

# Trapping the ATP binding state leads to a detailed understanding of the F<sub>1</sub>-ATPase mechanism

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The rotary motor enzyme F<sub>0</sub>F<sub>1</sub>-ATP synthase uses the proton-motive force across a membrane to synthesize ATP from ADP and P<sub>i</sub> (H<sub>2</sub>PO<sub>4</sub><sup>-</sup>) under cellular conditions that favor the hydrolysis reaction by a factor of 2 × 10<sup>5</sup>. This remarkable ability to drive a reaction away from equilibrium by harnessing an external force differentiates it from an ordinary enzyme, which increases the rate of reaction without shifting the equilibrium. Hydrolysis takes place in the neighborhood of one conformation of the catalytic moiety F<sub>1</sub>-ATPase, whose structure is known from crystallography. By use of molecular dynamics simulations we trap a second structure, which is rotated by 40° from the catalytic dwell conformation and represents the state associated with ATP binding, in accord with single-molecule experiments. Using the two structures, we show why P<sub>i</sub> is not released immediately after ATP hydrolysis, but only after a subsequent 120° rotation, in agreement with experiment. A concerted conformational change of the α<sub>3</sub>β<sub>3</sub> crown is shown to induce the 40° rotation of the γ-subunit only when the β<sub>E</sub> subunit is empty, whereas with P<sub>i</sub> bound, β<sub>E</sub> serves as a latch to prevent the rotation of γ. The present results provide a rationalization of how F<sub>1</sub>-ATPase achieves the coupling between the small changes in the active site of β<sub>DP</sub> and the 40° rotation of γ.

F<sub>1</sub>-ATPase | chemomechanical coupling | ATP waiting state | molecular dynamics | P<sub>i</sub> release

The molecular motor F<sub>0</sub>F<sub>1</sub>-ATP synthase is composed of two domains: a transmembrane portion (F<sub>0</sub>), the rotation of which is induced by a proton gradient, and a globular catalytic moiety (F<sub>1</sub>) that synthesizes and hydrolyzes ATP. The primary function of the proton-motive force acting on F<sub>0</sub>F<sub>1</sub>-ATP synthase is to provide the torque required to rotate the γ-subunit in the direction for ATP synthesis (1, 2). The catalytic moiety, F<sub>1</sub>-ATPase, has an α<sub>3</sub>β<sub>3</sub> “crown” composed of three α- and three β-subunits arranged in alternation around the γ-subunit, which has a globular base and an extended coiled-coil portion (3) (Fig. 1A). F<sub>1</sub>-ATPase by itself binds ATP and hydrolyzes it to induce rotation of the γ-subunit (in the opposite direction from that for synthesis) on the millisecond time scale under optimum conditions (4, 5). All of the α- and β-subunits bind nucleotides, but only the three β-subunits are catalytically active. The original crystal structure (3) of F<sub>1</sub>-ATPase from bovine heart mitochondria (MF<sub>1</sub>) led to the identification of three conformations of the β-subunit: β<sub>E</sub> (empty), β<sub>TP</sub> (ATP analog bound), and β<sub>DP</sub> (ADP bound); Fig. 1A. In the known structures of F<sub>1</sub>-ATPase, which apparently are near the “catalytic dwell” state, the state in which catalysis occurs (6, 7), the β<sub>E</sub> subunit conformation is partly to fully open and is very different from those of the β<sub>TP</sub> and β<sub>DP</sub> subunits, which are closed and very similar to each other (*SI Appendix, SII*).

## Searching for the ATP Waiting State

Because no X-ray structure is available for the ATP waiting state, we searched for it by molecular dynamics (MD) simulations with an external torque applied to the γ-subunit in the hydrolysis

direction while introducing different conformations of the β<sub>DP</sub> subunit in the α<sub>3</sub>β<sub>3</sub> crown, in accord with suggestions from single-molecule experiments (8). The results are shown in Fig. 2 (see *Methods* and *SI Appendix, S12* for details of the simulations). In Fig. 2, we refer 200° for the γ-rotation angle of the catalytic dwell state and 240° for the ATP waiting dwell state, respectively, to stress that the hydrolysis of an ATP, denoted as ATP\*, bound after the ATP waiting dwell at 0°, takes place at the 200° catalytic dwell state (see Fig. 1B for the rotation angle of γ relative to the α<sub>3</sub>β<sub>3</sub> complex). The initial simulation used the “Walker” crown structure [Protein Data Bank (PDB) ID code 1BMF] (3), in which the β<sub>DP</sub> subunit is closed with the angle (B^C) formed by helices B and C equal to 21.6°, and the γ-subunit structure of Gibbons et al. (PDB ID code 1E79) (9) (Fig. 1; see *Methods* for system preparation). It was represented by an all-atom model based on the CHARMM program (10), combined with a coarse-grained plastic network model (PNM) (11, 12). Even for an applied torque of 2,500 pN-nm, much higher than is generated in the normal function (13), the γ-subunit, which has an initial rotation angle of 200°, stalled at an angle of about 220°. In the present work, we define the rotation angle of γ as the angle formed between an instantaneous vector and a reference vector, each defining the orientation of γ relative to the three β-subunits for the instantaneous configuration from MD or the reference Walker structure, respectively (see *Methods* and *SI Appendix, S12* for angle definition). Major clashes between residues γS12-I16 and β<sub>DP</sub>L384-I388, near the DELSEED motif, prevented further rotation (*SI Appendix, S13* and Fig. S1). When the external torque was removed, the γ-subunit returned to within 2.5° of the crystal

## Significance

F<sub>1</sub>-ATPase is a motor protein that converts the free energy of binding of ATP and its hydrolysis products ADP and P<sub>i</sub> into a mechanical force for γ-subunit rotation. It is the catalytic moiety of F<sub>0</sub>F<sub>1</sub>-ATPase, which synthesizes ATP. There are two metastable states along each 120° rotation of the γ-subunit, one associated with ATP hydrolysis (the “catalytic dwell”) and the other with ATP binding (the “ATP waiting dwell”). We use molecular simulations to determine the ATP waiting dwell structure. With this structure and the catalytic dwell X-ray structure, we develop an atomic-level model of the coupling between ATP hydrolysis and γ-subunit rotation. The molecular-level understanding of this motor will aid in its use in nanomachines and cancer therapy.

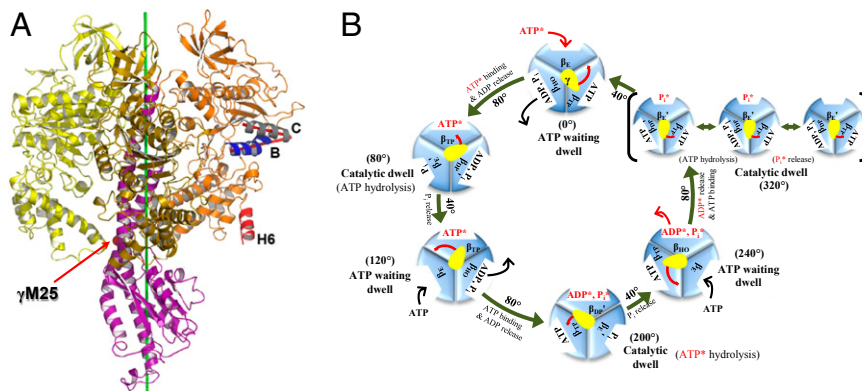
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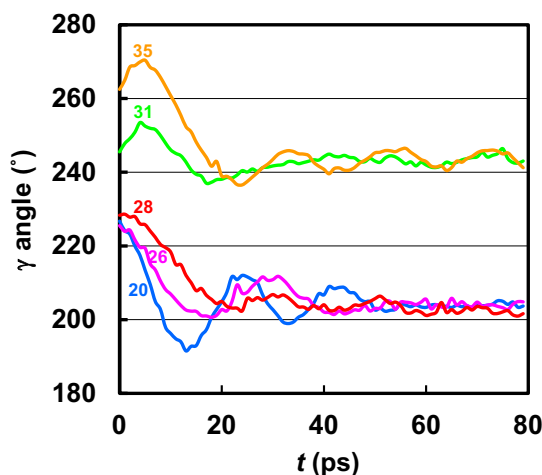
**Fig. 1.** (A)  $F_1$ -ATPase. The three  $\beta$ -subunits and the  $\gamma$ -subunit are shown ( $\alpha$ -subunits are not shown for clarity):  $\beta_E$  (yellow),  $\beta_{DP}$  (orange),  $\beta_{TP}$  (gold), and  $\gamma$  (purple). To define the  $\beta_{DP}$  subunit conformation we use the angle between helix B ( $\beta_{T163-A176}$ ) and helix C ( $\beta_{T190-G204}$ ). The two helices are highlighted: helix B (blue) and helix C (gray); the  $B^{\wedge}C$  angle is depicted as a red angle. The  $\beta_{DP}H6$  helix, whose orientation was reported to undergo a  $20^\circ$  change during the  $40^\circ$  substep  $\gamma$ -rotation, is highlighted as red. During the forced rotation simulations with an external torque, the force acts on the  $C_\alpha$  atom of  $MF_1:\gamma M25$  (shown as a red sphere). The direction of the force is determined as the cross-product of the radial vector of  $\gamma M25:C_\alpha$  and the rotational axis (green). (B) Proposed  $360^\circ$  rotation cycle of  $F_1$ -ATPase showing the subunit conformations, as well as the binding–release of ligands and the hydrolysis of ATP. Starting from the binding of an  $ATP^*$  to the  $\beta_E$  subunit in the ATP waiting state ( $0^\circ$ ), rotation of the  $\gamma$ -stalk by  $200^\circ$  ( $80^\circ$ ,  $40^\circ$ ,  $80^\circ$ ) leads to the transition of  $\beta_E$  ( $\gamma = 0^\circ$ ) via  $\beta_{TP}$  ( $\gamma = 80^\circ$ ) to  $\beta_{DP}$  ( $\gamma = 200^\circ$ ), the catalytic dwell state where hydrolysis of  $ATP^*$  takes place. The hydrolysis product  $P_i^*$  in the  $\beta_{DP}$  subunit is not released at this catalytic dwell ( $200^\circ$ ). Instead, the other hydrolysis product  $ADP^*$  is released first after a  $40^\circ$  rotation [ $\beta_{DP}$  ( $200^\circ$ )  $\rightarrow$   $\beta_{HO}$  ( $240^\circ$ )]. Then,  $\beta_{HO}$  is transformed to  $\beta_E$  and  $P_i^*$  is released after an additional  $80^\circ$  rotation to another catalytic dwell state ( $320^\circ$ ); the latter is shown in brackets outside the main cycle (see below). Finally, the release of  $P_i^*$  from  $\beta_E$  leads to a  $40^\circ$  rotation that completes the  $360^\circ$  cycle (21, 41). The other subunits are going through corresponding cycles offset by  $120^\circ$  ( $\beta_{DP}$ ) and  $240^\circ$  ( $\beta_{TP}$ ), respectively. Here, the prime symbol when it appears on the  $\beta_{DP}$  and  $\beta_E$  conformations indicates that the conformation of corresponding subunits change slightly in or near the specified reaction steps. The  $\gamma$ -subunit is shown as a yellow oval, and its rotation during the hydrolysis cycle is indicated by a red arrow. The reaction steps occurring in or near the catalytic dwell and corresponding changes of ligands in each  $\beta$ -subunit are also shown in the  $320^\circ$  catalytic dwell: The first state (Left in the  $320^\circ$  catalytic dwell) has a bound  $ATP$  in  $\beta_{DP}'$ , and is thus referred to as a prehydrolysis state (the state before the hydrolysis of  $ATP$  during the catalytic dwell). The second state (Middle) represents the state after  $ATP$  hydrolysis (posthydrolysis state), and the third state (Right) presents the state after the release of  $P_i$  bound in  $\beta_E'$  (postrelease state).

orientation in 200 ps, indicating that for the Walker crown structure, the  $\gamma$ -subunit orientation ( $200^\circ$ ) is a minimum.

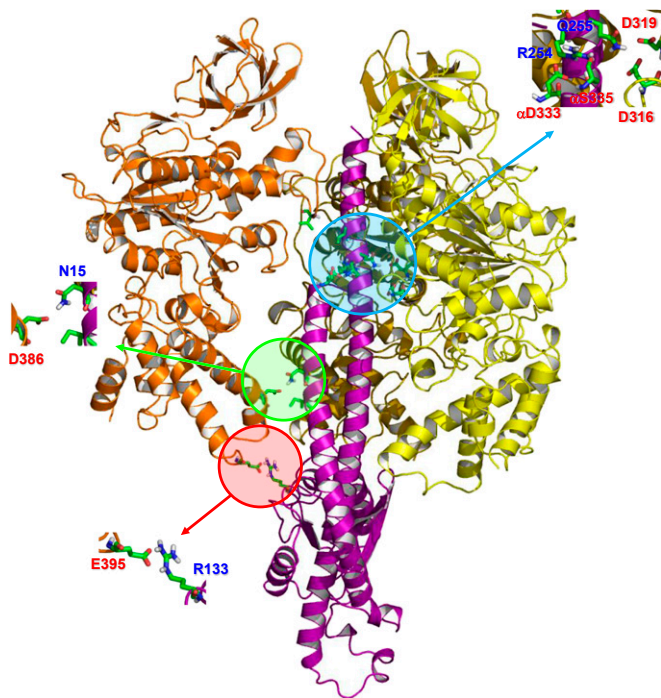
To find the ATP waiting state, we restarted the external torque simulations with more open conformations for the  $\beta_{DP}$  subunit, keeping the rest of the crown near its original structure with the PNM. Fig. 2 shows the  $\gamma$ -rotation angle and the ( $B^{\wedge}C$ ) angle of  $\beta_{DP}$  along the simulated conformational transition of  $\beta_{DP}$  to the more open conformation in the presence of the external torque. For a slightly more open  $\beta_{DP}$  subunit ( $B^{\wedge}C = 28^\circ$ ), similar to that of the half-open  $\beta_{HO}$  conformation ( $B^{\wedge}C = 23^\circ$ ; PDB ID code 2HLD\_I, where “I” denotes the first  $\alpha_3\beta_3\gamma$ -complex among the three complexes in the crystallographic asymmetric unit of 2HLD) (14), the  $\gamma$ -subunit returned to fluctuate around  $202^\circ$ . With the  $\beta_{DP}$  subunit having  $B^{\wedge}C$  equal to  $31^\circ$  and  $35^\circ$ , similar to that of the half-closed  $\beta_{HC}$  conformation ( $B^{\wedge}C = 32^\circ$ ) (see also below) in the Menz et al. structure (PDB ID code 1H8E) (15), the  $\gamma$ -subunit rotated to  $255^\circ$  and  $271^\circ$ , respectively, before it stalled again. When the torque was removed, the  $\gamma$ -subunit relaxed rapidly in both cases to near  $240^\circ$  and remained there during the rest of the simulation. The results show that there exists a locally stable state with the  $\gamma$ -rotation angle near  $240^\circ$  and the  $\alpha_3\beta_3$  crown with the catalytic subunits having conformations corresponding to  $\beta_E$ -like,  $\beta_{HC}$ -like (based on the  $B^{\wedge}C$  angle), and  $\beta_{TP}$ -like. We note that the  $B^{\wedge}C$  angles are  $\beta_E = 49^\circ$ ,  $\beta_{HC} = 32^\circ$ ,  $\beta_{HO} = 23^\circ$ ,  $\beta_{DP} = 22^\circ$ , and  $\beta_{TP} = 19^\circ$ .

To check the trapping simulation, we used an alternative protocol (SI Appendix, SI2) and experimental data from Masaïke et al. (8), who estimated that the helix-6 angle of  $\beta_{DP}$  is rotated by  $20^\circ$  in the ATP waiting state. The  $\beta_{DP}$  subunit and the  $\gamma$ -subunit were subjected to a biased simulation (16) and it was found that for the partly open structure of  $\beta_{DP}$  (helix-6 angle equal  $20^\circ$ ;  $B^{\wedge}C = 23^\circ$ ) the  $\gamma$ -subunit had rotated by  $40^\circ$  to reach the  $240^\circ$  state. A number of interactions stabilize the  $240^\circ$  state (see Fig. 3 and SI Appendix, SI4 for details and a comparison with the interactions in the trapped structure). The structure was then subjected to all-

atom explicit water MD simulations with no PNM (see SI Appendix, SI5 for details). Throughout the simulation (20 ns), the  $\gamma$ -stalk stayed near  $240^\circ$ , supporting the fact that it is a (locally) stable state. In SI Appendix, Fig. S2 A and B, we show the structure from the simulation, and compare it with the structure at the catalytic dwell. The conformations of  $\beta_{DP}$  for the two states differ as expected. Comparisons of  $\beta_{DP}$  with various



**Fig. 2.** Rotational angle of the  $\gamma$ -subunit as a function of time during the relaxation simulations (see text) for different  $B^{\wedge}C$  angle values of the  $\beta_{DP}$  subunit after the simulations with the applied torque. The values of the  $B^{\wedge}C$  angle in the  $\beta_{DP}$  subunit are shown for each trajectory to indicate the stage of binding left opening during the transition from the  $\beta_{DP}$  ( $B^{\wedge}C = 21.6^\circ$ ) to the  $\beta_E$  ( $B^{\wedge}C = 48.6^\circ$ ) conformation. The  $B^{\wedge}C$  angles were maintained at their initial values by the PNM restraining potentials during the simulations.



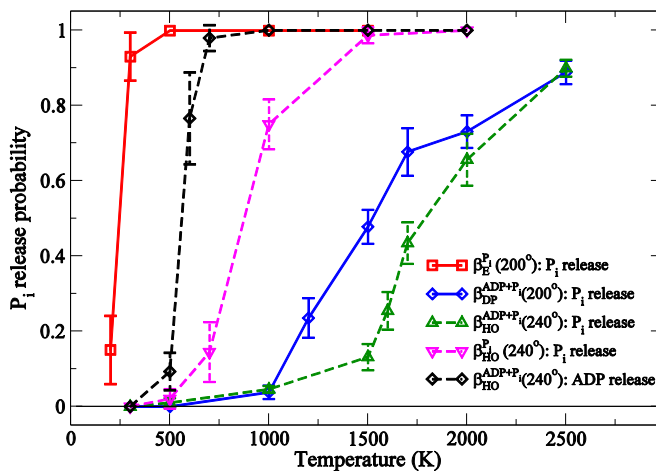
**Fig. 3.** Interactions between the  $\gamma$ -subunit and the surrounding subunits for a representative structure of the simulated ATP waiting state. The color scheme is the same as Fig. 1. Residues from the  $\gamma$ -subunit are labeled in blue, and residues from other subunits are labeled in red. Each residue involved in the interaction is shown in stick representation.

$\beta$ -conformations in crystal structures in terms of the rmsd values and the  $B^{\wedge}C$  angles (*SI Appendix, Fig. S2 C and D*) indicate that the conformation of the partly open  $\beta_{DP}$  structure is between  $\beta_{HO}$  and  $\beta_{HC}$ . Interestingly, the partly open  $\beta_{DP}$  subunit shares many structural features with  $\beta_{DP}$  in the recent  $F_1$ -ATPase structure of Rees et al. (PDB ID code 4ASU) (17); its  $B^{\wedge}C$  angle equals  $24^\circ$  and the helix-6 angle is close to  $20^\circ$ . In addition to the fact that the  $\gamma$ -subunit of the Rees et al. structure is rotated only by  $32^\circ$  relative to the reference Walker structure, the most significant difference between the present model structure and the structure by Rees et al. is that the C-terminal domain of  $\beta_{DP}$  of the model ATP waiting dwell structure clashes sterically with the  $\gamma$ -subunit of the Rees et al. structure (*SI Appendix, Fig. S2B*). One possible reason for the observed difference is that the structure trapped by Rees et al. contains nucleotide in all three active sites; therefore, it may represent another intermediate, rather than the ATP waiting dwell state modeled here. Although both the present work and Rees et al. (17) show a partial opening of  $\beta_{DP}$ , it is not observed in the model structure of the intermediate state proposed by Okazaki and Hummer (18). In addition, we observe that the C-terminal helix-turn-helix (hth) motif of  $\beta_E$  has closed slightly in the MD simulated structure (*SI Appendix, Fig. S2A*). Although Masaïke et al. (8) concluded in their single-molecule experiments that  $\beta_E$  does not change its C-terminal helix orientation during the  $40^\circ$  substep rotation, the large fluctuations of the orientation angle in the measurements suggest that the small change of  $\beta_E$  observed in the MD simulation is below their resolution. We use this experimentally derived structure for the analysis that follows. Because the structure of  $\beta_{DP}$  is closest in rmsd to  $\beta_{HO}$  (*SI Appendix, Fig. S2C*), we denote the conformation of the partly open  $\beta_{DP}$  as  $\beta_{HO}$ -like (or simply  $\beta_{HO}$  for brevity when no confusion arises).

### Timing of $P_i$ Release

The results from free-energy simulations and multiple MD simulations (*SI Appendix, SI6 and Fig. S3*) indicate that any of ATP hydrolysis, ADP and/or  $P_i$  release, can lead to the partly open  $\beta_{HO}$ -like conformation required to reach the ATP waiting state at  $240^\circ$  from the catalytic dwell ( $200^\circ$ ). All of these, as well as ADP and  $P_i$  repulsion, have been suggested as triggers for the  $\gamma$ -rotation (19–21). To investigate possibilities for the actual mechanism, we calculated the probability of ligand release, particularly of  $P_i$ , from different conformations of the  $\beta$ -subunits with different ligand occupancies. Given the results shown in Fig. 4 and additional experimental data [particularly Watanabe et al. (21)], we summarize in Fig. 1B the rotation cycle of the subunit conformations and their occupancies. For an ATP, denoted as ATP\*, bound after the ATP waiting dwell at  $0^\circ$ , the release of  $P_i^*$  generated from the ATP\* is shown to occur after an additional  $120^\circ$  rotation of the  $\gamma$ -subunit to  $320^\circ$  from the catalytic dwell at  $200^\circ$  where the ATP\* hydrolysis takes place. This contrasts with an earlier conclusion, also based on single-molecule experiments, that  $P_i$  is released immediately after ATP\* hydrolysis at  $200^\circ$  (20, 22, 23).

Because the  $P_i$  and ADP release takes place on the millisecond time scale, too long to be sampled in accessible simulation times at 300 K (nanoseconds), we use the high-temperature multicopy enhanced sampling (MCES) method (see *Methods*) to accelerate the events. This approach has been used previously to provide meaningful results on ligand release from proteins and its dependence on their conformation [e.g., CO from myoglobin by Elber and Karplus (24);  $P_i$  from myosin by Cecchini et al. (25)]. MCES was used here to explore the ligand release probabilities of  $P_i$  and ADP from subunits with the conformations  $\beta_E$ ,  $\beta_{DP}$ , and  $\beta_{HO}$ -like as part of the  $\alpha_3\beta_3\gamma$ -complex. For  $\beta_E$  and  $\beta_{DP}$ , we considered a structure at or near the catalytic dwell ( $200^\circ$ ), based on the X-ray structure of Braig et al. (PDB ID code 1E1R) (26); for  $\beta_{HO}$ , we considered the ATP waiting dwell ( $240^\circ$ ), based on the model structure reported here. Fig. 4 shows the probabilities of



**Fig. 4.** Probability of  $P_i$  release as a function of the temperature applied to  $P_i$  in the MCES simulations (see *Methods*):  $P_i$  release from  $\beta_E$  in the catalytic dwell ( $200^\circ$ ; red), and the release from ADP and  $P_i$  bound  $\beta_{DP}$  at  $200^\circ$  (blue). The angle definition is provided in Fig. 1B. The results test the two competing models for  $P_i$  release: one from  $\beta_{DP}$  immediately after its cleavage from bound ATP and the other from  $\beta_E$  after the rotation of  $\gamma$  by  $120^\circ$  to reach the next catalytic dwell, during which the  $P_i$  release from  $\beta_{DP}$  is prevented. The latter model corresponds to the reaction cycle proposed in the present paper and Watanabe et al. (21). We also show the release probabilities of  $P_i$  and ADP from the  $\beta_{HO}$  conformation at the ATP waiting dwell with different ligand contents:  $P_i$  release in the presence of ADP (green),  $P_i$  release in the absence of ADP (pink), and ADP release in the presence of  $P_i$  (black).

$P_i$  release from  $\beta$ -subunits in different conformational states as a function of the temperature of the multiple copies of  $P_i$ . In the Braig et al. structure used for the catalytic dwell state ( $200^\circ$ ),  $\beta_E$  is occupied by  $P_i$ ;  $\beta_{DP}$  by ADP,  $AlF_3$  (which was replaced with  $P_i$ ), and  $Mg^{2+}$ ; and  $\beta_{TP}$  by ATP analog and  $Mg^{2+}$ . The  $P_i$  present in  $\beta_E$  was produced during the catalytic dwell at  $80^\circ$  from an ATP bound at  $-120^\circ$ . Because  $P_i$  has a high release probability from  $\beta_E$  at a temperature as low as 250 K, it is very weakly bound. Interestingly, in one of the all-atom explicit water MD simulations (see below), we observed the spontaneous release and rebinding of  $P_i$  from  $\beta_E$  (Movie S1). The release and rebinding accompany a large fluctuation of the P-loop structure. This finding is consistent with the present  $P_i$  release data. By contrast, a much higher temperature (1,500 K) is required for a significant release probability of  $P_i$  from the closed  $\beta_{DP}$  subunit in the catalytic dwell structure ( $200^\circ$ ). This result indicates that in the  $200^\circ$  structure, release of  $P_i$  from  $\beta_E$  is the dominant process and that release of  $P_i^*$  from  $\beta_{DP}$  immediately after its cleavage from ATP\* does not occur to a significant extent, in accord with Fig. 1B and the proposal of Watanabe et al. (21).

In the ATP waiting state ( $240^\circ$ ), where  $\beta_E$  is empty (Fig. 1B),  $\beta_{DP}$  has opened more to become  $\beta_{HO}$ , but  $P_i^*$  release is even more hindered than in the  $200^\circ$  structure as long as ADP\* (and  $Mg^{2+}$ ) is present (Fig. 1B); i.e., release of  $P_i^*$  at  $240^\circ$  would be possible only after ADP\* has been released (also see SI Appendix, S17, S18, and Fig. S4). To confirm this result, we performed a set of MCES simulations for  $\beta_{HO}$  in the  $240^\circ$  structure, in which ADP and  $P_i$  were both represented by multiple copies and thus competed for release. As expected, ADP\* is released at a significantly lower temperature than  $P_i^*$  (Fig. 4). Once ADP\* is no longer present,  $P_i^*$  is released easily (SI Appendix, Fig. S5). However, as shown by Adachi et al. (20) and Martin et al. (27), ADP\* is released only during (or after) the rotation of the  $\gamma$ -subunit by another  $80^\circ$  to the catalytic dwell at  $320^\circ$  when  $\beta_{HO}$  has opened further to  $\beta_E$  (see Fig. 1B legend). Very recently, Czub and Grubmüller have shown by MD simulations that  $\beta_E$  closes spontaneously to  $\beta_{HO}$  during the  $80^\circ$  rotation in the synthesis direction in the absence of ligand (ADP or  $P_i$ ) in  $\beta_E$  (28). However, because changes of the ligand occupation of each  $\beta$ -subunit during the rotation were not taken into account in the simulations, an understanding of the entire sequence of events that occurs during the  $80^\circ$  rotation is not possible based on their results. Nevertheless, the results are consistent with the mechanism that the  $\beta_{HO} \rightarrow \beta_E$  conformational transition and the rotation of  $\gamma$  to the catalytic dwell occur during or after the release of ADP\*. Taken together, the  $P_i$  release simulations show that  $P_i^*$  is released after the  $\beta_{HO} \rightarrow \beta_E$  transition is completed as part of the rotation from  $320^\circ$  to  $360^\circ$ .

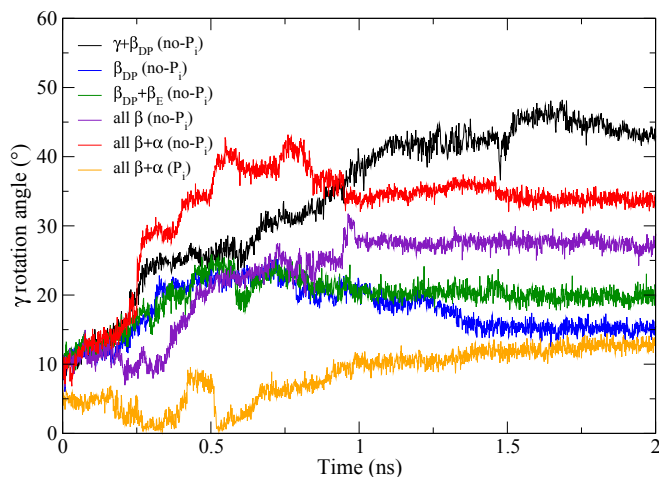
### Coupling Between ATP Hydrolysis, $P_i$ Release, and $\gamma$ -Rotation

An essential element for understanding  $F_1$ -ATPase function is knowledge of the mechanism by which the torque for  $\gamma$ -rotation is generated. The dominant factor in the  $80^\circ$  rotation from the waiting dwell is known to be ATP binding to a  $\beta_E$  subunit and its closure to form  $\beta_{TP}$  (12, 29), in which repulsive van der Waals interactions contribute dominantly in the generation of torque. On the other hand, our understanding of the  $40^\circ$  rotation is much more limited (20, 30). To explore the mechanism, we carried out targeted molecular dynamics (TMD) (31) simulations with the model generated in this paper for the ATP waiting structure as the target (see Methods). As a reference, a TMD simulation was performed, starting with the catalytic dwell structure, and a perturbation was applied to the  $\gamma$ - and  $\beta_{DP}$  subunits of the entire  $\alpha_3\beta_3\gamma$ -complex to induce the  $40^\circ$  rotation of  $\gamma$  and the transition of  $\beta_{DP}$  to  $\beta_{HO}$  required to reach the model ATP waiting structure. The simulation produces a structure with the  $\gamma$ -subunit rotated by  $40^\circ$  and  $\beta_{DP}$  partially open; the structure differs

slightly from the model ATP waiting structure in the orientations of the C-terminal hth motifs of  $\beta_E$  and all of the  $\alpha$ -subunits (SI Appendix, Fig. S6). The change of the  $\beta_E$  structure during or after the  $40^\circ$  rotation is in accord with the results of Watanabe et al. (30) that in the neighborhood of the ATP waiting state, the affinity for ATP changes with  $\gamma$ -rotation, implying a change in the  $\beta_E$  structure, and the all-atom explicit water MD simulation (SI Appendix, Fig. S24). Because the TMD perturbation was applied only to  $\gamma$  and  $\beta_{DP}$ , the structural changes of the other parts of the  $\alpha_3\beta_3\gamma$ -complex reflect their spontaneous response to the rotation of  $\gamma$  and the partial opening of  $\beta_{DP}$ . The resulting structure was used as the target structure in subsequent TMD simulations, where the perturbation was applied only to various parts of the  $\alpha_3\beta_3$  crown, with or without  $P_i$  in the  $\beta_E$  subunit (Fig. 5; see legend for details). With  $\beta_E$  empty, we obtained the striking result that transformations of all  $\alpha$ - and  $\beta$ -subunits are required to induce the  $40^\circ$  rotation (Fig. 5). As shown in the figure, when fewer elements of the  $\alpha_3\beta_3$  crown are transformed (e.g., all  $\beta$ -subunits), only intermediate rotation of the  $\gamma$ -subunits is produced during the simulation. Moreover, with  $P_i$  present in  $\beta_E$ , the  $P_i$  stays bound in the active site throughout the entire TMD simulation and only a  $10^\circ$  rotation of  $\gamma$  was achieved even with the full  $\alpha_3\beta_3$  transformation in the simulation (Fig. 5, orange; and see Movies S2 and S3). These results show that the presence of  $P_i$  in the  $\beta_E$  subunit blocks the  $\gamma$ -rotation.

### Dynamic Lock by $P_i$

To determine the mechanism of the  $\beta_E$  ( $P_i$ ) lock, the structure and dynamics of the  $\alpha_3\beta_3\gamma$ -complex in the catalytic dwell state with different occupations of  $\beta_{DP}$  and  $\beta_E$  were studied by all-atom explicit water MD simulations (see SI Appendix, S15 for details). In the simulations, the binding pockets of  $\beta_{TP}$  and all  $\alpha$ -subunit are occupied by ATP, whereas  $\beta_{DP}$  and  $\beta_E$  have different occupations: In the prehydrolysis state simulation, ATP occupies  $\beta_{DP}$  and  $P_i$  occupies  $\beta_E$ ; in the posthydrolysis state simulation, ADP and  $P_i$  occupy  $\beta_{DP}$  and  $P_i$  occupies  $\beta_E$ ; and in



**Fig. 5.**  $\gamma$ -Rotation angles from the TMD simulations (see text). The no- $P_i$  systems are systems with empty  $\beta_E$ :  $\gamma + \beta_{DP}$  simulation (black),  $\beta_{DP}$  simulation (blue),  $\beta_{DP} + \beta_E$  simulation (green), all  $\beta$  simulation (purple), all  $\beta + \alpha$  simulation (red). For the  $P_i$  system, which refers to the system with  $P_i$  bound in  $\beta_E$ : all  $\beta + \alpha$  simulation (yellow). The simulation time is shown in nanoseconds, and the  $\gamma$ -rotation angle is defined as in Pu and Karplus (12). Except for the  $\gamma + \beta_{DP}$  simulation, the TMD simulations continued for 1 ns and were followed by 1-ns unperturbed simulations to relax the system; during the latter all these systems reached a plateau for the rotation angle of  $\gamma$ . In the  $\gamma + \beta_{DP}$  TMD simulation, it took 2 ns before the rmsd distance to the  $240^\circ$  rotated structure fell below 0.75 Å; this was followed by a 1-ns relaxation simulation as in the other cases.

the postrelease state simulation,  $\beta_{DP}$  is occupied by ADP and  $P_i$  and  $\beta_E$  is empty. (See Fig. 1B and its legend for identification of the three states.) The structural comparisons reveal that during or after the hydrolysis of ATP in  $\beta_{DP}$  and the release of  $P_i$  from  $\beta_E$ , small changes occur in the C-terminal hth motif and at the intersubunit interfaces of the subunits (*SI Appendix*, Figs. S2E and S7, and *Movie S4*). In addition, there are significant differences in the dynamics, as evidenced in the cross-correlation maps of the  $\alpha_3\beta_3\gamma$ -complex; they are shown in *SI Appendix*, Fig. S8. Details of the structural and dynamic changes are given below.

*SI Appendix*, Fig. S8 shows the cross-correlation maps of the entire  $\alpha_3\beta_3\gamma$  complex for the prehydrolysis state (*SI Appendix*, Fig. S8A), posthydrolysis state (*SI Appendix*, Fig. S8B), and the postrelease state (*SI Appendix*, Fig. S8C). In comparing the simulation of the posthydrolysis state to that of the prehydrolysis state, there is a rigid-body rotation of the C-terminal hth motif of  $\alpha_{DP}$  toward  $\beta_{DP}$  in the former, relative to the latter. This rotation is caused by the cleavage of ATP into ADP and  $P_i$  in  $\beta_{DP}$  (*SI Appendix*, Fig. S7A). The rotation increases the contact between the two subunits and leads to a more closed  $\alpha_{DP}$ - $\beta_{DP}$  interface, as is evident from the increased buried surface area (*SI Appendix*, Fig. S9). It also leads to enhanced positive cross-correlation between the two subunits without a significant change of the intrasubunit cross-correlation of  $\beta_{DP}$  (compare *SI Appendix*, Fig. S8 A and B). A similar closure of the  $\alpha_{DP}$ - $\beta_{DP}$  interface is observed experimentally. In *SI Appendix*, Fig. S7B, the X-ray structure with the transition-state analog (26) is superimposed on the structure with the ATP analog (14). The superposition shows that the C-terminal domain of  $\alpha_{DP}$  is rotated toward  $\beta_{DP}$  for the transition-mimic state to make the interface tighter (*SI Appendix*, Fig. S7B, *Left*), in agreement with the simulations. The origin of this structural change appears to involve the displacement of  $\alpha_{DP}R373$ , which moves to interact with the  $P_i$  after it is cleaved from the ATP. *SI Appendix*, Fig. S7A shows the displacement of  $\alpha_{DP}R373$  upon the cleavage of ATP in the simulations and a similar displacement in the transition-state mimic structure (*SI Appendix*, Fig. S7B; also see *SI Appendix*, Fig. S7C for the changes of interactions at the interface between the two subunits). In this interpretation,  $\alpha_{DP}R373$  functions as a sensor that probes the progress of the hydrolysis reaction in  $\beta_{DP}$  and dynamically links the two subunits ( $\beta_{DP}$  and  $\alpha_{DP}$ ). This is consistent with mutation experiments, which suggested that  $\alpha_{DP}R373$  is involved in the rearrangement of the  $\alpha_{DP}$ - $\beta_{DP}$  interface upon ATP hydrolysis and the catalytic cooperativity of the enzyme (32).

We also find a noticeable difference between the cross-correlation maps of the posthydrolysis and postrelease states, i.e., there is an increase of intrasubunit correlation of both  $\alpha_E$  and  $\beta_E$  in the postrelease state (*SI Appendix*, Fig. S8C). The cross-correlation maps of  $\alpha_E$  and  $\beta_E$  in the postrelease state show cross-correlations that extend over the C-terminal and nucleotide binding domains (*SI Appendix*, Fig. S8 C and D), suggesting that the two domains behave like a rigid body. This difference in the dynamics of  $\beta_E$  is of interest because the differences between the  $\beta_E$  structures with or without  $P_i$  or a  $P_i$  analog are found to be negligible (*SI Appendix*, S19 and Fig. S10). The anticorrelation between  $\alpha_E$  and the C-terminal domain of  $\beta_E$  has also increased significantly (compare *SI Appendix*, Fig. S8 B and D). This result indicates that the two subunits move concertedly but in opposite directions. In this case,  $\alpha_ER373$  could play an important role in controlling the dynamics of  $\alpha_E$  and  $\beta_E$ , similar to the role of  $\alpha_{DP}R373$  in ATP hydrolysis. In this mechanism, the interaction between  $P_i$  in  $\beta_E$  and  $\alpha_ER373$  keeps  $\alpha_E$  close to  $\beta_E$  and away from  $\beta_{DP}$ , preventing  $\alpha_E$  from responding to the change of  $\beta_{DP}$ , thus blocking the rotation of  $\gamma$ . Once  $P_i$  leaves the binding pocket, the interaction is lost, so that  $\alpha_E$  and in particular its C-terminal domain are able to respond to the change occurring in  $\beta_{DP}$  and the rotation of  $\gamma$ .

To test the proposed mechanism, we have performed an additional TMD simulation. The simulation was carried out with the TMD perturbation applied to all  $\alpha$ - and  $\beta$ -subunits but without the interaction between  $P_i$  in  $\beta_E$  and  $\alpha_ER373$ . If these interactions were important in blocking the  $\gamma$ -rotation, it would be expected that  $\gamma$  would rotate further in their absence than when the interactions between  $P_i$  in  $\beta_E$  and  $\alpha_ER373$  were present. The simulation produced a  $\gamma$ -rotation that is larger (close to  $20^\circ$ ) than the simulation with the  $P_i$ - $\alpha_ER373$  interaction present, but then it falls back to the lower rotation angle during the subsequent relaxation simulation (*SI Appendix*, Fig. S11). The result confirms the proposed role of the interaction between  $P_i$  and  $\alpha_ER373$  in blocking  $\gamma$ -rotation. The result also suggests that interactions (within or between  $\beta_E$  and  $\alpha_E$ ), other than the interactions between  $P_i$  in  $\beta_E$  and  $\alpha_ER373$ , are important in preventing the rotation—for example, reducing the increase of the intrasubunit cross-correlation in the  $\beta_E$  and  $\alpha_E$  subunits and the increase of the anticorrelation between them, which occurs upon the release of  $P_i$ . In this regard, we note that  $\alpha_E$  is the subunit forming the most extensive surface contacts with  $\gamma$  among the  $\alpha$ -subunits and has an extensive surface contact with  $\beta_{DP}$  (*SI Appendix*, Fig. S9). The surface contacts of  $\alpha_E$  with  $\gamma$  are as extensive as the contact between  $\beta_{DP}$  and  $\gamma$ , which is the largest surface contact among all  $\beta$ s.

Taken together, the present analysis shows how the interactions between  $\beta_E$  and  $\alpha_E$ , including the interaction between  $P_i$  and  $\alpha_ER373$ , act as a “dynamic lock” to keep the protein in the prerotated catalytic dwell state. Only after  $P_i$  in  $\beta_E$  is released is  $\alpha_E$  freed from  $\beta_E$  and able to fully engage with  $\beta_{DP}$  to complete the concerted conformational transition of the  $\alpha_3\beta_3$  complex by which the  $\gamma$ -subunit rotates to reach the ATP waiting dwell state. Such dynamic locks have been proposed for different systems by Laity et al. for zinc finger proteins (33) and by Young et al. for c-Src (34).

## Concluding Remark

The present study provides a structural model for the ATP waiting state of  $F_1$ -ATPase, in agreement with single-molecule experiments which have suggested that it does not coincide with any of the known crystal structures. Knowledge of this structure, combined with that of the state in which catalysis takes place, makes possible the development of a detailed atomic-level description of the coupling between the binding and hydrolysis of ATP and the  $\gamma$ -subunit rotation induced by the conformational changes of the  $\alpha$ - and  $\beta$ -subunits. The suggested tests of the proposal structure and a possible method for trapping it in a crystallographically accessible conformation should stimulate experimental studies (see *SI Appendix*, S110 and S111 for details).

## Methods

**Forced Rotation Simulation for Finding the ATP Waiting State.** The structure of the minimal rotary complex  $\alpha_3\beta_3\gamma$  was prepared based on the  $\alpha_3\beta_3$  subcomplex of the 1BMF structure (3) and the  $\gamma$ -subunit of the 1E79 structure (9) by a procedure similar to that of Ma et al. (35). The CHARMM19 all-atom force field (36) and the EEF1 implicit solvation model (37) were used to describe the protein system and water solvation, respectively. In addition, the coarse-grained PNM (11, 12), in which each PNM node was assigned to the corresponding  $C_\alpha$  atom position of the protein, was used to stabilize the protein conformation in the presence of the high forces used in the simulation. The system was first heated from 0 K to 300 K in 60 ps and then equilibrated at 300 K for 300 ps (see details in *SI Appendix*, S12). The MD simulations were carried out with a 2-fs integration time step and SHAKE (38) applied to the bonds involving hydrogen atoms. The temperature was controlled using the Langevin thermostat.

After equilibration at 300 K for 300 ps, a large external torque was applied to drive the rotation of the  $\gamma$ -subunit in the hydrolysis direction (counterclockwise as seen from the membrane). Using the PULL command of the CHARMM program (10), an external force of 2,500 pN was applied to the  $C_\alpha$

atom of residue  $\gamma$ M25. Residue  $\gamma$ M25 was identified by Pu and Karplus to provide a key contact point for the torque generation (12). In the forced rotation simulation, the external torque was applied only when backward rotation is detected. In that way, the simulation was biased toward the hydrolysis direction only when  $\gamma$  rotates backward but not when the forward rotation occurs spontaneously; the  $\gamma$ -rotation angle was checked at each update step (at every 1 ps). The  $\gamma$ -rotation angle is defined as in Pu and Karplus (12) using the  $\alpha_3\beta_3(1\text{BMF})-\gamma(1\text{E79})$  Walker structure as the reference structure for the catalytic dwell state, and a similar definition was used in the work by Koga and Takada (39). The direction of the force was determined as the instantaneous cross-product between the radial vector of the residue  $\gamma$ M25 (perpendicular to the rotational axis) and the rotational axis itself (Fig. 1A). See *SI Appendix, S12* for details of forced rotation simulations and definition of the  $\gamma$ -rotation angle.

**P<sub>i</sub> Release Simulations.** The 200° rotated system was prepared using the  $\alpha_3\beta_3$  subcomplex of the 1E1R structure (26) and the  $\gamma$ -subunit from the 1E79 structure (9). For the 240° state, the starting structure was the present ATP waiting state model structure. For the P<sub>i</sub> release MCES simulation (25), the P<sub>i</sub> molecule was replicated 30 times by using the BLOCK module of the CHARMM program. In all simulations, P<sub>i</sub> was treated as doubly protonated (H<sub>2</sub>PO<sub>4</sub><sup>-</sup>), which was found to be favored in the active site of  $\beta$ -subunit (40). The interaction between the multiply copied P<sub>i</sub> and both the protein and the solvent was scaled by a factor that is inversely proportional to the number of P<sub>i</sub> copies, whereas each P<sub>i</sub> has no interaction with other P<sub>i</sub> molecules. The remaining interactions were not scaled. The temperature of P<sub>i</sub> was controlled by attaching each P<sub>i</sub> to a separate Langevin thermostat, while the remainder of the system was maintained at 300 K. At each P<sub>i</sub> temperature, the MCES simulation was repeated 40 times (*SI Appendix, S17*). Each

simulation was started with different initial velocities and ran for 2 ns with a 1-fs integration time step. SHAKE was applied to constrain bonds involving hydrogen atoms.

**Targeted MD Simulations of the Coordinated Conformational Transition of the  $\alpha_3\beta_3\gamma$ -Complex.** The 40° substep rotation was simulated by applying the TMD simulation method (31). The TMD simulation was first carried out with the 200° rotated catalytic dwell structure, which was prepared for the MCES P<sub>i</sub> release simulations. The TMD perturbation was applied to the nonhydrogen atoms of the  $\gamma$ - and  $\beta_{DP}$  subunits of the entire  $\alpha_3\beta_3\gamma$ -complex for the  $\beta_{DP} \rightarrow \beta_{HO}$  transition and the 40°  $\gamma$  rotation; the ATP waiting model structure was the target structure. Subsequently, using this TMD-produced structure as the target structure for the  $\alpha_3\beta_3$  complex, a set of TMD simulations was carried out with the TMD perturbation applied to various parts of the  $\alpha_3\beta_3$  crown with or without P<sub>i</sub> in  $\beta_E$  and without any perturbation to  $\gamma$  (see Fig. 5 legend for the notation of each TMD simulation). In the simulations the rms distance to the target structure was decreased by  $0.2 \times 10^{-6}$  Å at each MD step until the rms distance reached a value lower than 0.75 Å.

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