Mechanical Modulation of ATP-Binding Affinity of V1-ATPase*

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Background: ATP-binding reaction was hypothesized to be the main torque-generating step for V1-ATPase.

Results: Upon mechanical manipulation, ATP-binding reaction of V1-ATPase showed weaker angle dependency compared with that of F1-ATPase.

Conclusion: ATP-binding reaction is not the main torque-generating step in V1.

Significance: This external manipulation technique should be applied to other reaction steps of ATP hydrolysis to get the whole view of mechanochemical coupling mechanism in V1.

ABSTRACT

V1-ATPase is a rotary motor protein that rotates the central shaft in counterclockwise direction hydrolyzing ATP. Although the ATP-binding process is suggested to be the most critical reaction step for torque generation in F1-ATPase (the closest relative of V1-ATPase evolutionarily), the role of ATP binding for V1-ATPase in torque generation has remained unclear. In the present study, we performed single-molecule manipulation experiments on V1-ATPase from Thermus thermophilus to investigate how the ATP binding process is modulated upon rotation of the rotary shaft. When V1-ATPase showed an ATP-waiting pause, it was stalled at a target angle and then released. Based on the response of the V1-ATPase released, the ATP-binding probability was determined at individual stall angles. It was observed that the rate constant of ATP binding (k_on) was exponentially accelerated with forward rotation, whereas the rate constant of ATP release (k_off) was exponentially reduced. The angle-dependence of the k_off of V1-ATPase was significantly smaller than that of F1-ATPase, suggesting that the ATP-binding process is not the major torque-generating step in V1-ATPase. When V1-ATPase was stalled at the mean binding angle to restrict rotary Brownian motion, k_on was evidently slower than that determined from free rotation, showing the reaction rate enhancement by conformational fluctuation. It was also suggested that shaft of V1-ATPase should be rotated at least 277° in clockwise direction for efficient release of ATP under ATP-synthesis conditions.

The vacuolar proton pumps, V-ATPases (V_oV_1 type), are part of the ATPase/ATP synthase superfamily and share a common rotary catalytic mechanism with F_oF_1-ATPase (1-3). V_oV_1 consists of two rotary motors, that is, the membrane-embedded V_o subunit and the water-soluble V_1 subunit, each driven by a proton flux to create a proton-motive force (pmf) and by ATP hydrolysis, respectively. In cells, V_o and V_1 bind to one another via the central rotor stalk and peripheral stalks and interconvert the energy liberated from ATP hydrolysis and proton translocation down proton motive force into...
rotation of the central stalk (4,5). While \( V\alpha V1 \) is primarily known as an ATP-driven proton pump that acidifies the inside of vacuoles in eukaryotic cells, \( V\alpha V1 \) also catalyzes ATP synthesis driven by \( \text{pmf} \) in archaea and some eubacteria such as *Thermus thermophilus*.

The \( V\beta \) domain from *T. thermophilus*, termed \( V1\text{-ATPase} \), has been extensively investigated as a model enzyme for the bacterial type \( V\beta \) due to its conformational stability and ease of biochemical handling (6-8). \( V1\text{-ATPase} \) is composed of a hexameric stator ring of \( A3B3 \) subunits and the central rotary shaft of \( DF \) subunits. The \( A3B3 \) ring possesses 3 catalytic sites on each \( A-B \) interface, primarily on the \( A \) subunit. The \( D \) subunit is embedded inside the central cavity of the \( A3B3 \) ring, while the \( F \) subunit binds to a protruding segment of subunit \( D \) (9).

Although \( V1\text{-ATPase} \) has several rotation features in common with \( F1\text{-ATPase} \), such as rotation in the counterclockwise direction and a 120° stepping rotation, the rotational mechanism of \( V1\text{-ATPase} \) is distinct from that of \( F1\text{-ATPase} \) (10). One prominent difference between the 2 types of ATPase is that \( V1\text{-ATPase} \) does not show any rotational substep (7), while the elementary 120° step of \( F1\text{-ATPase} \) is composed of 80° and 40° substeps (11). Although the process by which the 3 catalytic \( A \) subunits participate in driving the unidirectional rotation remains unclear, it has been suggested that \( V1\text{-ATPase} \) executes all of the elementary reaction steps, that is, ATP binding, ATP hydrolysis, and product release, at each pausing position. This hypothesis implies that each elementary reaction step is responsible for contributing to the 120° rotation, in contrast to the torque-generation mechanism of \( F1\text{-ATPase} \), in which individual reaction steps induce either of the 80° or 40° substeps. Another difference between these two ATPases is that the torque of \( V1\text{-ATPase} \) is approximately 35 pN·nm, which is slightly smaller than that of \( F1\text{-ATPase} \) (40 pN·nm) (10,12). Comparative research on \( V1\text{-ATPase} \) and \( F1\text{-ATPase} \) could clarify the common working principles and unique mechanisms of these proteins.

We have previously conducted single-molecule stalling experiments to investigate how \( F1\text{-ATPase} \) modulates the chemical equilibrium and reaction rate of ATP-binding and ATP-hydrolysis steps via the rotation of the central rotary shaft (13). Although both reactions were exponentially enhanced during the forward rotation, the degree of reaction enhancement was distinctive; the ATP-binding rate was largely accelerated during the forward rotation, while the reaction enhancement of the ATP-hydrolysis step was only slight. The affinity of \( F1 \) for ATP was also exponentially enhanced, suggesting that the \( F1\text{-ATP} \) complex is stabilized upon rotation. This finding suggested that \( F1 \) generates a much greater torque during the binding-change process than during the hydrolysis step. The torque generated upon ATP-binding was quantitatively estimated from the angle-dependence of \( k\text{off} \).

Single-molecule manipulation with magnetic tweezers was also utilized in the experiment on \( V1\text{-ATPase} \) in order to attempt to activate \( V1\text{-ATPase} \) in the ADP-inhibited form by forcibly rotating the molecule with the magnetic tweezers (14). When rotated over +110°, \( V1\text{-ATPase} \) resumed active rotation. The activation probability was notably dependent on the angular displacement from the inhibitory pausing position, as observed in the mechanical activation of \( F1\text{-ATPase} \) in the ADP-inhibited form (15). This observation suggests that \( V1\text{-ATPase} \) also possesses the ability to modulate the catalytic reaction upon rotation, similar to that of \( F1\text{-ATPase} \). In the present study, we attempted to verify this hypothesis by examining how the ATP-binding process is modulated upon rotation of \( V1\text{-ATPase} \). Interestingly, \( V1\text{-ATPase} \) displayed demonstrably weaker angle-dependence of ATP-binding than \( F1\text{-ATPase} \), suggesting a smaller contribution of the ATP-binding process for torque generation than that observed for \( F1\text{-ATPase} \). The results have been discussed in the light of the current understanding of the mechanochemical coupling mechanisms of \( V1\text{-ATPase} \) and \( F1\text{-ATPase} \).

**EXPERIMENTAL PROCEDURES**

**Rotation assay** – Sample preparation and experimental procedures were performed essentially as described in our previous study (14). Wild-type \( V1 \) that had a minimal modification for the single-molecule rotation assay, \( A\text{His}10(C28S/C508S)3B(C264S)3D(E48C/Q55C)F \), was prepared and examined in the rotation assay. Streptavidin-coated magnetic beads (Thermo Scientific Seradyn, Indianapolis, IN, USA) were used as rotational markers. The beads showed relatively great diversity in diameter. The small particles (\( \phi \approx 200 \) nm) were selectively observed due to the low frequency of physical interaction...
with the glass surface. Rotation of the bead was observed under a phase-contrast microscope (IX70; Olympus, Tokyo, Japan) by using a 100× objective lens. Images were captured with a charge-coupled device camera (FC300M; Takenaka System Co., Kyoto, Japan) and recorded at 30 frames per sec (fps). A magnetic tweezers system was mounted on the specimen stage of the microscope and controlled with custom-designed software (Celery, Library, Tokyo, Japan). Analysis of rotation was also performed using the custom-designed software (Celery, Library, Tokyo, Japan). All the experiments were carried out at a temperature range of 23–25°C.

RESULTS

V1-ATPase was immobilized on the glass surface through His tags introduced at the N-termini of the A subunits of the stator A3B3 ring. Rotation of V1-ATPase was observed by attaching a streptavidin-coated magnetic bead to the D subunit. The magnetic beads were used also as the handle for manipulation by the magnetic tweezers. The rotation assay was conducted under ATP-limiting conditions (1 or 1.5 µM), well below the Michaelis-Menten constant (Km) of the rotation assay with magnetic beads (8.1 µM) (14). Under these conditions, V1-ATPase demonstrated a 120° stepping rotation (Fig. 1A). The mean times of the ATP-waiting pause were 0.57 and 0.32 sec at 1 and 1.5 µM, respectively (Fig. 1B). Note that the mean time for catalysis on V1-ATPase was 2.5 msec (7), which was much shorter than the ATP-waiting dwell or the mean time for catalysis on V1-ATPase (likely to be caused by the ADP-inhibited form). However, the occurrence rate of minor behaviors was very low (less than 5%); therefore, they were omitted from the analysis.

The Pon obtained at limiting concentrations of ATP (1 µM) was plotted against the stall angle (Fig. 4A). It is evident from the graph that Pon significantly depended on the stall angle. Here, we defined 0° as the mean angle for ATP-waiting pause and the “plus” direction as the rotational one (i.e., counterclockwise). When the data points were re-plotted against the stall time, the time course of Pon was obtained for individual stall angles (Fig. 4B). The values of Pon at -100° were too low to provide a reliable time-dependent increment of Pon; therefore, these data points were omitted. The time courses displayed simple saturation curves, suggesting that the single event i.e. ATP binding triggers rotation. This supports the above expectation that the time constant of other reactions such as ATP hydrolysis step is too short to affect Pon. Note that all of the time courses displayed plateau levels below 100%, except for the +100° stall. This result implies that the ATP-binding
event is reversible; during a long period of stalling, V1-ATPase releases ATP into the medium. This process was nearly identical to the stall-and-release experiment of F1-ATPase (13). The time courses were fitted on the basis of a reversible reaction scheme to determine the rate constant of ATP binding (k_on), as well as the rate constant of ATP release (k_off). The dissociation constant of ATP (K_d) was also determined as the ratio of k_off to k_on. The kinetic parameters were plotted in semi-log plots (red data in Fig. 5A-C). In Fig. 5A, k_on from free rotation was also designated (red open circle). It is evident that k_on exponentially increased upon V1-ATPase rotation, while k_off was exponentially reduced (Fig. 5A and 5B), resulting in an exponential reduction in K_d (Fig. 5C). From a -60° to +60° rotation, k_on increased by approximately 22 fold, while k_off decreased by 8 fold, resulting in an decrease in K_d by approximately 173 fold. For comparison, kinetic parameters of F1-ATPase were also included in Fig. 5A-C (gray data) (13).

DISCUSSION

In our previous study, the rate constants k_on, k_off, and K_d were determined in the stall-and-release experiments of F1-ATPase (gray circles and lines; Fig. 5) (13). A comparison of V1-ATPase and F1-ATPase data suggests that the ATP-binding process does not contribute to torque generation in V1-ATPase as much as it does in F1-ATPase. One of the distinctive features of V1-ATPase data is that the ATP-binding site of V1-ATPase has significantly lower affinity to ATP than the ATP-binding site of F1-ATPase (6). In kinetic terms, the k_on of V1-ATPase is lower than that of F1-ATPase over all stall angles, while k_off and K_d are higher. However, the individual data points do not provide any indication of the contribution of ATP-binding process to torque generation. More important in understanding the contributions to torque generation is the angle dependence of the kinetic parameters. In our previous report on the angle dependence of F1-ATPase (13), we estimated the contributions of ATP binding to torque generation from the angle dependence of k_off, comparing it to that of the ATP-hydrolysis step. Torque generated by ATP binding corresponds to the slope of the rotary potential of the ATP-bound state (16). Because $-k_a T \frac{d \ln k_{on}}{d \theta}$ represents the relative energy difference between the ATP-bound state and the transition state of ATP binding/release, the differential function, $-k_a T \frac{d \ln k_{on}}{d \theta}$ indicates the magnitude of torque generated by ATP binding. The assumption behind this estimation is that only the free energy of the ground state changes upon rotation, while the activation energy level remains constant. The magnitude of $-k_a T \frac{d \ln k_{on}}{d \theta}$ for V1-ATPase is only 38% that of F1-ATPase. The absolute values of estimated torque generation during the ATP-binding process were 4 pN·nm and 11 pN·nm for V1-ATPase and F1-ATPase, respectively. In practice, these values must be underestimated due to the elasticity of the rotary shaft (17-19). Despite this qualification, the estimates still suggest that the role of ATP binding in torque generation is not as important in V1-ATPase as in F1-ATPase, considering that the torsional rigidity of the rotor shaft estimated from the rotary fluctuation during the pausing state does not greatly differ between V1-ATPase and F1-ATPase (10,19). The conformational changes upon ATP binding of the A subunit are expected to be small compared with those of the β subunit in F1-ATPase. The structural analysis of the A subunit with or without bound nucleotides is highly anticipated.

Interestingly, the ATP-binding rate determined from free rotation (open circle in Fig. 5A) was slightly (but noticeably) higher than the k_on determined from the stalling experiment at +0°. The essentially same observation was reported for F1-ATPase. This observation is attributable to rate enhancement by thermal agitation; the rotary shaft of V1-ATPase always undergoes a thermally agitated rotary fluctuation, and an occasional large rotary fluctuation in the forward direction (counterclockwise) triggers ATP binding.

The present results also have implications regarding ATP synthesis. The K_d determined at +0° was 0.7 µM, which is too low to release ATP under physiological conditions where the ATP concentration is in the millimolar range. The angle dependence of K_d predicts that when rotated over -162°, the K_d increases to the millimolar range and V1-ATPase is able to release ATP into the medium. Moreover, the k_off determined at +0° was 0.46 s⁻¹, which was also too slow to explain the maximum turnover rate of ATP synthesis (67–73 s⁻¹) (6). The angle dependence of k_off suggests that the rotary shaft would have to be rotated over -277° to achieve the maximum turnover rate. Therefore, the
present results suggest that the reaction scheme of ATP synthesis is not simply the reverse reaction of the hydrolysis scheme and that the angular dependence of kinetic and thermodynamic parameters must be taken into account (note that these values are also overestimated, considering the possible elasticity of the rotary shaft). The torsional rigidity of the rotary shaft remains to be clarified for a more precise estimation of the contribution of ATP-binding to torque generation.
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FIGURE LEGENDS

Figure 1 – Rotation of V1 molecule at 1 µM ATP. A, Trajectory of a single V1 molecule at 1 µM ATP. Inset, X–Y trajectory of the same V1 molecule for which the trajectory was shown. B, Dwell-time histogram of V1 at 1 µM ATP. Data are well fitted with a single exponential, providing the time constant as 0.57 sec. The number of molecules was 3, and the number of trials used for dwell-time analysis was 654.

Figure 2 – V1 showed two general behaviors upon mechanical manipulation with magnetic tweezers. The left figures display the X–Y trajectories, and the right figures display the revolution vs time graphs. A, “on” event. When the molecule entered into the ATP-waiting pause, we switched on the magnetic tweezers to generate a magnetic field and rotated the magnetic bead and therefore the shaft to the target angle. Here, the bead was rotated almost 60° from the original waiting angle, stalled there for 0.5 sec, and then released. Upon release, the shaft directly proceeded to the next ATP-waiting angle. This behavior implies that V1 was bound ATP at the time of release, which generated the torque necessary for advancing to the next step. B, “off” event. The same molecule from A was stalled again at 60° from another ATP-waiting angle. Here, shaft returned to the original (0°) angle, indicating that V1 was not bound ATP at the time of release.

Figure 3 – Dwell time analysis of spontaneous ATP binding immediately after an ‘off’ event (A) or an ‘on’ event (B). To determine the effect of mechanical manipulation on kinetics of V1-ATPase, dwell time analysis was performed for both ‘off’ and ‘on’ events.

Figure 4 – Angle and time dependency of the ATP-binding event in V1. A, Angle dependency of ATP binding. The stalling experiment was performed in the range of -120° to +120° for the stalling times of 0.5 (orange), 1 (red), 3 (blue), and 6 sec (green). Each data point was obtained from the analysis of 17–201 trials by using a total of 20 molecules. B, Time course of ATP binding. The same dataset from A was used to demonstrate the stall time dependency of ATP binding. Data were fitted according to a reversible reaction scheme. The error bars represent the standard deviation.

Figure 5 – Rate constants determined from the time course of ATP binding: $k_{on}(A)$, $k_{off}(B)$ and $K_d(C)$ were fitted with single exponentials. Data of F1-ATPase were also included for comparison (gray-colored) (13). Both in V1 and F1, angle dependencies are apparent for all the kinetic parameters. Open circle in $k_{on}$ graph (A) designates the $k_{on}$ value in case of free rotation. From the slope of $k_{off}$, the torque of ATP binding for V1-ATPase was calculated to yield 4 pN·nm/radian, which is smaller than 11 pN·nm/radian of F1-ATPase.
Figure 1

(A) Graph showing the relationship between Revolution and Time (sec).

(B) Graph showing the distribution of Number of Events over Time (sec) with a fitted line.

\[ \tau_{\text{ATP}} = 0.57 \pm 0.01 \text{ sec} \]
Figure 2
Figure 3
Figure 4
Figure 5
