Molecular Basis of ADP Inhibition of Vacuolar (V)-type ATPase/Synthase*

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Background: ADP inhibition of rotary ATPases is a common mechanism to avoid wasteful ATP hydrolysis.

Results: Domain swap approaches in V1 showed that domain interaction plays a key role in sensitivity of ADP inhibition.

Conclusion: Increasing the affinity of V1 for phosphate correlates with reducing sensitivity to ADP inhibition.

Significance: The molecular basis of ADP inhibition of V0V1 is clarified.

Reduction of ATP hydrolysis activity of vacuolar-type ATPase/synthase (V0V1) as a result of ADP inhibition occurs as part of the normal mechanism of V0V1 of Thermus thermophilus but not V0V1 of Enterococcus hirae or eukaryotes. To investigate the molecular basis for this difference, domain-swapped chimeric V1, consisting of both T. thermophilus and E. hirae enzymes were generated, and their function was analyzed. The data showed that the interaction between the nucleotide binding and C-terminal domains of the catalytic A subunit from E. hirae V1 is central to increasing binding affinity of the chimeric V1 for phosphate, resulting in reduction of the ADP inhibition. These findings together with a comparison of the crystal structures of T. thermophilus V1 with E. hirae V1 strongly suggest that the A subunit adopts a conformation in T. thermophilus V1 different from that in E. hirae V1. This key difference results in ADP inhibition of T. thermophilus V1 by abolishing the binding affinity for phosphate during ATP hydrolysis.

Vacuolar-type ATPase/synthase (V0V1)3 functions as an ATP hydrolysis-driven proton pump that carries out acidification of cellular compartments in eukaryotes (1). A family of V0V1, sometimes referred to as the A-type ATPases or A3B3 (2), is also found in archaea and some eubacteria (the prokaryotic V0V1 family) (3). In most prokaryotes, such as Thermus thermophilus, the V0V1 functions as an ATP synthase. However, it can also act as a primary ion pump as seen in Enterococcus hirae (4).

Similar to F-type ATP synthase (F0F1), V0V1 consists of two distinct motor subcomplexes: hydrophilic V1, which catalyzes either ATP hydrolysis or synthesis, and hydrophobic V0, which is responsible for proton translocation across membranes (see Fig. 1a). The two motors are coupled through a central rotor complex that rotates against a surrounding stator apparatus (3, 5–7).

ADP inhibition caused by entrapment of ADP at a catalytic site is believed to be a regulatory mechanism of F0F1 to prevent wasteful ATP consumption when proton motive force is lost (8, 9). V0V1 from T. thermophilus (TthV0V1) also exhibits sensitivity to ADP inhibition, resulting in rapid decay of the ATPase activity of the V1 subcomplex (10, 11). ADP inhibition has been investigated in F0F1, but the precise molecular mechanism remains poorly understood (8).

The prokaryotic V1 is composed of four different subunits with a stoichiometry of A3B3D (A3B3DG in E. hirae) (3). A3B3 forms a hexameric ring in which three A and B subunits are alternately arranged (12). The D and F (G in E. hirae) subunits form the rotary shaft; the D subunit penetrates the central cavity of the A3B3 ring, and the F subunit binds to the protruding part of subunit D (13–15). The catalytic reaction center resides at the A-B interface mainly on the A subunit. The A subunit consists of three subdomains given the abbreviations NT (N-terminal), NB (nucleotide-binding), and CT (C-terminal) based on their individual structures and functions (see Figs. 1b and 2) (12–14). The NT domain forms a β-barrel structure with adjacent subunits and is thought to be important for the formation of the A3B3 hexamer (12). The NB domain includes the Walker A and B motifs that form a nucleotide-binding site (Fig. 2). Thus, the NB domain functions as a catalytic center. The CT domain interacts with the rotary shaft during rotary catalysis (13, 14).

The primary sequences of the V1 from T. thermophilus (TthV1) and E. hirae (EhiV1) are highly conserved (for example, the two A subunits share 57.8% sequence identity and 79.5% sequence similarity, respectively; see Fig. 2). Furthermore, the overall structures of the two V1 subcomplexes are almost identical (13, 14). However, there is a significant functional difference between the two V1 subcomplexes. The TthV1 rapidly lapses into an ADP inhibition state during ATP hydrolysis and ultimately loses all ATPase activity (10). This ADP inhibition mechanism of TthV0V1 is advantageous as it prevents consumption of ATP when proton motive force is lost. In contrast, because the E. hirae and eukaryotic V0V1 enzymes function as ion pumps coupled with continuous ATP hydrolysis, they do
not exhibit sensitivity to ADP inhibition. Indeed, a rapid decrease of ATPase activity of the \textit{E. hirae} and eukaryotic V0V1 has not been reported (1, 16).

In this study, domain swapping was applied to the V1-A subunit to investigate the factors that define sensitivity to ADP inhibition using V1 from \textit{T. thermophilus} and \textit{E. hirae}. The results indicate that sensitivity to ADP inhibition is not due to differences in the NB domains of the two V1-ATPases. We have experimentally demonstrated the relationship between the affinity for Pi and sensitivity to ADP inhibition of V1. With recent structural studies of both V1-ATPases (13, 14), we discuss the molecular basis of the ADP inhibition mechanism of V0V1.

**EXPERIMENTAL PROCEDURES**

Preparation of Chimeric V1.—The expression plasmids for chimeric V1 were constructed by an overlap PCR-based method. DNA fragments for a subunit gene for chimeric V1 were amplified from \textit{TthV1} expression plasmid (17) and pCemtp18 (18), which contains \textit{EhiV1} (ntp) operon, by PCR using oligonucleotide primers, respectively. These PCR fragments contain sequences complementary to each other. They were mixed and submitted to a further round of amplification using the 5’- and 3’-terminal primers used in the first PCR to produce a single fragment containing the chimeric gene sequence. The chimeric gene sequences were then digested with appropriate restriction enzymes and inserted into the corresponding region of the \textit{TthV1} expression plasmid (17) (see Fig. 3). Escherichia coli strain BL21-CodonPlus-RP (Stratagene) was used for expression of chimeric V1. The chimeric V1 constructs were isolated as described previously (17). Cells containing the expressed proteins were suspended in equilibration buffer (20 mM imidazole/HCl (pH 8.0) containing 300 mM NaCl and disrupted by sonication. After removal of debris by centrifugation at 4,500 \(\times\) g for 30 min, the supernatant was applied to a nickel-affinity column (Qiagen; 3 \(\times\) 5 cm), the column was washed thoroughly with equilibration buffer, and bound protein was eluted with 200 mM imidazole/HCl (pH 8.0) containing 300 mM NaCl. The V1 was exchanged into 20 mM Tris/HCl (pH 8.0), 1 mM EDTA by ultrafiltration (Millipore, Amicon Ultra), and the dialysis solution was applied to a UNOQ column (BioRad) for further purification. The fractions containing chimeric
V1 were concentrated, and contaminating proteins were removed on a Superdex HR-200 column equilibrated with MOPS buffer (20 mM MOPS, pH 7.0, 100 mM NaCl).

Single Molecule Observation for ATP Hydrolysis—For single molecular observation experiments, cysteine residues were introduced into the D subunit in the V1-A011. Then the modified V1-A011 was biotinylated as described previously (5, 11). The single molecule observation system using magnetic beads was described previously (11). Briefly, the biotinylated chimeric V1 was bound to Ni2+-nitrilotriacetic acid-coated coverslips via their His tag. Then streptavidin-coated magnetic beads (nominal diameter, 200 nm; Thermo Fisher Scientific) were bound to the chimeric V1, and finally, observation of the rotation was initiated after infusion of buffer containing Mg-ATP. To observe the rotation under a low viscous load, instead of the magnetic beads, we used gold beads (nominal diameter, 80 nm; BBInternational, Cardiff, UK) that functionalized with Neutravidin (Pierce) and polyethylene glycol (molecular weight, 1214; Quanta BioDesign, Powell, OH) (19). The rotation was observed on an inverted microscope (IX71, Olympus, Tokyo, Japan) using a 100× objective lens (UPlanApo; numerical aperture, 1.35; Olympus) and a condenser unit (U-UCD8, U-TLD, Olympus) with a stable microscope stage (KS-O, ChuukoushaSeisakujo, Tokyo, Japan) that reduces thermal drift. The bead images were captured with an sCMOS camera (Neo, Andor, Tokyo, Japan) at 1000 frames s−1 and analyzed by NIH ImageJ with home-made plug-ins (created by Dr. K. Adachi) (20). The centroids of the bead images were calculated as described previously (21).

Other Assays—Protein concentrations of the purified V1 constructs were determined from UV absorbance calibrated by quantitative amino acid analysis; 1 mg/ml gives 0.88 OD at 280 nm. ATPase activity was measured at 25 °C with an enzyme-coupled ATP-regenerating system. The ATPase assay solution contained 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 6 mM MgCl2, 2 mM phosphoenolpyruvate, 100 μg/ml lactate dehydrogenase, 100 μg/ml pyruvate kinase, 0.2 mM NADH, and a range of concentrations of Mg-ATP. The reactions were initiated by the addition of enzymes. ADP removal treatments for chimeric V1 were carried out by methods described previously (11). Polyacrylamide gel electrophoresis in the presence of SDS or alkyl ether sulfate (AES) was carried out as described previously (22).

**Figure 2. Sequence alignment of the A subunits of T. thermophilus and E. hirae V0V1.** The primary sequences of the A subunits of V0V1 were aligned. TthA and EhiA represent T. thermophilus (NCBI Gene ID Q72172.1) and E. hirae (Q08636.1), respectively. NT, NB, and CT domains are shown in green, red, and blue, respectively. The P-loop (motifs A and B) is indicated by the rectangles. An arrow indicates the fusion point in V1-A011. An asterisk indicates same residues; a colon and period indicate high and low similarity residues, respectively.
the C terminus of subunit L as described previously (23). The reconstituted chimeric V0V1 was then incorporated into liposomes by a freeze-thaw method (24). The liposomes were used for ATP synthesis activities driven by acid-base transition as described previously (11). The condition used is as follows: pH\text{in} = 4.9, pH\text{out} = 8.5, [K^+]\text{in} = 1 \text{ mM}, [K^+]\text{out} = 100 \text{ mM}, 25 \degree \text{C}. Figures of structural models were prepared using the UCSF Chimera package (25).

RESULTS

ATP Hydrolysis of Chimeric V1—All chimeric V1 constructs were generated using the basic \textit{Tth} V1 (Figs. 1, b and c, and 3). The subunit stoichiometry and complex formation of all the purified chimeric V1 constructs were confirmed by both SDS- and native PAGE (Fig. 1, d and e). The NB domain is thought to be critical for entrapment of inhibitory ADP at a catalytic site. ADP inhibition of \textit{Tth} V1 can be overcome by introduction of the TSSA mutation in the P-loop region in the NB domain of the A subunit (S232A/T235S; see Refs. 5 and 11). In this case, entrapped inhibitory ADP in the catalytic site of TSSA mutated \textit{Tth} V1 was easily detached from the enzyme during turnover because of its lower affinity for nucleotide (ADP and/or ATP) than that of wild-type \textit{Tth} V1. Assuming that the insensitivity of \textit{Ehi} V1 to ADP inhibition is due to a difference in the molecular characteristics of the NB domain of the two enzymes, chimeric V1-A010 containing the NB domain (residue numbers 194–434; Figs. 1, b and c, 2, and 3) of the A subunit of \textit{Ehi} V1 should show insensitivity to ADP inhibition. However, the ATP hydrolysis activity of isolated V1-A010 was very low due to the presence of inhibitory ADP bound to the enzyme (Fig. 4). This inhibitory ADP in V1-A010 was successfully removed by phosphate/EDTA treatment as described previously (17). After the treatment, V1-A010 showed an apparent ATP hydrolysis activity of 34.4 \pm 0.6 \text{ s}^{-1}, which is almost equivalent to that of \textit{Tth} V1 (39.9 \pm 0.3 \text{ s}^{-1}) (11). However, the ATPase activity of V1-A010 rapidly decayed due to transition into the ADP inhibition state. The inhibition rate of the V1-A010 was 1.4 \times 10^{-2} \text{ s}^{-1}, which is higher than that of \textit{Tth} V1 (7.9 \times 10^{-3} \text{ s}^{-1}; see Ref. 11) (Fig. 4). These results clearly indicate that the different sensitivities of the two V1 enzymes to ADP inhibition are not solely dependent on the properties of the NB domain.

A CT domain chimera, V1-A001 (containing residue numbers 435–593 of the A subunit of \textit{Ehi} V1; Figs. 1c and 3), was constructed and purified (Fig. 1, c and d). The isolated V1-A001 exhibited extremely low ATP hydrolysis activity likely as the result of bound inhibitory ADP (Fig. 4). This inhibitory ADP in V1-A001 was successfully removed by phosphate/EDTA treatment as described previously (17). After the treatment, V1-A001 showed an apparent ATP hydrolysis activity of 5.0 \pm 0.2 \text{ s}^{-1}, which is significantly lower than that of V1-A010 or \textit{Tth} V1. The ATPase activity of V1-A001 also decayed. The results indicate that sub-
substitution of the CT domain of TthV₁ to that of EhiV₁ is not sufficient to suppress ADP inhibition of V₁.

In contrast, the chimeric V₁-A₀₁₁ containing both the NB and CT domains of EhiV₁ maintained nearly continuous ATP hydrolysis activity of 57.7 ± 0.8 s⁻¹ without ADP removal treatment (Fig. 4). These results from three chimeric V₁ indicate that substitution with both the NB and CT domains of EhiV₁-A subunit is required to suppress the ADP inhibition of the TthV₁.

The first and second helices of the CT domain lie in close proximity to the lower side of the NB domain in the crystal structure of TthV₁ (Fig. 1b), suggesting that these helices play an important role in the interaction between the two domains. To investigate the inherent function of the CT domain helices in ADP inhibition, chimeric construct V₁-A₀₁₀₁₁, which contains both the NB domain and the first and second CT helices (residue numbers 435–481; Figs. 1c and 3) of the A subunit of EhiV₁, was constructed and purified (Fig. 1, c and d). V₁-A₀₁₀₁₁ showed continuous ATP hydrolysis activity without ADP removal treatment (Fig. 4), indicating that both of these regions of the A subunit of EhiV₁ are required to overcome ADP inhibition of TthV₁. In other words, interdomain interaction between the NB domain and the first and second helices of the CT domain is likely to be important for suppression of ADP inhibition.

**Kinetic Parameters of Chimeric V₁ for ATP Hydrolysis**—Affinity of V₁ for nucleotides seems to be key for sensitivity of ADP inhibition because the ADP inhibition is due to entrapment of ADP in a catalytic site of the V₁. In fact, TSSA mutated TthV₁ exhibited insensitivity to ADP inhibition because of its lower affinity for nucleotide (ADP and/or ATP) than that of wild-type TthV₁ (5, 11). Thus, we examined kinetic parameters of V₁-A₀₁₁, which exhibits insensitivity to ADP inhibition, and other chimeric V₁ by both bulk phase assay and single molecule observation. The ATP hydrolysis rates of V₁-A₀₁₀ and V₁-A₀₁₁ obeyed simple Michaelis-Menten kinetics (Fig. 5a). The V_max values of V₁-A₀₁₀ and V₁-A₀₁₁ were calculated to be 34.4 ± 0.6 and 57.7 ± 0.8 s⁻¹ (mean ± S.E.), respectively (Table 1), which are similar to that of TthV₁. The K_m for ATP (K_m(ATP)) of V₁-A₀₁₁, 21.5 ± 1.8 μM (mean ± S.E.), was 10-fold lower than that of TthV₁ (205 ± 7 μM (mean ± S.E.)) and 6-fold lower than that of V₁-A₀₁₀ (132 ± 11 μM (mean ± S.E.)) (Table 1). The V_max/K_m value of V₁-A₀₁₁ was ~14-fold higher than that of TthV₁, despite that V_max of V₁-A₀₁₀ was 1.5-fold higher than that of TthV₁. These results suggest that V₁-A₀₁₁ has a higher affinity for ATP compared with TthV₁ and V₁-A₀₁₀.

To obtain the kinetic parameters of chemical reactions in the ATP hydrolysis, we observed the rotations of single molecule V₁-A₀₁₁ (Fig. 5b) under an optical microscope. A bead (nominal
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diameter, ~200 nm) attached to the D subunit of V_{1, A011} rotated stepwise at 2 and 0.5 μM ATP concentration, pausing every 120°, as observed for TthV_{1} (11) (Fig. 5b). The dwell time between successive 120° steps in V_{1, A011} at low ATP concentrations is a result of the enzyme waiting to bind ATP because binding of ATP is the rate-limiting step under these conditions (11). Based on dwell time analysis (Fig. 5, c–d), the apparent binding rate, k_{on} for ATP of V_{1, A011} was estimated to be (3.5 ± 0.1) × 10^6 and (5.1 ± 0.2) × 10^6 M⁻¹ s⁻¹ (mean ± S.E.) at 2 and 0.5 μM ATP, respectively. These values are 3–5-fold higher than that reported previously for TthV_{1} (11). These results indicate a higher affinity of V_{1, A011} for ATP, consistent with the result obtained for the bulk ATP hydrolysis assay (Fig. 5a and Table 1).

**ATP Synthesis by Chimeric Enzymes**—For the ATP synthesis assay, V_{1, A010}, V_{1, A011}, and V_{1, A001} were individually reconstituted with TthV_{0} respectively. Reconstitution of each chimeric V_{1} and TthV_{0} was confirmed by alkyl ether sulfate-PAGE (Fig. 1e). Each reconstituted chimeric V_{0}V_{1} complex was individually incorporated into liposomes, and the ATP synthesis activities were measured. All the chimeric V_{0}V_{1} complexes exhibited ATP synthesis activity (Fig. 6 and Table 2). The ATP synthesis rates were plotted against ADP concentration ([ADP]). The plots obeyed roughly Michaelis-Menten behavior (Fig. 6, a–d, and Table 2). The apparent K_{m} values for ADP (K_{m(ADP)} of V_{0}V_{1, A010} (14.0 ± 3.0 μM) and V_{0}V_{1, A001} (13.5 ± 2.3 μM) were slightly higher than that of TthV_{0}V_{1} (5.4 ± 1.3 μM). In contrast, the K_{m(ADP)} value of V_{0}V_{1, A011} (1.3 ± 0.3 μM) was lower than those obtained for TthV_{0}V_{1} and the other chimeric V_{0}V_{1} complexes. These results indicate that V_{0}V_{1, A011} has a high affinity for ADP, suggesting that there is no direct relationship between sensitivity to ADP inhibition of V_{1} and the affinity for nucleotides.

The rates of ATP synthesis of the chimeric V_{0}V_{1} were also measured as a function of the phosphate (P_{i}) concentration (Fig. 6, e–h, and Table 2). Interestingly, the apparent K_{m} value for P_{i}(K_{m(P_{i})}) for V_{0}V_{1, A011} (10.6 ± 3.5 μM) was markedly lower than those of TthV_{0}V_{1} and the other two chimeric V_{0}V_{1} complexes, indicating that V_{1, A011} has a much higher affinity for P_{i} than the other constructs.

**P_{i} Effect on ATP Hydrolysis of Chimeric V_{1}**—To confirm the change in affinity for P_{i} of V_{1, A011}, the effects of P_{i} on ATP hydrolysis were examined by both bulk phase and single molecule observation. The ATPase activities of V_{1, A011} were measured in the presence of various P_{i} concentrations. The ATPase activities of TthV_{1}, V_{1, A010}, and V_{1, A001} were not affected at low P_{i} concentrations (1–30 mM). In marked contrast, the ATPase activity of V_{1, A011} was strongly inhibited at low P_{i} concentrations with an IC_{50} of ~10 mM (Fig. 7). This result indicates that V_{1, A011} has higher affinity for P_{i} than the other constructs, consistent with the results of ATP synthesis experiments.

V_{1, A011} was subjected to single molecule measurement using an 80-nm gold bead for which viscous drag is very low (19, 26). ATP dependence of the time-averaged rotation rate of V_{1, A011} follows simple Michaelis-Menten kinetics (Fig. 8a). The V_{max} value was 28.3 ± 0.5 revolutions/s, which is comparable with a turnover rate of almost 90 s⁻¹ (3 × V_{max}). The K_{m(ADP)} value of 28.7 ± 2.8 μM is almost identical to the K_{m(ADP)} value of 21.5 ± 1.8 μM estimated from the bulk ATP hydrolysis assay (Table 1). At 2 mM ATP, the rotation rate (28.0 ± 1.7 revolutions/s) decreased to 14.8 ± 0.7 revolutions/s upon addition of 20 mM P_{i}.

### TABLE 2

| V_{max} | K_{m} |
|---------|-------|
| s⁻¹     | μM    |
| TthV_{1} |   |
| ADP     | 44.8 ± 2.1 | 5.4 ± 1.3 |
| P_{i}    | 35.1 ± 2.3 | 322 ± 90 |
| V_{1, A010} |   |
| ADP     | 1.8 ± 0.1 | 14.0 ± 3.0 |
| P_{i}    | 1.7 ± 0.1 | 402 ± 40 |
| V_{1, A011} |   |
| ADP     | 1.0 ± 0.1 | 13.3 ± 0.3 |
| P_{i}    | 1.0 ± 0.1 | 10.6 ± 3.5 |
| V_{1, A011} |   |
| ADP     | 1.6 ± 0.1 | 13.5 ± 2.3 |
| P_{i}    | 1.8 ± 0.1 | 457 ± 68 |

FIGURE 6. ATP synthesis by reconstituted chimeric V_{0}V_{1} as a function of ADP and phosphate concentrations. The reconstitution of V_{0}V_{1} and the ATP synthesis reaction were performed as described under “Experimental Procedures.” a–d, [S]-V plot of ATP synthesis rate catalyzed by reconstituted V_{0}V_{1} (a), V_{0}V_{1, A010} (b), V_{0}V_{1, A011} (c), and V_{0}V_{1, A001} (d) at various ADP concentrations in the presence of 10 mM sodium phosphate. The solid lines show fit with the Michaelis-Menten equation; e–h, [S]-V plot of ATP synthesis rate catalyzed by reconstituted V_{0}V_{1} (e), V_{0}V_{1, A010} (f), V_{0}V_{1, A011} (g), and V_{0}V_{1, A001} (h) at various phosphate concentrations in the presence of 1 mM ATP. The solid lines show fit with the Michaelis-Menten equation. The apparent kinetic values are summarized in Table 2. Error bars represent S.D.
into the rotation assay buffer (Fig. 8, a and b). This indicates that addition of P_i prolongs the dwell time probably due to rebinding of P_i to the enzyme. At saturating ATP concentrations, V_1-A011 showed 120° steps, but no additional substep was observed as for the TthV_1 (26) both in the absence and presence of P_i (Fig. 8, c and d). The results suggest that release of P_i from the V_1 occurs at the same angles as that of ATP waiting to bind.

**DISCUSSION**

In this study, we investigated the molecular mechanism of ADP inhibition of V_1 by a domain swap approach using combinations of subunit A from ADP inhibition-sensitive TthV_1 and from ADP inhibition-insensitive EhiV_1. The chimeric V_1-A010 containing the NB domain of EhiV_1 was highly sensitive to ADP inhibition, indicating that sensitivity of V_1 to ADP inhibition is not defined solely by the NB domain. In contrast, the chimeric V_1-A011 consisting of both NB and CT domains of EhiV_1 exhibited insensitivity to ADP inhibition without decreasing the binding affinity for nucleotides (Fig. 4 and Tables 1 and 2). Thus, the V_1-A011 has a mechanism of reducing sensitivity to ADP inhibition that is different from that of the ADP inhibition-insensitive mutant TSSA V_1, which was previously shown to have decreased binding affinity for nucleotides as a result of two point mutations in the P-loop region of the NB domain of the A subunit (5, 11).
For the ATP synthesis reaction, the affinity of $V_1A_{011}$ for $P_i$ was over an order of magnitude higher than that of $V_0A_{1010}$ even though both contain the NB domain from $EhV_1$. The high affinity for $P_i$ of $V_1A_{011}$ was confirmed using the ATP hydrolysis assay (Fig. 7). In addition, single molecular analysis of $V_1A_{011}$ using a low viscous drag probe clearly indicates that
addition of P_i prolongs the dwell time at every 120° dwell position (Fig. 8). These results strongly suggest a tight correlation between the insensitivity to ADP inhibition of V_{1-A011} and an increased affinity for P_i.

Feniouk et al. (8) proposed a mechanism for ADP inhibition of F_i: when P_i release from a catalytic site happens before ADP release during turnover, the ADP is stochastically and tightly entrapped in the catalytic site. As a result, the F_i lapses into the ADP-inhibited state (8). Based on this assumption and our results, we think it is safe to conclude that the delayed release of ADP-inhibited state (8). Based on this assumption and our increased affinity for Pi.

between the insensitivity to ADP inhibition of V_{1-A011} and an increased affinity for Pi. Here we propose the molecular basis of ADP inhibition of V_0V_1 by comparing the recently reported crystal structures of EhiV_1 and TthV_1 (13, 14).

In the crystal structure of TthV_1, the three A subunits of TthV_1 adopt three different conformations, namely “TthAT” containing ADP, “TthAD” containing ADP and, and nucleotide-free “TthAO” (referred to as A_N, A_N, and A_W, respectively in Ref. 13) (Fig. 9a and Table 3). In contrast, there are only two structurally distinct A subunit conformations in EhiV_1: two A subunits including bound nucleotide (referred as A_C and A_C in Ref. 14) and one A subunit without nucleotide (referred to as A_O) (Fig. 9b). The structure of A_C is almost identical to that of A_C (Table 4). In this study, we call the two A subunits with bound nucleotide in EhiV_1 (A_C and A_C) EhiAT_1 and EhiAT_2, respectively, and the one A subunit with no bound nucleotide EhiAO hereafter.

The structure of TthAO is almost identical to that of EhiAO (Table 5). In addition, the overall structure of TthAT is highly similar to that of EhiAT_1 and EhiAT_2 (Table 5). In contrast, the catalytic site of TthAT is significantly different from that of the TthAT and the EhiAT_1 and EhiAT_2 structures. The P-loop residues of the TthAT, TthAD, and EhiAT were superimposed, and the positions of the catalytic residues were compared (Fig. 9, c and d). The amino group of Lys^234 is 2.9 Å from the β-P group of bound nucleotide in TthAT, but 3.8 Å distant in the TthAT (Fig. 9, e and f). The narrow space between the amino group of Lys^234 and the β-P group blocks further binding of P_i in the catalytic site of the TthAT. Together with the data from our biochemical analysis of the chimeric V_1 complexes, these findings indicate that the higher affinity of V_{1-A011} for P_i is due to the A subunit adopting the EhiAT form with ADP (but no P_i), which is capable of binding P_i. In contrast, TthV_1 or V_{1-A010} can lapse into the ADP-inhibited form when one A subunit in V_1 stochastically adopts the TthAD form with bound ADP, which is then incapable of binding P_i. This leads to inhibition of catalysis.

**TABLE 3**

r.m.s.d. values between A subunits from TthV_1

|        | TthA_{13} (A_N) | TthA_{13} (A_W) | TthA_{13} (A_W) |
|--------|-----------------|-----------------|-----------------|
| TthA_{13} (A_N) | 11.410          | 6.424           | 5.711           |

**TABLE 4**

r.m.s.d. values between A subunits from EhiV_1

|        | EhiA_{10} (A_C) | EhiA_{10} (A_N) | EhiA_{10} (A_C) |
|--------|-----------------|-----------------|-----------------|
| EhiA_{10} (A_C) | 13.119          | 15.705          | 2.872           |

**TABLE 5**

r.m.s.d. values between A subunits from TthV_1 and EhiV_1

|        | T. thermophilus |        |        |
|--------|-----------------|--------|--------|
| E. hirae | TthA_{19} (A_N) | TthA_{19} (A_C) | TthA_{19} (A_C) |
| E. hirae | 2927            | 14.510 | 16.920 |
| E. hirae | 14.510          | 4.588  | 6.244  |
| E. hirae | 16.920          | 6.424  | 10.687 |

**FIGURE 10. Interaction between NB and CT domains in TthV_{1-A} subunits.** The structures of TthAT (gray) and TthAO (color) were superimposed at the NB domain of the A subunit (residues 190–428). NB and CT domains are colored yellow and pink, respectively. H1 and H2 indicate the first and second helix of CT domain, respectively. a, upper part of the CT domain in TthA_{19} (gray) and TthA_{19} (color). b, lower part of the CT domain of TthA_{19} (gray) and TthA_{19} (color). Cyan lines show possible H-bonds in TthA_{19} and TthA_{19}.
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The overall structure of TtH A_{v} is also significantly different from that of TtH A_{p}. This difference is caused by movement of the CT domain relative to the NB domain in the A subunit. Some hydrogen bonds (Val^{214}-Arg^{451}, Leu^{215}-Thr^{432}, and Asn^{225}-Gln^{459}) observed between NB and CT domains in TtH A_{p} are absent in TtH A_{v} by the movement (Fig. 10). This strongly suggests that the interaction between the NB and CT domains plays an important role in the conformation of the A subunit in V_{1}. In the crystal structure of TtH V_{1}, the first and second helices of domain CT lie in close proximity to the lower part of NB domain (Fig. 10), suggesting that these two helices are important mediators of the interaction between the two domains. The chimeric V_{1,A010} containing both the NB domain and the first and second helices of the CT domain from Ehi V_{1} exhibits insensitivity to ADP inhibition. However, the V_{1,A010} shows sensitivity to ADP inhibition despite containing the NB domain of Ehi V_{1}. Taken together, these findings strongly indicate that the interaction between the NB and CT domains of the A subunit that prevents the A subunit from adopting the A_{D} form during turnover is critical for insensitivity to ADP inhibition. This ADP inhibition mechanism of TtH V_{1} is advantageous as it prevents consumption of ATP when proton motive force is lost. In contrast, because the E. hirae and eukaryotic V_{0} V_{1} enzymes function as ion pumps coupled with continuous ATP hydrolysis, they do not exhibit sensitivity to ADP inhibition.

Eukaryotic V_{0} V_{1} functions as an ion (proton) pump like Ehi V_{0} V_{1}. Thus, we speculate that eukaryotic V_{0} V_{1} is also insensitive to ADP inhibition to sustain continuous ATP hydrolysis. A similar interaction between the NB and CT domains of the A subunit as seen in the Ehi V_{1} is also likely to exist in the eukaryotic enzymes.

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REFERENCES

1. Forgac, M. (2007) Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. Nat. Rev. Mol. Cell Biol. 8, 917–929
2. Muench, S. P., Trinick, J., and Harrison, M. A. (2011) Structural divergence of the rotary ATPases. Q. Rev. Biophys. 44, 311–356
3. Yokoyama, K., and Imamura H. (2005) Rotation, structure, and classification of prokaryotic V-ATPase. J. Bioenerg. Biomembr. 37, 405–410
4. Murata, T., Igarashi, K., Kakinuma, Y., and Yamato, I. (2000) Na+ binding of V-type Na+-ATPase in Enterococcus hirae. J. Biol. Chem. 275, 13415–13419
5. Imamura, H., Nakano, M., Noji, H., Muneyuki, E., Ohkuma, S., Yoshida, M., and Yokoyama, Y. (2003) Evidence for rotation of V_{1}-ATPase. Proc. Natl. Acad. Sci. U.S.A. 100, 2312–2315
6. Yokoyama, K., Nakano, M., Imamura, H., Yoshida, M., and Tamakoshi, M. (2003) Rotation of the proteolipid ring in the V-ATPase. J. Biol. Chem. 278, 24255–24258
7. Lau, W. C., and Rubinstein, J. L. (2012) Subnanometer-resolution structure of the intact Thermus thermophilus H+-driven ATP synthase. Nature 481, 214–218
8. Feniouk, B. A., Suzuki, T., and Yoshida, M. (2007) Regulatory interplay between proton motive force, ADP, phosphate, and subunit epsilon in bacterial ATP synthase. J. Biol. Chem. 282, 764–772
9. Jault, J. M., Dou, C., Grodsky, N. B., Matsu, T., Yoshida, M., and Allison, W. S. (1996) The α_{B}β subcomplex of the F_{1}-ATPase from the thermophilic Bacillus PS3 with the βT1655 substitution does not entrap inhibitory MgADP in a catalytic site during turnover. J. Biol. Chem. 271, 28818–28824
10. Yokoyama, K., Muneyuki, E., Amano, T., Mizutani, S., Yoshida, M., Ishida, M., and Ohkuma, S. (1998) V-ATPase of Thermus thermophilus is inactivated during ATP hydrolysis but can synthesize ATP. J. Biol. Chem. 273, 20504–20510
11. Nakano, M., Imamura, H., Toei, M., Tamakoshi, M., Yoshida, M., and Yokoyama, K. (2008) ATP hydrolysis and synthesis of a rotary motor V-ATPase from Thermus thermophilus. J. Biol. Chem. 283, 20789–20796
12. Maher, M. J., Akimoto, S., Iwata, M., Nagata, K., Hori, Y., Yoshida, M., Yokoyama, S., Iwata, S., and Yokoyama, K. (2009) Crystal structure of A_{B} B_{3} complex of V-ATPase from Thermus thermophilus. EMBO J. 28, 3771–3779
13. Nagamatsu, Y., Takeda, K., Kuranaga, T., Numoto, N., and Miki, K. (2013) Origin of asymmetry at the intersubunit interfaces of V_{1}-ATPase from Thermus thermophilus. J. Mol. Biol. 425, 2699–2708
14. Arai, S., Saijo, S., Suzuki, K., Mizutani, K., Kakinuma, Y., Ishizuka-Katsura, Y., Ohswa, N., Terada, T., Shirouzu, M., Yokoyama, S., Iwata, S., Yamato, I., and Murata, T. (2013) Rotation mechanism of Enterococcus hirae V_{1}-ATPase based on asymmetric crystal structures. Nature 493, 703–707
15. Saijo, S., Arai, S., Hassain, K. M., Yamato, I., Suzuki, K., Kakinuma, Y., Ishizuka-Katsura, Y., Ohswa, N., Terada, T., Shirouzu, M., Yokoyama, S., Iwata, S., and Murata, T. (2011) Crystal structure of the central axis DF complex of the prokaryotic V-ATPase. Proc. Natl. Acad. Sci. U.S.A. 108, 19955–19960
16. Kettner, C., Obermeyer, G., and Berti, A. (2003) Inhibition of the yeast V_{1} ATPase by cytosolic ADP. FEBS Lett. 535, 119–124
17. Imamura, H., Ikeda, C., Yoshida, M., and Yokoyama, K. (2004) The F subunit of Thermus thermophilus V_{1}-ATPase promotes ATPase activity but is not necessary for rotation. J. Biol. Chem. 279, 18085–18090
18. Murata, T., Takase, K., Yamato, I., Igarashi, K., and Kakinuma, Y. (1999) Properties of the V_{0} V_{1} Na+-ATPase from Enterococcus hirae and its V_{0} moiety. J. Biochem. 125, 414–421
19. Furuike, S., Adachi, K., Sakaki, N., Shimo-Kon, R., Itoh, H., Muneyuki, E., Yoshida, M., and Kinosita, K., Jr. (2008) Temperature dependence of the rotation and hydrolysis activities of F_{1}-ATPase. Biophys. J. 95, 761–770
20. Adachi, K., Oiwa, K., Nishizaka, T., Furuike, S., Noji, H., Itoh, H., Yoshida, M., and Kinosita, K., Jr. (2007) Coupling of rotation and catalysis in F_{1}-ATPase revealed by single-molecule imaging and manipulation. Cell 130, 309–321
21. Yasuda, R., Noji, H., Yoshida, M., Kinosita, K., Jr., and Itoh, H. (2001) Resolution of distinct rotational substeps by submillisecond kinetic analysis of F_{1}-ATPase. Nature 410, 898–904
22. Yokoyama, K., Nagata, K., Imamura, H., Ohkuma, S., Yoshida, M., and Tamakoshi, M. (2003) Subunit arrangement in V-ATPase from Thermus thermophilus. J. Biol. Chem. 278, 42686–42691
23. Tamakoshi, M., Uchida, M., Tanabe, F., Kukuyama, S., Yamasaki, A., and Oshima, T. (1997) A new Thermus-Excherichia coli shuttle integration vector system. J. Bacteriol. 179, 4811–4814
24. Kishikawa, J., and Yokoyama, K. (2012) Reconstitution of vacuolar-type rotary H+-ATPase/synthase from Thermus thermophilus. J. Biol. Chem. 287, 24597–24603
25. Petersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612
26. Furuike, S., Nakano, M., Adachi, K., Noji, H., Kinosita, K., Jr., and Yokoyama, K. (2011) Resolving stepping rotation in Thermus thermophilus H+-ATPase/synthase with an essentially drag-free probe. Nat. Commun. 2, 233