{\text{max}}$ = 14.6 ± 0.5 s$^{-1}$, $K_m$(Pi) = 1.06 ± 0.11 mM (TSSA-V-ATPase) (mean ± S.E.).

### DISCUSSION

In this study, we investigated the detailed enzymatic properties of *T. thermophilus* V-ATPase using biophysical and biochemical methods. A comparison of ATP-driven rotation of wild type V-ATPase with that of the V$_1$ domain indicates that both rotate stepwise, pausing roughly every 120°. The histograms of dwell time between steps, which corresponds to time waiting for ATP, revealed that the binding rate constant $k_{on}$ of wild type V-ATPase is nearly identical to that of the wild type V$_1$. In addition kinetic parameters of V-ATPase measured by bulk phase analysis were similar to those of V$_1$ (Table 1). These results indicate that the enzymatic properties of V$_1$ are not affected by association with V$_0$.

The ATP hydrolysis activity of WT-V-ATPase from *T. thermophilus* is easily abolished by Mg-ADP, whereas the enzyme is capable of effective ATP synthesis from ADP and phosphate using pmf (Fig. 5). The F$_0$F$_1$ from a thermophilic eubacterium *Bacillus* sp. PS3 also entraps an inhibitory Mg-ADP in a catalytic site, resulting in enzyme inhibited for ATP hydrolysis (31). However, this entrapped Mg-ADP in the F$_0$F$_1$ is capable of being released from the catalytic site (32, 33). Thus, the F$_0$F$_1$ from PS3 can catalyze both the ATP synthesis/hydrolysis directions. In this respect, the V-ATPase of *T. thermophilus* seems to be more similar to the F$_0$F$_1$ from *Paracoccus denitrificans*, which catalyzes rapid ATP synthesis coupled to the pmf, whereas ATP hydrolysis occurs but at a very low rate (19).

Single molecule analysis for ATP hydrolysis by V-ATPase, indicated a binding rate constant $k_{on}$ of 1.1 × 10$^6$ M$^{-1}$ s$^{-1}$ for ATP. This value is ~30-fold lower than that of the F$_0$F$_1$ from PS3 (3.6 × 10$^7$ M$^{-1}$ s$^{-1}$) (30), suggesting a much slower rate of ATP binding by V-ATPase from *T. thermophilus* during ATP hydrolysis.

We also determined kinetic parameters for the ATP synthesis reaction. If V-ATPase is evenly oriented within the liposomes, the $V_{\text{max}}$ of ATP synthesis is estimated to be ~140 s$^{-1}$. This value is nearly equal to the $V_{\text{max}}$ of 147 s$^{-1}$, a product of $k_{on}$ (1.1 × 10$^6$ M$^{-1}$ s$^{-1}$, from single molecule analysis) and $K_m$ (134 mM, from bulk phase analysis) obtained from this study. The estimated $k_{on}$ ($V_{\text{max}}/K_m$) for ADP is 1.4 × 10$^7$ M$^{-1}$ s$^{-1}$, ~13-fold higher than the $k_{on}$ for ATP during ATP hydrolysis. These two enzymatic properties of the V-ATPase from *T. thermophilus*, 1) lower $k_{on}$ for ATP and 2) irreversible inactivation because of Mg-ADP inhibition, might prevent wasteful consumption of ATP when the pmf has collapsed because of inhibition of respiration, for example, at mesophilic temperatures.

We have also investigated the enzymatic properties of the TSSA mutant enzymes, which have low propensity for Mg-ADP inhibition during ATP hydrolysis. Single molecule analysis for ATP hydrolysis revealed that the TSSA mutants of V$_1$ and V-ATPase have apparently lower $k_{on}$ values for ATP than the wild type enzymes. In addition, TSSA-V-ATPase has an

### TABLE 2

| Protein          | Substrate | $V_{\text{max}}$ | $K_m$ | $V_{\text{max}}/K_m$ |
|------------------|-----------|------------------|-------|----------------------|
| WT-V-ATPase      | ADP       | 67.4 ± 1.5       | 4.7 ± 0.5 μM | 1.43 × 10$^7$ |
|                  | P$_i$     | 73.2 ± 1.3       | 0.46 ± 0.03 mM | 1.59 × 10$^5$ |
| TSSA-V-ATPase    | ADP       | 14.9 ± 0.5       | 17.1 ± 3.1 μM | 8.71 × 10$^5$ |
|                  | P$_i$     | 14.6 ± 0.5       | 1.06 ± 0.11 mM | 1.38 × 10$^6$ |

The ATP synthesis reaction was performed as described under “Experimental Procedures.” $A$, [S]-v plot of ATP synthesis rate catalyzed by WT-V-ATPase (filled circles, WT) and TSSA-V-ATPase (open circles, TSSA) at various ADP concentrations in the presence of 10 mM sodium phosphate. The solid lines show fit with the Michaelis-Menten equation. $V_{\text{max}}$ = 67.4 ± 1.5 s$^{-1}$, $K_m$(ADP) = 4.7 ± 0.5 μM (WT-V-ATPase), and $V_{\text{max}}$ = 14.9 ± 0.5 s$^{-1}$, $K_m$(ADP) = 17.1 ± 3.1 μM (TSSA-V-ATPase) (mean ± S.E.). $B$, [S]-v plot of ATP synthesis rate catalyzed by WT-V-ATPase (filled triangles, WT) and TSSA-V-ATPase (open triangles, TSSA) at various phosphate concentrations in the presence of 1.1 mM ADP. The solid lines show fit with the Michaelis-Menten equation. $V_{\text{max}}$ = 73.2 ± 1.3 s$^{-1}$, $K_m$(Pi) = 0.46 ± 0.03 mM (WT-V-ATPase) and $V_{\text{max}}$ = 14.6 ± 0.5 s$^{-1}$, $K_m$(Pi) = 1.06 ± 0.11 mM (TSSA-V-ATPase) (mean ± S.E.).
Rotary Catalysis of V-ATPase in Both Directions

~20-fold lower $V_{\text{max}}/K_m$ value for ADP than wild type in the ATP synthesis reaction (Table 2). The bound-ADP in TSSA-V$_{1}$ was mostly removed by one phosphate/EDTA treatment even though the wild type enzyme still contains ~0.3 mol of ADP per 1 mol of enzyme after five successive phosphate/EDTA treatments (data not shown). These results indicate that the binding affinity to ADP is decreased in the TSSA mutants. Noticeably, the $V_{\text{max}}$ rate of TSSA V-ATPase for ATP synthesis was about five times lower than that of wild type V-ATPase. This suggests that one or more of the elementary step(s) involved in ATP synthesis are prolonged by the TSSA mutation.

In TSSA-V-ATPase, the ATP hydrolysis rate of ~35 s$^{-1}$ calculated from the $K_m$ from the bulk phase analysis and $k_{\text{on}}$ of the single molecule analysis is nearly equal to the $V_{\text{max}}$ value (~30 s$^{-1}$) of bulk phase analysis. Indeed, the ATP hydrolysis rate of TSSA-V-ATPase estimated from single molecule analysis is consistent with that obtained from bulk phase analysis at a range of from 4 to 1000 µM ATP (Fig. 4A). These results indicate that most of the TSSA-V-ATPase molecules are active for ATP hydrolysis. In contrast, ATP hydrolysis by wild type V-ATPase has a $V_{\text{max}}$ value (~41 s$^{-1}$) from bulk phase analysis much lower than 143 s$^{-1}$, a product of $K_m$ (bulk phase) and $k_{\text{on}}$ (single molecule). The $V_{\text{max}}$ value of wild type V$_{1}$ (~40 s$^{-1}$) of bulk phase analysis was also much lower than ~270 s$^{-1}$, a product of $K_m$ (bulk phase) and $k_{\text{on}}$ (single molecule). This inconsistency is likely caused by contamination with Mg-ADP-inhibited wild type V-ATPase.

In this study, we investigated the ATP synthesis reaction of the V-ATPase only by bulk phase analysis, and the kinetic parameters obtained indicate that the enzyme avoids wasteful consumption of ATP when the pmf has collapsed in vivo. Future work will aim to observe the ATP synthesis reaction using single molecule techniques which will further clarify the kinetic parameters for ATP synthesis.

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