Mechanism of the $\alpha\beta$ Conformational Change in $F_1$-ATPase after ATP Hydrolysis: Free-Energy Simulations

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ABSTRACT One of the motive forces for $F_1$-ATPase rotation is the conformational change of the catalytically active $\beta$ subunit due to closing and opening motions caused by ATP binding and hydrolysis, respectively. The closing motion is accomplished in two steps: the hydrogen-bond network around ATP changes and then the entire structure changes via B-helix sliding, as shown in our previous study. Here, we investigated the opening motion induced by ATP hydrolysis using all-atom free-energy simulations, combining the nudged elastic band method and umbrella sampling molecular-dynamics simulations. Because hydrolysis requires residues in the $\alpha$ subunit, the simulations were performed with the $\alpha\beta$ dimer. The results indicate that the large-scale opening motion is also achieved by the B-helix sliding (in the reverse direction). However, the sliding mechanism is different from that of ATP binding because sliding is triggered by separation of the hydrolysis products ADP and Pi. We also addressed several important issues: 1), the timing of the product Pi release; 2), the unresolved half-closed $\beta$ structure; and 3), the ADP release mechanism. These issues are fundamental for motor function; thus, the rotational mechanism of the entire $F_1$-ATPase is also elucidated through this $\alpha\beta$ study. During the conformational change, conserved residues among the ATPase proteins play important roles, suggesting that the obtained mechanism may be shared with other ATPase proteins. When combined with our previous studies, these results provide a comprehensive view of the $\beta$-subunit conformational change that drives the ATPase.

INTRODUCTION

The rotation of $F_1$-ATPase, an ATP-driven rotary motor enzyme, is performed by the catalytically active $\beta$ subunits (1–12). This rotation can be reversed via ATP synthesis/hydrolysis and is coupled to an electrochemical gradient for diffusion across a membrane-embedded unit, $F_0$ (Fig. 1 A) (13). In the 3D structure of $F_1$-ATPase, the $\alpha_3\beta_3$ subunits are arranged hexagonally around the central $\gamma$-subunit stalk (Fig. 1 B) (14). Because the $\beta$ subunit changes its conformation during nucleotide perturbations (nucleotide binding/ release and ATP hydrolysis), most crystal structures show the three $\beta$ subunits in different states: two closed states ($\beta_{TP}$ and $\beta_{TP}$) and one open state ($\beta_E$), which corresponds to the catalytic dwell (15).

The scheme of the structural change of the $\beta$ subunit associated with the nucleotide changes has been elucidated by single-molecule experiments using the bacterial $F_1$-ATPase. One step of the rotation is 120° (16), which can be further decomposed into 40° and 80° substeps (Fig. 1 D) (17). The 40° rotation after ATP hydrolysis (18) is supposedly induced by the conformational change of the $\beta$ subunit (closed → half-closed) caused by the ATP hydrolysis (19) and the release of Pi from the $\beta_E$ subunit (20). In the ATP-binding dwell state, the three $\beta$ subunits in the $F_1$-ATPase complex take on closed, open, and half-closed conformation states (19). The half-closed structure was previously observed in a single-molecule experiment (19), but the atomic-scale structure has not been determined. Next, the subsequent 80° rotation is induced by ATP binding and ADP release, which are accompanied by structural changes (open → closed, and half-closed → open, respectively) (19,21). Very recently, the rotation scheme of the human $F_1$-ATPase was identified. It is slightly different from those of the bacterial $F_1$-ATPase; one more rotation pause has been identified before the catalytic dwell (three rotation pauses in total). The Pi release occurs in the newly found rotation pause, which is called as “the Pi dwell”. This means that one of the nucleotide events in the 40° substep occurs in the Pi dwell separately. Nevertheless, in either case (bacteria or human), the conformational changes of the $\beta$ subunit are the engine of the molecular motor.

The consumption of one molecule of ATP involves opening and closing motions for the $\beta$ subunit conformational changes. The closing motion is induced by the ATP binding in the phase during which $\gamma$ rotates from 0° to 80°, as shown in Fig. 1 C (19). This mechanism, which was determined in our previous study, is accomplished in two steps: the hydrogen-bond network around ATP changes and then the entire structure changes via sliding of the B-helix (22). After ATP hydrolysis, whose chemical reaction mechanism has been elucidated by using the quantum mechanics/molecular mechanics (QM/MM) method (23,24), the $\beta$ subunit...
becomes open again in the phase of rotation from 200° to 320° (19), as shown in Fig. 1, C and D (cyan circles). Important issues regarding this opening motion remain to be elucidated: 1), the timing for the product Pi release; 2), the half-closed structure of the β subunit in atomic detail; and 3), the mechanism of the ADP release. All of these issues are crucial for elucidating the mechanism of motor rotation. Hence, in this study, we investigated the opening motion caused by ATP hydrolysis.

With respect to the first issue, it has been debated whether Pi, which is produced by ATP hydrolysis, is released at the rotation of γ from 200° or 320° (Fig. 1, C and D), because it is extremely difficult to obtain direct proof of the rapid phosphate release. To overcome this difficulty, stochastic estimation has been applied to a single-molecule experiment. This experiment utilized the reversibility of ATP hydrolysis and demonstrated that Pi is released from the αβDP interface after an additional 120° rotation (at 320°), as shown in Fig. 1, C (red) and D (the model with the large brackets) (20). Although this scheme is supported by a recent theoretical study (25), we cannot completely rule out an alternative hypothesis, namely, that Pi is released from the αDPβDP catalytic site immediately after the ATP hydrolysis, as shown in Fig. 1, C (in gray at 200°) and D (with the small bracket) (26–30). This hypothesis is supported by observations of the nucleotide occupancy of all three sites during the catalytic dwell, because Pi is released immediately after hydrolysis, and the absence of Pi in the αεβE site (the empty αεβE in the small bracket in Fig. 1 D) allows a third nucleotide to bind there (21,31). It is important to elucidate the timing of Pi release because the two scenarios imply different mechanisms for generating the torque for the 40° rotation. In the case of Pi release at 320°, the two different sites can contribute to the rotational torque, because ATP hydrolysis and Pi release occur at αDPβDP and αεβE, respectively, as shown in the large bracket of the 40° substep in Fig. 1. D. In contrast, in the case of immediate Pi release (at 200°), only the αDPβDP site contributes to the torque because both the hydrolysis and Pi release occur at the αDPβDP subunits (Fig. 1 D, the small bracket). To assess these two possibilities, the quantitative stability of the structural changes both with and without Pi must be investigated, and the mechanistic reason for requiring the presence or absence Pi to take advantage of the structural change should be elucidated.

With regard to the second issue, although the existence of the half-closed structure at the ATP binding dwell (Fig. 1 D, 240°) was previously identified by a single-molecule experiment (19), the atomic-scale structure has not yet been determined. A definitive half-closed structure would provide a rational reason for why such an intermediate appears at the rotation pause (the ATP binding dwell). Additionally, an evaluation of the relative energy of the half-closed state with respect to the other open and closed structures would give us insight into the energetics of the opening pathway (e.g., downhill or uphill), which ultimately provides the driving force for the rotation after hydrolysis.

Finally, with respect to the third issue, although a single-molecule experiment identified ADP release after formation of the half-closed structure (rotation of γ from 240° to 320° in Fig. 1 C) (21), how the structure of the nucleotide binding

**FIGURE 1** General information about F1-ATPase. (A and B) Structure of the F0F1-ATPase complex (A) and the cross-sectional arrangement of the subunits in the F1 moiety (B), viewed from the side of the C-terminal domains. This schematic figure corresponds to the 200° rotation angle in C. The list indicates abbreviations assigned to the nucleotide states and the β subunit conformations, which correspond to each other. In the crystal structures of F1-ATPase, αβDP subunits have been referred to as αεβE. However, in this paper, in order to distinguish the Pi-bound form from the empty state, we refer to the Pi-bound form as αεβE. (C) Reaction scheme of the bacterial F1-ATPase for the 360° rotation of the γ subunit. For convenience, nucleotide events are indicated in only one αε subunit pair, which is colored cyan. The Pi-retaining model is indicated in red, and the model with immediate Pi release after hydrolysis is shown in gray after 200°. (D) Reaction scheme for the 120° rotation of the γ subunit (this angle corresponds to the phase between 200° and 320° in C). The figures inside the brackets indicate each intermediate for the 40° and 80° substeps. The large and small brackets indicate the Pi-retaining and immediate-release models, respectively. To see this figure in color, go online.
site changes upon the ADP release and why it occurs after the formation of the half-closed state are barely understood. The release of ADP with the opening motion is found not only in the β subunit of F₁-ATPase but also in a variety of ATPase proteins. Therefore, elucidating the concerted functions in the β subunit (the opening motion and the reduction of the ADP binding affinity) may provide a clue as to their exact relationship, which may be shared across the ATPase family.

To examine these issues, we studied the structural transition pathway after ATP hydrolysis using a theoretical method. Here, we used all-atom free-energy simulations. We adopted a combination of the nudged elastic band (NEB) method and umbrella sampling molecular-dynamics (MD) simulations (32–33) using the ΔRMSD reaction coordinate (the difference in the root mean-square deviation (RMSD) between the open and closed structures) (32–35). The results show the behavior of the system during the transition from the closed form to the open form, along with the free-energy variations along the reaction coordinate. The hydrolysis-induced structural change requires residues in the α subunit (e.g., the arginine finger, αArg73) (23,36,37). Hence, the simulations of the β opening motion were performed with the inclusion of the α subunit. The revealed conformational change of the αβ dimer addresses all of the issues listed above. Because these issues are fundamental to motor function, the rotational mechanisms of the entire F₁-ATPase complex were also elucidated by this αβ study. Moreover, because strongly conserved residues among the ATPase protein family, such as the Walker A/B motifs and the P-loop, play important roles during the conformational change, we suggest that the obtained mechanism may be shared with other ATPase proteins. When combined with our previous studies on the β closing motion (22) and the hydrolysis reaction (23,24), these results present a comprehensive view of the atomic-level β-subunit conformational change that drives the ATPase.

MATERIALS AND METHODS

Outline of computational methods

To determine the timing of the Pi release, a pair of free-energy profiles for the ADP + Pi and the ADP-bound states is required. Therefore, we first generated two initial paths, one with ADP + Pi and one with ADP, between the open and closed states of the αβ dimer using the NEB method (38,39) implemented with CHARMM (40). The NEB method can be used to find a minimum energy path between two given reference structures. Subsequently, each configuration along the path was subjected to umbrella sampling (41), which involved MD simulations with restraints on the ΔRMSD order parameter. The ΔRMSD reaction coordinate has previously provided functionally important information, such as the side-chain conformational changes that cause an overall structural transition (32–35). Next, the free-energy surface along the ΔRMSD order parameter was calculated from a series of the umbrella sampling simulations using a weighted histogram analysis method (WHAM) (42). The details of this procedure are described in the following sections.

Reference structures

In both the path with ADP + Pi and the path with ADP, the αDP (A and E chain) and αDP_C (C and D chain) dimers in the crystal structure (Protein Data Bank (PDB) 2JDI) (43) were used for the two endpoints for the open and closed states, respectively. In the 2JDI crystal structure, the αDP pair is in the nucleotide-free state, whereas the αDP_CDP binds AMP-PNP, which represents a structure in the catalytic dwell, rather than the ADP-inhibited state. Using the following procedures, we modeled the two endpoint structures in the two paths for a total of four structures (αDP_CDP with ADP + Pi, αDP_E with ADP + Pi, αDP_C with ADP, and αDP with ADP). First, to build the closed αDP_CDP structure with ADP + Pi after hydrolysis, the positions and orientations of the ADP and Pi molecules were obtained from the product geometry of a QM/MM study (23). To model the subsequent open αDP subunits binding ADP + Pi, the coordinates of the only ADP in that modeled closed αDP_CDP structure were transplanted into the adenine-binding pocket of the open αDP structure, where the fit was carried out over the C-terminal domain of the β subunit. A previous NMR study showed that mutant monomer β subunits can bind a nucleotide at the adenine-binding pocket even in the open conformation (44); thus, this position of the bound ADP in the adenine pocket in the open αDP structure is plausible. Then, we used the position of Pi in the open αDP structure from a crystal structure reported in 2006 (45) because this structure contains Pi in the β open subunit. For the reference structures in the path with only ADP, the Pi molecule was simply removed from the modeled αDP and αDP_C subunits of the ADP + Pi state.

Regarding the protonation state of Pi, we adopted doubly protonated Pi (H₂PO₄⁻), which is consistent with the product state in QM/MM studies (22,24). On the other hand, a recent MD simulation indicated that a singly protonated Pi (HPO₄⁻) is released from the αDP subunit (25). However, in that study, it was suggested that the migration of one proton from H₂PO₄⁻ occurs after the ADP release (Fig. 1D, middle), since when ADP (the negatively charged molecule) is released after the half-closed state, the electrostatic environment of the nucleotide-binding site shifts to positive, enabling the proton to be withdrawn. Therefore, for our main focus (to explore the structural transition from hydrolysis to the half-closed state), H₂PO₄⁻ is suitable.

Initial path

Sixty-one initial structures, including the endpoint states, were generated via linear interpolation between the open and closed reference structures for each path (with ADP + Pi and with ADP). This initial path was minimized using the steepest descent (SD) method with 4,000 steps and the replica path method with a spring constant of 500,000 kcal/mol/Å². Subsequently, NEB using the adopted basis Newton-Raphson (ABNR) minimization with a force constant of 10,000 kcal/mol/Å² was performed for 5,000 steps until convergence occurred. These details regarding the NEB method are taken from Arora et al. (32).

Umbrella sampling simulations

The 61 initial structures along the ADP + Pi pathway and the ADP pathway were subjected to umbrella sampling MD simulations with the restraint w_i on the ΔD_RMSD order parameter (34), w_i = K_RMSD(ΔD_RMSD − ΔD_min)², where ΔD_min is the value around which ΔD_RMSD is restrained, and K_RMSD is a force constraint. The ΔD_RMSD order parameter is the difference in the RMSD values of each intermediate structure from the reference open and closed states. It is given by ΔD_RMSD = rmsd(Xi, X_open)− rmsd(Xi, X_closed), where Xi is the instantaneous structure during the simulation, and X_open and X_closed are the two reference structures, i.e., the open αDP and closed αDP_CDP dimers, respectively. ΔD_RMSD has been successfully used as an order parameter to characterize the transition pathways between two structures in various proteins and DNA (32–35). The advantage of using the ΔD_RMSD restraint is that intermediates on the transition path can simultaneously evolve away from both the open and closed structures (34).
61 structures obtained from the NEB path optimization cover an RMSD range of ~9.5 Å (the $\Delta_{RMSD}$ value ranges from ~4.7 to 4.7 Å). The structures were separated by an interval of ~0.15 Å in the $\Delta_{RMSD}$ order parameter space. These 61 structures formed 61 windows for umbrella sampling runs.

In each window, the structure was solvated in a box of water and neutralized with counterions (Na$^+$ and Cl$^-$). The total number of atoms was 137,164 for the ADP- Pi-bound state and 137,743 for the ADP-bound state. These states included 122,196 (ADP + Pi) and 122,781 (ADP) atoms for the water molecules and 222 (Na$^+$: 120 and Cl$^-$: 102, ADP + Pi) and 223 (Na$^+$: 120 and Cl$^-$: 103, ADP) atoms for the counterions. The initial distance between periodic images of the protein was 28.0 Å. After minimization of the initial structure in each window for 6000 steps using SD, heating from 1 to 300 K was performed with harmonic constraints of 1.2 kcal/mol on the nonhydrogen atoms of the solute (total of 14,746 (ADP + Pi) and 14,739 (ADP) atoms) under NVT ensemble conditions. The system was then equilibrated for 100 ps with constraints of 1.0 kcal/mol on the nonhydrogen atoms under an NPT ensemble (300 K and 1 atm) condition. Subsequently, the RMSD force constraint, $K_{RMSD}$, was gradually reduced from 7300 to 140 kcal/mol Å$^2$ over a period of 0.5 ns in the NVT MD simulation. After equilibration, sampling simulations were performed. Initially, the sampling simulations of each window for both the ADP + Pi and ADP pathways were performed for 10 ns. In the ADP-bound pathway, the energy profiles of three phases (0–3.3 ns, 3.3–6.6 ns, and 6.6–10 ns) were converged. However, in the ADP + Pi-bound pathway, the energy profiles of three phases were different. Therefore, the calculations of all the windows for the ADP + Pi-bound pathway were extended until 40 ns. In the last 10 ns (30–40 ns), the differences in the energy profiles for the three phases were satisfactorily small, as shown by the error bars in Fig. 2 A. This means that the only ADP pathway is not much different from the path found by the NEB, whereas the ADP + Pi pathway, which is fairly different from the NEB path, is successfully obtained by the umbrella sampling simulations. Accordingly, we used the last 10 ns of the trajectories for the data analysis of the ADP + Pi-bound pathway. The structures were simulated with a weak 1D $\Delta_{RMSD}$ restraint of 140 kcal/mol Å$^2$ on the heavy atoms of the solute using NVT MD simulations.

After all sampling simulations, the biased distribution along the reaction coordinate was checked. At several points of the $\Delta_{RMSD}$ reaction coordinate, the distribution overlap between adjacent windows was insufficient to explore the free-energy surface. Therefore, additional windows were appended. Those initial structures were generated via NEB using the neighbor, the distribution overlap between adjacent windows was insufficient.

Free-energy calculation

The 1D potential of mean force (PMF) (55) along the $\Delta_{RMSD}$ Reaction coordinate was calculated using WHAM (42) from a set of 10 ns umbrella sampling windows (ADP + Pi: 30–40 ns; ADP: 1–10 ns). Before the computationally intensive 2D umbrella sampling simulations were performed, the 2D free-energy profile along local conformational changes (e.g., the RMSD for a local structure, the distance between two atoms, and the dihedral angle of residues) were obtained from the trajectories of the 1D umbrella sampling simulations. The full 2D PMF was also calculated using WHAM.

![Figure 2](https://example.com/figure2.png)

**Figure 2** (A and B) Free-energy profiles associated with the conformational transitions of the αβ dimer subunits after ATP hydrolysis. The 1D energy profiles along the $\Delta_{RMSD}$ reaction coordinate in the ADP + Pi- and ADP-bound pathways, respectively, are shown. The error bars represent the standard error of the mean energy values determined from the 10 ns trajectory divided into three phases. The $\Delta_{RMSD}$ value of the open (αδγβp) and closed forms (αδγβpδj) in the 2JDI crystal structure is ~4.7 and 4.7, respectively. (C) The 2D landscape shows the changes of the probe angle along with the $\Delta_{RMSD}$ reaction coordinate. The unit of energy is kcal/mol. The half-closed structure (20º) is indicated by the magenta dotted line. (D and E) The 2D landscapes between the RMSD values from the half-closed structure of the PDB 1HBE crystal structure and the $\Delta_{RMSD}$ reaction coordinate. Judging from F–E, the half-closed structure is at approximately $\Delta_{RMSD} = -0.6$. This structure position is marked in green in A. To see this figure in color, go online.
RESULTS
Timing of Pi release

After ATP hydrolysis, there are two possible nucleotide states. One is that Pi is retained at the catalytic site and released from the αβDP interface in Fig. 1, C (in red) and D (the scheme with the large brackets), after an additional 120° rotation of the γ subunit (at the 320°). Alternatively, immediately after ATP hydrolysis, Pi is released from the αDPβDP interface at the 200°, as shown in Fig. 1, C (in gray) and D (with the small bracket). To assess these two possibilities, we calculated the structural changes with and without Pi (ADP + Pi/only ADP) in the β subunit and determined their quantitative stabilities.

The energy profiles indicate that Pi should be present in the binding site for the spontaneous structural change because there is no barrier between the open and closed forms in the profile of the ADP + Pi-bound state (Fig. 2 A). The results agree well with observation in the single-molecule experiment of the reversibility of ATP hydrolysis (20) and a recent theoretical study (25). By contrast, in the profile of the structural change with only ADP, the open and closed states are separated by an energy barrier of ~7 kcal/mol (Fig. 2 B). This means that when Pi escapes immediately after ATP hydrolysis, the structure remains closed. We interpret this as a result of ADP inhibition: during the rotation of ATP hydrolysis, the motor stops at the catalytic waiting dwell due to the inhibition of the product, i.e., ADP (56–59), because the inhibited timing and the nucleotide-binding state shown in the simulation results of the state bound with only ADP are consistent with this ADP inhibition. Moreover, an observation related to this ADP inhibition, where adding Pi into inhibited F1-ATPase reactivates the rotation (60), can be explained by the two energy profiles. Upon addition of Pi, the free-energy landscape shifts from the only-ADP-bound state to the ADP + Pi-bound state, so that the inhibited closed β subunit regains the ability to become the open form, resulting in the restart of the motor rotation. The consistency with experimental results (the 320° timing of the Pi release (20) and the ADP inhibition (56–60)) indicates that the energy profiles obtained for the two different binding states are reasonable.

The half-closed structure

The section above clarifies that for continual motor rotation (without entering the inhibition state), the β subunit must retain Pi in the binding site after ATP hydrolysis; thus, the structural details of the transition pathway are discussed only for the ADP + Pi-bound state hereafter.

For this pathway, we determined whether the half-closed structure is obtained during the transition from the closed form (αDPβDPγ) to the open form (ααβγ) because it was found at the ATP binding dwell (Fig. 1 D) in the single-molecule experiment (19). In this experiment, to reveal the β subunit conformation, the dipole orientations of a fluorophore introduced into helix 6 (residues from E464 to D471) of the β subunit were monitored. The orientations of the fluorophore were projected onto the N-terminal plane, which passes through the center-of-mass coordinates of the N-terminal domain in the three β subunits. The projected angles, 0°, 20°, and 45°, correspond to the open, half-closed, and closed structures, respectively (19). To compare the simulation results with the experimentally measured angles of the probe, we projected the orientations of helix 6 (the line connecting its Cβ atoms) of the simulated structures onto the same N-terminal plane. The projection was calculated using the superimposition of the whole structure of 2JDI onto the simulated trajectory (the fit was performed over the N-terminal domain of the αβ dimer subunits) because the simulation was performed with only the αβ dimer. The 20° angle corresponding to the half-closed structure is observed at ΔDRMSD = −0.6 (Fig. 2 C), which is the global minimum of the energy surface (Fig. 2 A). The F1-ATPase rotation is paused at the half-closed state (the ATP binding dwell) (16,17,19); thus, it is reasonable that the half-closed structure appears at the global minimum in the energy profile.

To further validate the obtained structure at ΔDRMSD = −0.6 (the global minimum), we compared the simulated structures with the 1H8E crystal structure (61). The 1H8E structure of the αβγ complex is not supposed to represent the intermediate along the reaction pathway because its half-closed form is found in the position of the β0 subunit, and the other two β subunits bind a nucleotide and adopt the closed form (the other two should be the open and closed forms at the ATP binding dwell, as illustrated in Fig. 1 D). Due to these differences, the position of the half-closed αβ in 1H8E inevitably also differs with respect to the γ axis from that of the ATP binding dwell. Nonetheless, only the part of the half-closed αβ dimer that has thus far only been found in the 1H8E structure is a good indicator for comparing the open-closed bending angle of the β subunit and the αβ relative subunit arrangement (the interface configuration). Therefore, the Cα RMSD values between the simulated structure and the half-closed form in 1H8E were calculated for both the β subunit and the αβ dimer subunits. At ΔDRMSD = −0.6, both the RMSD values are under 2.0 Å (Fig. 2, D and E), meaning that the structure obtained at ΔDRMSD = −0.6 is similar to the half-closed form of 1H8E in terms of the opening angle of the β subunit and the relative arrangement of the αβ subunit. Consequently, the data (the helix 6 angle and the RMSD values) indicate that the half-closed structure is successfully obtained at ΔDRMSD = −0.6 on the pathway of the structural transition.

Atomic characteristics of the half-closed structure

Next, we report details of the obtained half-closed structure. Although the half-closed structure was detected in the
single-molecule experiment (19), the definitive structure has not been determined. Here, we investigated the portions that are essential for the structural change (44,62). The experimental results and the comparison between the open and closed β subunits in the crystal structure indicate that the following local structural changes should occur after hydrolysis: flipping of the main-chain dihedral angle in the hinge region (H177 and G178), opening of the β3/7 sheet, and switching of the hydrogen bond of the Walker A/B motifs. In the conformational change in the Walker A/B motif, the Walker B residue (D256) changes the hydrogen bond from one of the Walker A residues (T163) to the other one (K162).

Fig. 3, A and B, show the values of the backbone dihedral angles, ψ and φ, of the hinge residues, H177 and G178, respectively. The main chain of the hinge in the half-closed structure (at $\Delta D_{\text{RMSD}} = -0.6$) is already flipped from the conformation found in the closed form and shows the same values as in the open form. The conformation is shown in Fig. 3 C. However, the opening of the β3/7 sheet and the switching of the hydrogen bond in the Walker A and B motifs are not completed in the half-closed state ($\Delta D_{\text{RMSD}} = -0.6$), as shown by the RMSD values in Fig. 3, D and E. Specifically, D256 has already lost the hydrogen bond with T163, but has not yet formed the new hydrogen bond with K162 (Fig. 3, C and F). The completion of the structural change of the β3/7 sheet and the Walker A/B motifs occurs at approximately $\Delta D_{\text{RMSD}} = -1.0$ (Fig. 3, D–F).

Consequently, the half-closed structure can be summarized as follows: the hinge adopts the conformation found in the open form, but the β3/7 sheet and the Walker A/B motifs do not reach the conformation found in the open structure. The reason for the incompleteness of the structural change of the β3/7 sheet and the Walker A/B motifs in the half-closed structure is described in the following sections.

Conformational change mechanism for the opening motion

Our previous study elucidated the mechanism of the closing motion caused by ATP binding (22), where the large movement of the C-terminal domain corresponding to the overall structural change was achieved by the B-helix displacement. To investigate whether the mechanism of the opening motion due to ATP hydrolysis is the same as that found in the previous study, we monitored the displacement of the B-helix along the $\Delta D_{\text{RMSD}}$ reaction coordinate. The resulting RMSD value of the B-helix is gradually changed along the reaction coordinate (Fig. 4 A). Together with the alteration of the probe angle along the reaction coordinate (Fig. 2 C), which can be used as an indicator of the change of the entire structure, the large-scale conformational change after ATP hydrolysis is also induced by the B-helix movement. Fig. 4 B shows more clearly that the B-helix displacement relative to the upper portion leads to opening

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**FIGURE 3** (A, B, D, and E) 2D energy maps indicate the local conformational changes (vertical) associated with the structural transition of the αβ dimer subunits after hydrolysis (horizontal) in the ADP + Pi-bound pathway. The unit of energy is kcal/mol. (A and B) Changes in the dihedral angles of His-177 and Gly-178 (the hinge residues), respectively. The dihedral angles of the open, half-closed, and closed forms in the crystal structures (PDB 2JDI and 1H8E) are indicated in each graph. (D and E) RMSD changes for the β3/7 sheets and the Walker A and B motifs, respectively. The best fit for the RMSD values is obtained over the N-terminal domain. (C and F) Structures at $\Delta D_{\text{RMSD}} = 0.0$, $-0.6$, and $-1.0$ (marked in A); these structures are superimposed and depicted in pink, green, and orange, respectively. This local structure is the same part as the green rectangle region of Fig. 4, B and D. To see this figure in color, go online.
of the closed form. In a recent free-energy simulation (63) that used the β subunit without a substrate and a different reaction coordinate, a B-helix movement involving a large-scale conformational change was observed.

The opening motion induced by the B-helix slide is decomposed further into two steps, closed → half-closed and half-closed → open, because the half-closed state appears at the global energy minimum (Fig. 2A). In the following sections, the two steps are analyzed separately. Their mechanisms are summarized in Fig. 7.

**Closed → half-closed**

The B-helix movement that induces the large structural change is in fact derived from the separation between the hydrolysis products, ADP and Pi. The ADP is bound directly to the P-loop, which is located beside the B-helix in the lower portion, as shown in Fig. 4D. In contrast, Pi is bound to the upper (dark) portion (Fig. 4D). The displacement of the ADP-binding region (ADP, the P-loop, and the B-helix) relative to the Pi-binding region (Pi and the Pi-binding residues) is shown using the RMSD value in Fig. 4C. R189 and E188 are the Pi-binding residues of the β subunit because they continually contact Pi during the conformational change (the details of the thresholds for the selection of these residues are described in Figs. 4, S1, and S2). The RMSD value gradually changes along the reaction coordinate, suggesting that after hydrolysis, the ADP- and Pi-binding regions move away from each other. Moreover, this RMSD value changes simultaneously with the RMSD value relative to N-terminal domain (Fig. 4A), indicating that the separation between Pi and ADP makes the adjacent B-helix slide relative to the upper portion. In short, the B-helix displacement corresponding to the entire opening motion is attributed to the separation between the ADP- and Pi-binding regions (see Fig. 7, [C′ → HC]).

With the sliding of the B-helix due to the separation between ADP and Pi, K162 (Walker A), which plays crucial
roles throughout the conformational change, forms a hydrogen bond with Pi rather than with ADP (Figs. 5, A and C, and S1; the graph for the K162-Pi distance). K162 resides in the P-loop of the lower portion (the second to last residue of the common sequence: GXXXGXKT). However, the side chain of K162 is long enough to contact the Pi that is bound to the upper portion, until the half-closed state is reached (see Fig. 7, [C’→HC]). In the half-closed structure at the global minimum state, the side chain of K162 is fully stretched, bringing the Pi–K162 interaction to the point just before breaking (see Fig. 7, 240°). The same detail is found in the half-closed structure of 1H8E (61). This K162 condition (K162 retaining Pi) explains the incompleteness of the hydrogen bond formation of the Walker A/B motifs in the half-closed form, as shown in the section “Atomic characteristics of the half-closed structure” (Fig. 3 E). The opening of the β3/7 sheet was also incomplete in the half-closed state (Fig. 3 D), because the β3/7 sheet that is located just beside the Walker A/B motifs, as shown in Fig. 3 F, opens concomitantly with the conformational change of the Walker A/B motifs. Conversely, the hinge is already flipped in the half-closed structure, as shown in Fig. 3, A–C.

Half-closed → open

The β subunit opens further after the formation of the half-closed structure in the subsequent 80° substep, as shown in Fig. 7, [HC→O']. As shown in the free-energy profile in Fig. 2 A, the half-closed structure appears at the global minimum state. To evolve from this stable half-closed state, the structural change from half-closed to the open state must be coupled to the neighboring conformational change, i.e., the closing motion (βE→βT) due to the ATP binding (Fig. 1 D), which is energetically downhill (22). This is consistent with the fact that the Fi1-ATPase rotation pauses in the half-closed state until ATP binds to the neighboring subunit (16,17,19). When ATP binds to the neighboring αβ interface at the ATP binding dwell, an external perturbation (i.e., the inward αβ motion and its induced γ axis rotation) makes the half-closed structure slightly more open, meaning that the structure evolves from the global minimum. This further opening motion breaks the Pi–K162 hydrogen bond, which was already stretched to its limit in the half-closed state (Fig. 5, A and C). With the loss of its hydrogen-bonding partner, K162 eventually replaces Pi with D256 (Walker B) (Fig. 5, B and C). (Although K162 does not interact with Pi anymore, the Pi is bound by the E188 and R189 located in the upper portion (Figs. S1 and S2), and is mostly located at a distance of ~6 Å from K162, as shown in Fig. 5 A. Therefore, it is still inside the nucleotide-binding cavity, as illustrated in Fig. 7, [HC→O'].$\$

During the structural change from the half-closed to the open state, ADP is released, as shown in Fig. 1 C (from 240° to 320° in the rotation angle of the γ subunit) and Fig. 1 D (the 80° substep) (21,64). Unexpectedly, even in the 40 ns time length for each sampling window, we observed a consistent result in our simulation: the ADP escapes from the binding pocket in some running windows during the phase of ΔDRMSD < −0.6 (forms that are more open than the half-closed structure). To investigate the ADP release more clearly, we added one more dimension, the ADP-P-loop distance, to the umbrella sampling simulations and examined the positions of the ADP at the atomic level during the conformational transitions of the αβ dimer subunit.

Fig. 6 A shows the full 2D PMF. The color map indicates that in the phase of ΔDRMSD > −0.6 (the forms that are more closed than the half-closed structure), the area for the short distance between the ADP and the P-loop shows a lower energy state, meaning that ADP tends to stay on the P-loop. In contrast, in ΔDRMSD < −0.6 (forms that are more open than the half-closed structure), the lower-energy regions are spread over a wider range of distance values, indicating that ADP is easily released from the binding site. In addition to this ADP release, Fig. 6, B and C, show switching of the hydrogen bonds of K162 at the half-closed state (ΔDRMSD = −0.6). This switching was previously found in the 1D umbrella sampling simulations (Fig. 5, A–C) and is involved in the structural change from

![Image](https://example.com/image.png)

FIGURE 5 (A and B) 2D landscapes indicate the distance changes between K162 (NC) and Pi (P) and between K162 (NC) and D256 (CY), respectively, along the ΔDRMSD reaction coordinate in the ADP + Pi-bound pathway. The unit of energy is kcal/mol. (C) The local structure shown is the same part as in Figs. 2, B and D, and 3, C and F. To see this figure in color, go online.
the stable half-closed state. These data indicate that the local change at K162 also somehow leads to the ADP release.

By further analyzing this ADP release, we found that it is achieved by many concerted structural rearrangements around K162. First, the establishment of the K162–D256 bond (Fig. 6, B and C) rotates the K162 side chain from the orientation for binding to Pi to the one for binding to D256 (Fig. 6, D and F). This K162 rotation induces a P-loop conformation change (Fig. 6, D and E) because it resides on the P-loop. At the end of the cascade, the conformational change of the P-loop, which binds the ADP directly, necessarily decreases the ADP binding affinity and eventually leads to ADP release (Fig. 7, [HC → O']). Once ADP is released after the formation of the half-closed structure, the entire structure will become open spontaneously because, without any ligand binding, the open form is generally more stable than the closed. This drives the 80° substep rotation of the γ axis in concert with the conformational change induced by the ATP binding, as shown in Fig. 1 D.

Comparison with the rotation scheme of the human F₁-ATPase

So far, our simulation results based on the bovine F₁-ATPase have been compared with experimental data from the thermophilic Bacillus. Recently, Suzuki et al. (65) reported the rotational scheme of the human mitochondrial F₁-ATPase, which is evolutionarily close to the bovine F₁-ATPase. Surprisingly, the angles of substeps in the human F₁-ATPase are slightly different from those of the bacterial F₁-ATPase, and there is another rotation pause between the ATP binding and catalytic dwell. They insisted that most of the crystal structures of the bovine F₁-ATPase, which have been thought to be the catalytic dwell from the rotation...
scheme of the Bacillus F$_1$-ATPase, correspond to that new rotation pause (Pi-releasing pause, Pi dwell). Even so, this does not change the results presented here. First of all, the catalytic dwell lies on the path between this Pi dwell (the referenced structure) and the open state. In our previous studies of the yeast F$_1$-ATPase, the α$_{gD}$β$_{gP}$ interface became slightly loose after the Pi dwell. In our study, although the β subunits at Δ$D_{RMSD}$ = 2–3 in both paths are still closed (Figs. 2 C and S3), the αβ interface becomes looser (Fig S4). Therefore, the structures at around Δ$D_{RMSD}$ = 2–3 may be the catalytic dwell. In particular, the energy minimum in the ADP pathway appears at Δ$D_{RMSD}$ = 2–3, which strongly supports that idea. Consequently, our proposed mechanism still works out for the new scheme of the human F$_1$-ATPase.

**DISCUSSION**

One of the most prominent features in this work is that the half-closed structure appears at the global minimum in the structural transition pathway, which is simulated by referencing only the open and closed structures. This implies that during the αβ structural change, the β open-closed bending motion determines the αβ interface open-closed configuration, and vice versa. Additionally, the successful attainment of the half-closed structure in the simulation conditions, where the simulated system does not include the γ subunit, suggests that the formation of α$_{HC}$β$_{HC}$ should be independent of the existence of the γ subunit. This notion is supported by the presence of α$_{HC}$β$_{HC}$ in the 1H8E crystal structure, where the γ subunit orientation is different from the ATP binding dwell (61). This αβ self-formation without the γ subunit agrees well with our proposed rotational mechanism, i.e., that the conformational change of the α$_3$β$_3$ hexamer precedes the γ axis rotation (66–68).

The mechanism of the β-subunit conformational change is completely elucidated by our series of studies (22,23). The large-scale changes for both the closing and opening motions are induced by the B-helix sliding. Because the large-scale conformational change (open-closed) of the β subunit rotates the central stalk (66,67,69,70), the rotational torque can be attributed to this B-helix sliding. To experimentally test this hypothesis (i.e., whether the rotational torque is derived from the B-helix), single-molecule experiments involving the simultaneous observation of the B-helix sliding and the rotation of the central stalk are currently being conducted. Although both the opening and closing motions are induced by the B-helix sliding, their mechanisms are not simply reversed, because these slides are driven by different nucleotide perturbations (ATP binding and hydrolysis). During the closing motion caused by ATP binding, the slide of the B-helix occurs to relieve the frustration induced by the binding of ATP in its binding site (22). In contrast, this study shows that during the opening motion, the B-helix sliding is triggered by the separation between the hydrolysis products, ADP and Pi. The ADP-Pi separation inducing the large-scale structural change via the B-helix sliding is the mechanistic reason underlying the requirement for the presence of both ADP and Pi to take advantage of the structural change. Once the structure loses Pi (i.e., one of the leading factors for the opening motion), the structural transition has a 7 kcal/mol barrier and the structure remains in the closed, ADP-inhibited form (Fig. 2 B).

The structural change induced by the ADP-Pi separation is preceded by the ATP hydrolysis reaction, that is, the chemical cleavage of the γ phosphate group, which is exothermic by only 1.6 kcal/mol (23). This reaction energy is too small to contribute to the motor function. However, because the subsequent structural change induced by the ADP-Pi separation is barrierless (Fig. 2 A), the ATP hydrolysis does not have to produce a large reaction energy. Supposedly, the hydrolysis reaction serves to control the rotational direction of the motor, thus avoiding any unproductive reverse rotations caused by severe thermal fluctuations (23). Combined with this ATP hydrolysis reaction, the ADP-Pi separation triggering the large outward motion may be the mechanism that converts the chemical reaction energy to the mechanical work. Moreover, this chemical-mechanical conversion is simply performed by the hydrolysis products (ADP and Pi) and strongly conserved residues (as shown in Fig. 7 [C’→HC]). Therefore, we assume that it could apply to other ATPase proteins.

Although the ADP-Pi separation contributes to the opening motion of the β subunit, after the formation of the half-closed state, the ADP is released. Despite the direct observation in the single-molecule experiment (21), how the structure of the nucleotide-binding site changes upon ADP release and why this change occurs after the half-closed state were not well understood. Our study reveals that the local structural change of K162 (i.e., K162 (Walker A) switches its hydrogen bond from the product Pi to D256 (Walker B)) simultaneously induces both the opening motion from the half-closed to the open state and the ADP release (Figs. 5, A–C, and 7), resulting in ADP release after the formation of the half-closed state. The concerted function (i.e., the nucleotide release with the opening motion) is found not only in the β subunit of the F$_1$-ATPase but also in a variety of the ATPase proteins. This common phenomenon in the ATPase protein family might also be induced by a conserved structural change because the local change of K162 is accomplished independently by the strongly conserved residues in the ATPase proteins, such as the hydrolysis products (ADP and Pi), the Walker A/B motifs, and the P-loop, suggesting that the local change may also be conserved among various ATPase proteins.

The other hydrolysis product, Pi, is released from the α$_{gD}$β$_{gP}$ interface with an additional 120° rotation (at 320°); thus, the torque for the 40° rotation is induced by two sites,
Finally, although the mechanism is different, the same conserved regions around K162 also induce the large-scale closing motion in response to ATP binding, via the B-helix slide (22). This allows us to propose a view of the conformational change shared across the ATPase proteins. Conceivably, depending on whether ATP binds or hydrolyzes, a group of conserved residues in the ATPase proteins undergo a specific pattern of structural change that displaces a neighboring structure (such as the B-helix slide), leading the entire structure to a different state to perform its functions. Together with results from our previous studies (22,23), the findings presented here provide a comprehensive view of the β-subunit structural change that drives the ATPase.

**SUPPORTING MATERIAL**

Five figures are available at [http://www.biophysj.org/biophys/supplemental/S0006-3495(14)03063-X](http://www.biophysj.org/biophys/supplemental/S0006-3495(14)03063-X).

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