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ADP-Bound States of Cytoplasmic Dynein: Cryo-electron Microscopy Reveals a Two-Step Post-Power-Stroke Transition and Roles of Regulatory ATPase Sites

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Abstract

Cytoplasmic dynein is essential for intracellular transport and cell division, and its dysfunction is implicated in severe neurological disorders. Its motility is driven by the primary ATPase site in the AAA1 module and allosterically modulated by regulatory ATPase sites in the AAA3 and AAA4 modules. Despite recent structural advances, post-force-generating (post-power-stroke) transitions and the structural basis of AAA3/AAA4-mediated regulation remain elusive. Using cryo-electron microscopy, we captured intermediate structures of *Dictyostelium discoideum* cytoplasmic dynein. We identified two distinct ADP-bound states and two apo states, the latter comprising a partially nucleotide-free state with ADP retained at the AAA3 and AAA4 ATPase sites and a fully nucleotide-free state. For the AAA1 ATPase site, our structures and molecular dynamics simulations suggest a dynamic equilibrium between two ADP-bound states. In this model, linker swing and docking onto the AAA5 module can be driven by thermal fluctuations while ADP remains bound at the AAA1 ATPase site, shifting the motor from a canonical ADP-bound state to a metastable apo-like conformation with reduced ADP affinity. This transition could provide a framework for tension-sensing coordination. For the AAA3 and AAA4 ATPase sites, comparison of the partially and fully nucleotide-free apo structures indicates that ADP release induces localized outward tilting of the corresponding large submodules. We propose that this rearrangement displaces the AAA4 Pre-Sensor-I insert from the linker, thereby disfavoring the recovery stroke and stabilizing an inactive state. Together, our findings provide a mechanistic model in which coordinated conformational dynamics across multiple ATPase sites govern dynein's chemomechanical cycle.

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Introduction

Cytoplasmic dynein 1 (hereafter referred to as cytoplasmic dynein) is a major cytoskeletal motor protein complex in eukaryotes [1,2]. Cytoplasmic dynein drives a wide variety of cellular functions by utilizing the free energy derived from ATP hydrolysis to move processively along microtubules toward their minus ends. Although most plus-end-directed intracellular transport is mediated by members of the diverse kinesin superfamily, which comprises approximately 45 members in humans [3], long-distance transport toward the microtubule minus end is driven almost exclusively by cytoplasmic dynein [4–6]. Consequently, cytoplasmic dynein is essential for the transport of diverse cargoes (e.g., protein complexes, mRNAs, organelles, and viruses) from the cell periphery to the cell center [1,2]. Cytoplasmic dynein also plays a central role in cell division by contributing to key mitotic processes such as bipolar spindle assembly, chromosome congression, and spindle assembly checkpoint silencing [2,7]. To enable a single motor type to transport and position such diverse cargoes at the appropriate time, dynein motor activity requires strict regulation.

Cytoplasmic dynein is a massive protein complex with a total molecular mass of approximately 1.4 MDa [2,8,9]. Its core consists of a homodimer of heavy chains associated with intermediate chains, light intermediate chains, and three classes of light chains [9]. Functionally, each heavy chain is divided into two major regions. The N-terminal tail region, which comprises approximately one-third of the chain, mediates dimerization and binding to other subunits and cargo adapters [9]. The remaining C-terminal motor domain, comprising approximately two-thirds of the chain, is responsible for motor activity [1,9]. The motor domain possesses a highly organized structure, featuring at its center an ATP-hydrolyzing ring consisting of six AAA⁺ (ATPases associated with diverse cellular activities) modules arranged in a ring [1,9,10].

The stalk, a long coiled-coil structure of approximately 150 Å with a microtubule-binding domain at its tip, protrudes from the ATP-hydrolyzing ring [1,5,11]. Additionally, the linker, positioned across the ring, acts as a mechanical unit that amplifies force generation [1,9]. Among the six AAA⁺ modules that constitute the ring, the AAA1–AAA4 modules can bind nucleotides, and the AAA1, AAA3, and AAA4 ATPase sites have been shown to exhibit ATP hydrolysis activity [10,12–15]. The nucleotide bound at the AAA2 site, however, is not hydrolyzed and is thought to serve primarily a structural role by stabilizing the closed AAA2–AAA3 interface [10,16]. Specifically, the ATPase site in the AAA1 module serves as the primary ATPase site, where hydrolysis-driven confor-

mational changes directly power the dynein motor [1,10,12–14,17]. In contrast, the ATPase sites in the AAA3 and AAA4 modules are thought to function as regulatory ATPase sites that can modulate the activity of the primary ATPase site and dynein's microtubule-binding affinity [10,12–15,18–21], although the details remain incompletely understood.

Dramatic advances in X-ray crystallography and cryo-electron microscopy (cryo-EM) have enabled visualization of the structures of intermediates in the chemomechanical cycle of cytoplasmic dynein [9,12,16,22–33]. The dynamic behavior of cytoplasmic dynein, including linker bending upon ATP binding (recovery stroke), force generation upon linker straightening (power stroke), and microtubule affinity regulation via stalk registry changes, is becoming understood from a structural perspective at near-atomic resolution [12,16,22–24,26,30,32,33]. However, it remains unclear exactly how the ADP-bound state and subsequent ADP release, crucial steps in the chemomechanical cycle of dynein, affect the structure and activity of the dynein motor. Specifically, dynamic structural transitions driven by the primary ATPase site in the AAA1 module during the ADP-bound phase, as well as the structural basis for how the nucleotide states in the regulatory ATPase sites in the AAA3 and AAA4 modules influence chemomechanical processes, remain unclear.

Here, we used the dynein motor domain from the cellular slime mold *Dictyostelium discoideum*, a representative model system for cytoplasmic dynein [34–41], to comprehensively analyze key intermediate states in the chemomechanical cycle [23,42]. We performed high-resolution structural analyses using cryo-EM under three nucleotide conditions: in the presence of ATP and orthovanadate (Vi), which traps the motor in an ADP–Vi state (hereafter, the ADP–Vi condition); in the presence of ADP; and in the absence of nucleotides. We identified three distinct intermediate structures in the presence of ADP, including two states in which ADP is bound at the primary ATPase site in the AAA1 module but the motor domain adopts distinct conformations. The structural features of these two ADP-bound states support a model in which dynein adopts a dynamic equilibrium between these conformations, which could provide a structural basis for tension-sensing coordination. Furthermore, we captured conformational changes associated with ADP release from the regulatory ATPase sites in the AAA3 and AAA4 modules, allowing us to propose a new model for how such changes contribute to the regulation of the dynein motor. Our findings provide new structural insights into the ADP-bound state and transition to the apo state, contributing to a more comprehensive understanding of the complex regulatory mechanisms of cytoplasmic dynein.

Results and Discussion

Cryo-EM analysis defines pre- and post-force-generating states of the dynein motor domain

To elucidate the detailed mechanism whereby cytoplasmic dynein generates force, particularly the processes that occur after force production, we overexpressed and purified the 380-kDa motor domain of cytoplasmic dynein from the cellular slime mold, *D. discoideum*. This motor domain retains high microtubule-activated ATPase activity and robust motility along microtubules, making it an excellent construct for dissecting the essential mechanisms that generate and regulate dynein motor activity [13,23,37,42,43]. Using single-particle cryo-EM, we determined five different conformational states of this motor domain at near-atomic resolution (2.20–3.20 Å) across three different nucleotide conditions.

To establish a reference structure for subsequent analyses of dynein after force generation, we first determined the structure of the dynein motor domain under conditions that trap the motor in its pre-force-generating state, namely the ADP–Vi condition [24,38,44,45]. The resulting motor domain structure (2.20 Å resolution; [Supplementary Figures 1–3](#)) showed a markedly bent force-generating linker, with its tip located between the AAA2 and AAA3 modules of the AAA⁺ ATP-hydrolyzing ring. This overall feature is consistent with pre-force-generating (pre-power-stroke) structures of dynein from other species [24,28,29]. However, our analysis also revealed several previously undescribed interactions between the force-generating linker and the ATP-hydrolyzing ring ([Supplementary Figure 2](#)). Notably, the construct used to express the motor domain lacked the tail region that mediates dimerization and was designed to produce monomeric particles; however, approximately 93% of the observed particles assembled into a characteristic dimeric configuration known as the φ particle [28,29,46], with only approximately 7% being monomeric ([Supplementary Figure 4](#)). To date, φ particle-like structures have been reported only for dynein proteins containing tail regions that mediate dimerization or where dimerization was forced by fusion with dimerizing glutathione S-transferase tags or DNA linkers [28,29,41,43,46–49]. In contrast to these previous examples, the present observations demonstrate that the isolated motor domain has an intrinsic capacity to assemble into the dimeric φ -particle configuration, even in the absence of the tail and any artificial dimerization motif. Furthermore, this φ -particle assembly was specific to the pre-force-generating ADP–Vi condition: under the post-force-generating conditions analyzed below (the ADP and nucleotide-free conditions), essentially all particles were monomeric, and no φ -particle-like dimers were detected during two- or three-dimensional classification.

Next, we structurally analyzed the dynein motor domain in the presence of ADP, a condition expected to trap the motor in post-force-generating states [23,42]. During the initial stages of image processing, we noticed marked structural heterogeneity among the dynein particles, particularly in the force-generating linker and at the primary ATPase site in the AAA1 module of the ring. To address this heterogeneity, we performed unsupervised three-dimensional clustering [50], followed by independent structural refinement of the resulting classes. This analysis classified the dynein particles into three distinct conformational states. We obtained near-atomic-resolution structures for each state ([Supplementary Figure 5](#); [Supplementary Table 1](#)). Based on structural features described below, we refer to these conformations as ADP state 1, ADP state 2, and Apo state 1 (2.61 Å, 3.20 Å, and 2.76 Å resolutions, respectively) ([Figure 1A](#); [Supplementary Figures 5–8](#)); in this nomenclature, the first descriptor denotes the nucleotide state of the primary ATPase site in the AAA1 module. In the presence of ADP, the relative populations of the three conformations were approximately 3:1:2 [2,128,136 particles for ADP state 1; 596,990 particles for ADP state 2; and 1,347,494 particles for Apo state 1]. Taken together, these results suggest that ADP state 1, ADP state 2, and Apo state 1 represent major intermediate structural states populated after ATP hydrolysis and phosphate release during dynein's chemomechanical cycle.

ADP state 2 combines ADP-bound and apo-like structural features

We then performed a detailed structural analysis of the overall architectures of the three conformational states obtained under ADP conditions to clarify their structural interrelationships. ADP state 1 is characterized by ADP bound at the primary ATPase site in the AAA1 module ([Figure 1B](#)) and closely resembles the previously reported ADP-bound structure of *D. discoideum* cytoplasmic dynein (PDB: 3VKG; [Supplementary Figure 9](#)) [23]. In contrast, Apo state 1 displays no detectable nucleotide density at the AAA1 ATPase site ([Figure 1B](#)) and most closely resembles the previously reported nucleotide-free structure of yeast cytoplasmic dynein (PDB: 4AKI; [Supplementary Figure 9](#)) [16]. The third conformation, ADP state 2, contains ADP at the AAA1 ATPase site ([Figure 1B](#)); however, as described below, its architecture exhibits features intermediate between ADP state 1 and Apo state 1.

Regarding the ATP-hydrolyzing ring, structural comparisons based on the C α root-mean-square deviation (RMSD) indicate that ADP state 2 possesses structural features that are more similar to ADP state 1 than to Apo state 1 ([Figure 1C](#)). Nonetheless, the arrangement of the large (L) submodules of the ring in ADP state 2

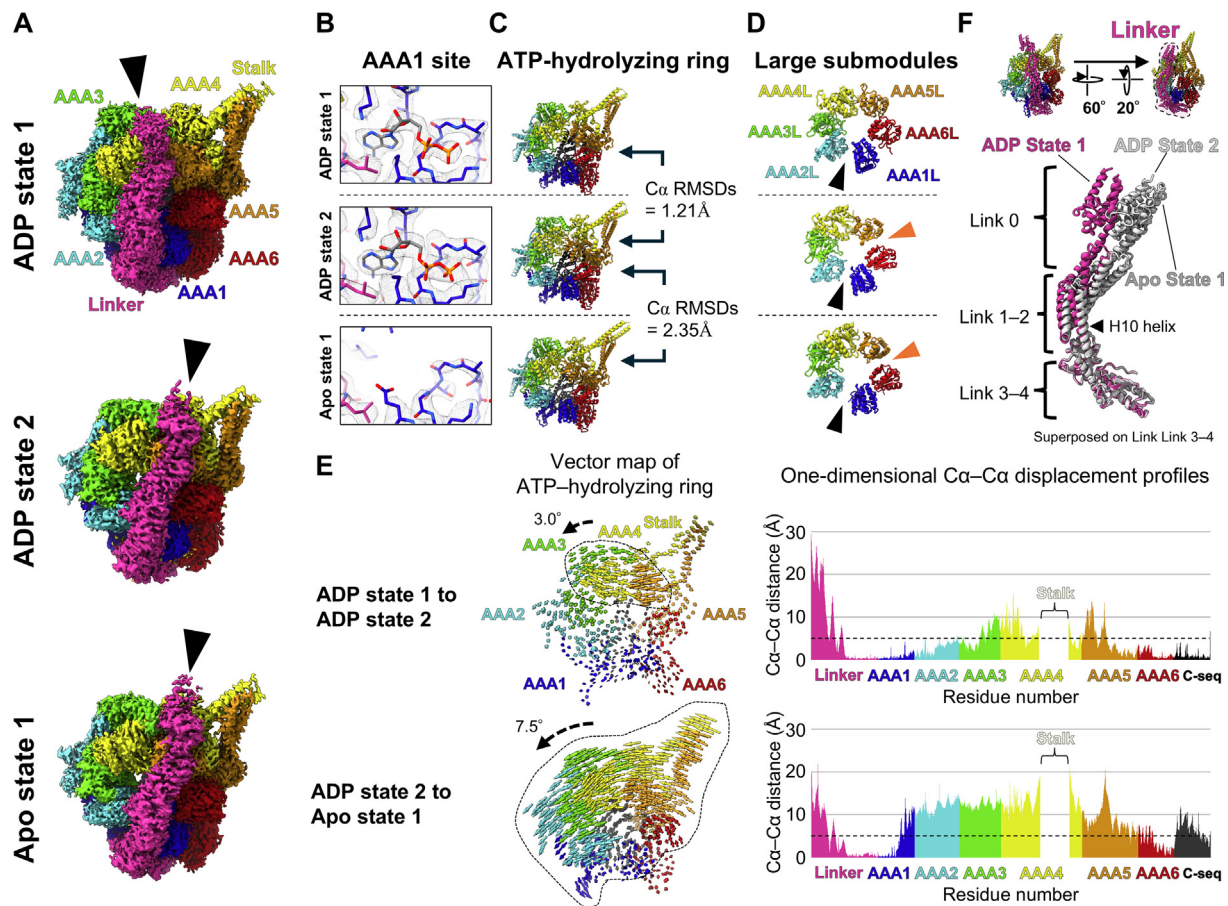


Figure 1. Cryo-EM structures reveal three conformational states of cytoplasmic dynein. (A) Cryo-EM reconstructions of *Dictyostelium discoideum* cytoplasmic dynein motor domains in ADP state 1, ADP state 2, and Apo state 1, shown in the same orientation after alignment on the ATP-hydrolyzing ring. Arrowheads indicate linker positions. (B) Close-up views of the primary ATPase site in the AAA1 module. ADP density is observed in ADP state 1 and ADP state 2, whereas no detectable nucleotide density is observed at the AAA1 ATPase site in Apo state 1. (C) Cartoon representations of the ATP-hydrolyzing ring with $C\alpha$ root-mean-square deviations (RMSDs) indicated. (D) Large (L) submodules of the ATP-hydrolyzing rings. Black arrowheads indicate gaps between AAA1L and AAA2L, whereas orange arrowheads indicate gaps between AAA5L and AAA6L. (E) Pairwise $C\alpha$ – $C\alpha$ displacement analyses comparing ADP state 1 with ADP state 2 and ADP state 2 with Apo state 1. Vector maps of the ATP-hydrolyzing ring, sampled at every third residue, are shown after alignment on AAA1L; linker vectors are omitted for clarity. Vectors point from the first to the second state in each comparison. Curved dashed arrows indicate counterclockwise rotations of the indicated ring regions as viewed from the linker side: 3.0° from ADP state 1 to ADP state 2 and 7.5° from ADP state 2 to Apo state 1. One-dimensional displacement profiles are shown on the right; structural regions are color-coded as indicated, and dashed lines represent 5 Å. (F) Linker conformations in ADP state 1, ADP state 2, and Apo state 1. The top panel indicates the viewing direction, and the bottom panel shows linker structures superposed on Link 3–4 (residues 1766–1950).

displays structural characteristics reminiscent of those of Apo state 1, including distinct azimuthal gaps at two positions in the ring: between AAA1L and AAA2L and between AAA5L and AAA6L (Figure 1D) [24]. When the ring structures of ADP state 1 and ADP state 2 are compared using AAA1L as a reference, the major conformational changes ($C\alpha$ displacement > 5 Å) are largely confined to the upper region of the ring, spanning the AAA3–AAA5 modules (Figure 1E). When viewed from the linker side, this ring region undergoes a counterclockwise rotation of 3.0°. In contrast, the transition

from ADP state 2 to Apo state 1 involves more pronounced ring rearrangements: using AAA1L as a reference, almost the entire ring, spanning from AAA1S to AAA6L, exhibits substantial structural changes ($C\alpha$ displacement > 5 Å; Figure 1E). These changes involve complex local rearrangements, but the ring as a whole rotates counterclockwise by 7.5°.

For the force-generating linker, ADP state 2 adopts a conformation that is more similar to that observed in Apo state 1 than to that in ADP state 1 (Figure 1A and F; Supplementary Figure 1).

When viewed from the linker side of the ring, the linker in ADP state 1 extends nearly straight across the ring, from the AAA1 side toward the opposite AAA4 side, and is supported primarily by the H2 insert protruding from the AAA2 module, which interacts with the central region of the linker (H6–H7; Figure 2A and B). In contrast, in Apo state 1, the linker bends clockwise by approximately 10° at its central hinge region (H10), and the linker tip (H1–H6) approaches the AAA5 module rather than the AAA4 module (Figure 1A and F; Supplementary Figure 1). This conformation is stabilized mainly by a strong interaction between the linker tip and the L submodule of AAA5, whereas the interaction between the central portion of the linker and the AAA2 module, observed in ADP state 1, is no longer detected (Figure 2A–C). In ADP state 2, the linker conformation, its position on the ring,

and the engagement of its tip with the AAA5 module are essentially the same as those in Apo state 1 (Figure 2A and C; Supplementary Figure 1). Importantly, however, ADP state 2 also retains the hallmark feature of ADP state 1, namely the interaction between the central region of the linker and the AAA2 module (Figure 2A and B).

Structural and computational analyses suggest a two-step post-force-generating transition pathway

Based on the structural features of the three conformations obtained under the ADP conditions described above, we propose the following model for dynein's structural transitions after force generation (Figure 5A): (i) following force-generating phosphate release from the AAA1

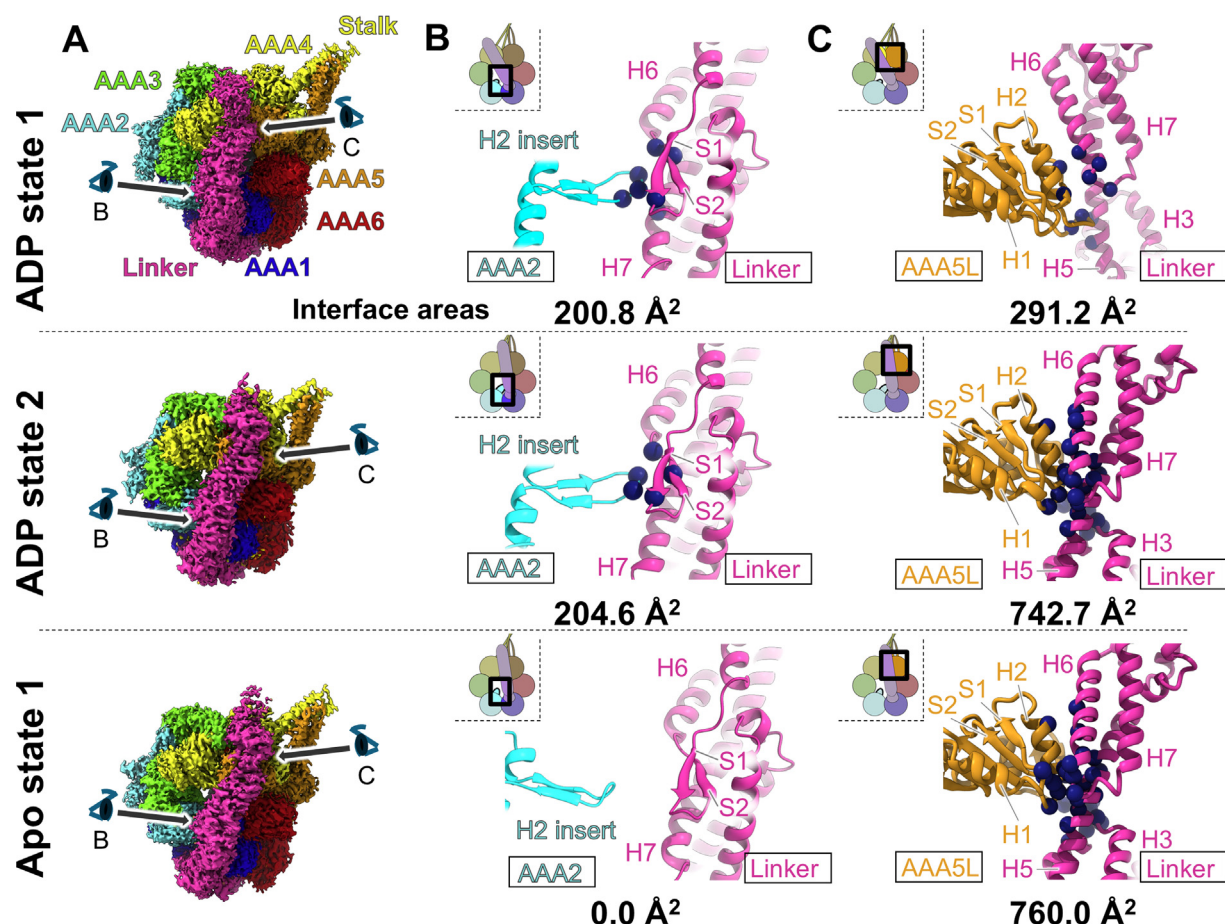


Figure 2. ADP state 2 combines linker association at AAA2 with docking on AAA5L. (A) Cryo-EM reconstructions of ADP state 1, ADP state 2, and Apo state 1. Arrows indicate the viewing directions for the close-up views shown in (B) and (C). (B) Close-up views of linker interactions with the AAA2 H2 insert. The linker H6–H7 region and S1–S2 strands interact with the AAA2 H2 insert in ADP state 1 and ADP state 2, but not in Apo state 1. (C) Close-up views of linker interactions with AAA5L. The linker tip forms a larger interface with AAA5L in ADP state 2 and Apo state 1 than in ADP state 1. The cartoon representations at the upper left of (B) and (C) indicate the regions shown in the corresponding close-up views. Interface areas are indicated in (B) and (C). C α atoms of interface residues are shown as dark blue spheres.

ATPase site, the primary ATPase site of dynein, dynein would first adopt the ADP state 1 conformation; (ii) next, while retaining ADP at the AAA1 ATPase site, dynein could swing its linker by approximately 10° clockwise and dock the linker tip onto the AAA5 module, thereby transitioning to the ADP state 2 conformation; and (iii) finally, dynein would release ADP from the AAA1 ATPase site while rotating the entire ATP-hydrolyzing ring, resulting in the Apo state 1 conformation. A key point of this model is that the structural transition between steps (i) and (ii) is not driven by conformational changes in the AAA1 ATPase site itself but, rather, is most likely driven by thermal fluctuations. This interpretation is supported by the observation that, although the comparison of ADP state 1 and ADP state 2 revealed substantial structural changes in the linker region (Figure 1F; Supplementary Figure 1), no clear differences were detected in the local structure of the primary ATPase site or in its ADP-binding mode (Supplementary Figure 10; Supplementary Table 2). The novelty of our model lies in the proposal that two distinct structural changes after force generation—the swing of the linker from the vicinity of the AAA4 module to that of the AAA5 module and the docking of the linker tip onto the AAA5 module—occur while ADP remains bound at the AAA1 ATPase site. This view differs from conventional models, which posit that these structural changes occur during the transition from the ADP-bound state to the apo state [5].

To evaluate this model further, we performed all-atom molecular dynamics (MD) simulations for each cryo-EM structure. Because cryo-EM requires sample exposure to extremely low temperatures, the resulting structures may deviate from those observed at physiological temperature (e.g., [51]). Therefore, our simulations were also intended to assess the extent of relaxation. For each of the three post-force-generating conformations obtained by cryo-EM (ADP state 1, ADP state 2, and Apo state 1), we performed five independent 1000-ns all-atom MD simulations under conditions of 300 K, 1 bar, and 50 mM KCl. The RMSD of each trajectory plateaued, on average, after approximately 500 ns, confirming that each system had reached a stable equilibrated state (Supplementary Figure 11). To characterize the dominant conformations, we performed clustering for the final 500 ns of each trajectory and obtained representative MD structures. Compared with their corresponding cryo-EM structures, all three representative MD structures exhibited a common structural change: a local rearrangement of the ATP-hydrolyzing ring accompanied by increased separation between the AAA4S/AAA5L and AAA5S/AAA6L submodules (Supplementary Figure 12). This structural change is likely attributable to thermal relaxation at physiological temperature.

In addition, the ADP state 1 and ADP state 2 systems exhibited distinct state-specific structural transitions. The representative MD structure originating from ADP state 1 deviated markedly from the corresponding cryo-EM structure, characterized by bending of the distal portion of the force-generating linker around Q1522 in the H5 helix of Link 0 (Figure 3A and B) and docking of the bent tip onto the AAA3S/AAA4L submodules of the ring (Figure 3C). These results suggest that during the ADP-bound phase of the chemomechanical cycle, one major intermediate conformation of dynein is a metastable state wherein the linker simultaneously engages two ring-docking sites: AAA2 and AAA3S/AAA4L. This conformation corresponds to ADP state 1 in our schematic model of the structural transitions, as shown in Figure 5A(i). In contrast, the representative MD structure originating from ADP state 2 was strikingly more similar to that of Apo state 1. Specifically, although ADP remained bound at the AAA1 ATPase site (Supplementary Figure 13), the overall architecture of the motor domain, the pattern of interactions between the linker and ring, and the local structure of the AAA1 ATPase site shifted toward a conformation similar to that of Apo state 1 (Figure 3D–F). These findings support our model that, if the post-force-generating structural transitions of dynein proceed sequentially, then ADP state 1 $\rightarrow$ ADP state 2 $\rightarrow$ Apo state 1 represents the most likely pathway.

To further test our model, we analyzed the ADP-binding mode at the AAA1 ATPase site in each representative MD structure. The number of hydrogen bonds involved in ADP–protein interactions was approximately 30% lower in ADP state 2 (nine hydrogen bonds) than in ADP state 1 (13 hydrogen bonds) (Supplementary Figure 13). We then estimated the ADP-binding free energy by enhanced sampling of the ADP-release process using dissociation Parallel Cascade Selection MD (dPaCS-MD) [52–55] (Supplementary Figure 14). The ADP-binding free energy for ADP state 2 was -10.2 ± 0.2 kcal/mol (mean $\pm$ SEM, $n = 10$), indicating weaker binding than that of ADP state 1 (-11.1 ± 0.4 kcal/mol; mean $\pm$ SEM, $n = 10$). This analysis quantitatively demonstrates that ADP state 2 has structural features that facilitate ADP dissociation, allowing ADP state 2 to transition to the apo state more readily than does ADP state 1. Together, these results strongly suggest that, during the ADP-bound phase of the chemomechanical cycle, dynein can adopt another major intermediate conformation, i.e., an apo-like state that readily releases ADP and proceeds to the apo conformation. This conformation corresponds to ADP state 2 in our schematic model of the structural transitions, as shown in Figure 5A (ii) and (iii). It should be noted, however, that the proposed ADP state 1 $\rightarrow$ ADP state 2 $\rightarrow$ Apo state 1 pathway is inferred from structures resolved from

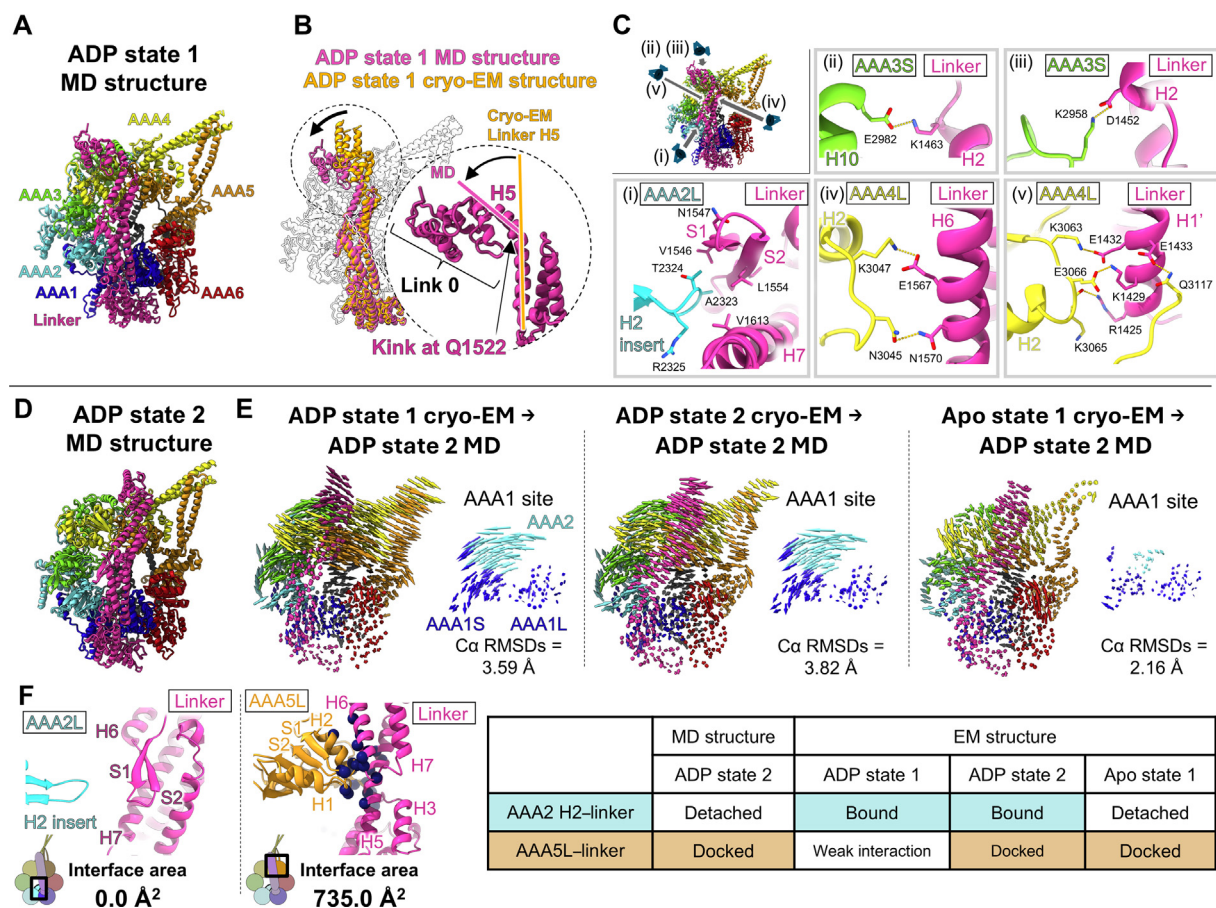


Figure 3. MD simulations support a transition pathway from ADP state 1 through ADP state 2 to Apo state 1. (A) Overall view of the representative MD structure for ADP state 1. (B) Linker conformations in ADP state 1, comparing the cryo-EM structure with the corresponding representative MD structure. The cryo-EM and MD linkers are shown in orange and magenta, respectively. The close-up view shows a kink near Q1522 in H5 and counterclockwise bending of Link 0, viewed from the linker side. (C) Atomic details of linker–ring interactions in the representative MD structure for ADP state 1. Interacting residues are shown as sticks; hydrogen bonds, where present, are indicated by dashed lines. (D) Overall view of the representative MD structure for ADP state 2. (E) Structural comparisons of the representative MD structure for ADP state 2 with the three cryo-EM structures (ADP state 1, ADP state 2, and Apo state 1). C α –C α displacement vector maps for the whole motor domain and the AAA1 ATPase site, together with C α RMSDs for the AAA1 ATPase site, are shown after alignment on AAA1L. Vector maps were sampled at every third residue; arrows indicate displacements from each cryo-EM structure to the representative MD structure for ADP state 2. (F) Close-up views of linker interactions with the AAA2 H2 insert and AAA5L in the representative MD structure for ADP state 2. The accompanying table compares the corresponding interactions across the three cryo-EM structures and the representative MD structure for ADP state 2. Interface areas are indicated in the close-up panels. C α atoms of interface residues are shown as dark blue spheres.

a single equilibrium cryo-EM dataset; establishing its temporal order, relative fluxes, and possible alternative routes will require future kinetic or time-resolved structural studies.

ADP-bound intermediates suggest a structural basis for tension-dependent inter-motor coordination

A natural question is whether the two metastable ADP-bound states adopted by dynein (ADP state 1 and ADP state 2) have physiological significance. We propose that these conformations could

contribute to a tension-sensing mechanism of dynein. Cytoplasmic dynein is a large motor protein complex containing two to four motor domains [56–63], and its minus-end-directed motility along microtubules is thought to arise from stochastic forward stepping of these motor domains [64,65]. However, when the distance between a leading and a trailing motor domain becomes substantially extended, the trailing motor has been proposed to sense the tension transmitted through the linkers and actively step forward, thereby increasing the transport efficiency [64,65]. Under such conditions, the linker of the leading motor would be pulled

backward, whereas that of the trailing motor would be pulled forward [18,41,66]. We hypothesize that these distinct mechanical constraints could bias the leading motor toward the ADP state 1 conformation and the trailing motor toward the ADP state 2 conformation (Figure 5B). Consequently, the leading motor would be more likely to remain in the ADP-bound state, whereas the trailing motor would more readily undergo structural transitions to the apo state and subsequently to the ATP-bound state, thereby promoting forward stepping of the trailing motor.

During the preparation of this manuscript, multiple cryo-EM structures of human and yeast cytoplasmic dynein at different stages of their chemomechanical cycles have been reported [67–70]. The ADP-bound structures described in this study are broadly consistent with these independent findings in revealing multiple motor-domain conformations during the ADP-bound phase. Specifically, the linker positions in our ADP state 1 and ADP state 2 resemble those in the ADP post-2 and post-1 conformations, respectively [67–70]. Related post-1 and post-2 linker-docking positions have also been reported for ciliary outer-arm dyneins [71], suggesting that these two post-force-generating linker positions may be conserved across dynein classes.

ADP release from the AAA3 and AAA4 ATPase sites induces localized ring remodeling

Next, we focused on the remaining two ATPase sites within the dynein motor domain: the AAA3 and AAA4 ATPase sites. Evidence from genetic and biochemical studies of cytoplasmic dynein in yeast and *Dictyostelium* has shown that these sites are important for motor activity and are thought to regulate the AAA1 ATPase site, the primary ATPase site, allosterically, either directly or indirectly [13,14,18–21,25,72]. In yeast cytoplasmic dynein, the nucleotide bound at the AAA3 ATPase site is a critical determinant of dynein motor function, and normal motor activity is observed when this site is ADP-bound. Once the AAA3 ATPase site becomes nucleotide-free via ADP release from this site, dynein enters an inactive state in which it remains tightly bound to microtubules but does not translocate them [18–20]. Despite these functional insights, the structural mechanism by which the AAA3 and AAA4 ATPase sites regulate dynein motor activity has remained unknown.

To elucidate the structural basis of the regulatory roles of the AAA3 and AAA4 ATPase sites, we determined the structure of the dynein motor domain in the absence of nucleotide. The resulting Apo state 2 structure (2.66 Å resolution) showed no detectable nucleotide density at any of the AAA1, AAA3, or AAA4 ATPase sites, distinguishing it from Apo state 1, in which ADP

density was clearly resolved at the AAA3 and AAA4 ATPase sites (Figure 4A and B; Supplementary Figures 7, 8, and 15). Matched contour-level comparisons and local-resolution analyses support the nucleotide-density interpretations used here, although low or partial nucleotide occupancy at sites lacking detectable density cannot be excluded (Supplementary Figures 7 and 8).

Comparison of the Apo state 2 and Apo state 1 structures therefore enabled, for the first time, a direct evaluation of the structural consequences of ADP binding and release at the AAA3 and AAA4 ATPase sites. Although a crystal structure of yeast cytoplasmic dynein in a nucleotide-free state has been reported previously (PDB: 4AKI) [16], this structure differs markedly from the Apo state 2 structure and instead more closely resembles the Apo state 1 structure (Supplementary Figures 9H and 16A–E).

We first conducted a detailed comparison of the ATPase sites in Apo state 1 and Apo state 2 to assess the local effects of ADP binding and release at the AAA3 and AAA4 ATPase sites. The AAA3 ATPase site is formed at the interface of the AAA3L, AAA3S, and AAA4L submodules (reviewed in [10]). During the structural transition from Apo state 1 to Apo state 2, the relative positions of these submodules change substantially (Supplementary Figure 17A). When viewed in the orientation shown in Supplementary Figure 17A with the AAA3L submodule as a reference, the AAA3S and AAA4L submodules move cooperatively in a counterclockwise direction along the outer perimeter of the AAA3L submodule by 32.6° and 24.4°, with displacements of 14.6 Å and 16.1 Å, respectively. Consequently, the AAA3 ATPase site converts from a semi-open to a fully open conformation. Similar ADP-release-coupled structural changes occur at the AAA1, AAA3, and AAA4 ATPase sites within the dynein motor domain, although the magnitude of these structural changes is site-dependent (Supplementary Figure 17A–C).

We then examined how the local conformational changes associated with ADP release from the AAA3 and AAA4 ATPase sites affect the overall architecture of the dynein motor domain. When the structural transition from Apo state 1 to Apo state 2 is examined with AAA1L as a reference, major structural rearrangements ($C\alpha$ positional shifts ≥ 5 Å) are restricted to the AAA3 module and its neighboring AAA2 and AAA4 modules (Figure 4C). The most prominent conformational change is the outward tilting of the L submodules of the AAA2, AAA3, and AAA4 modules toward the ring periphery in a coordinated manner when viewed from the linker side of the ATP-hydrolyzing ring (Figure 4C). In contrast to ADP release from the AAA1 ATPase site, which induces extensive conformational changes throughout the motor domain, ADP release from the AAA3 and AAA4

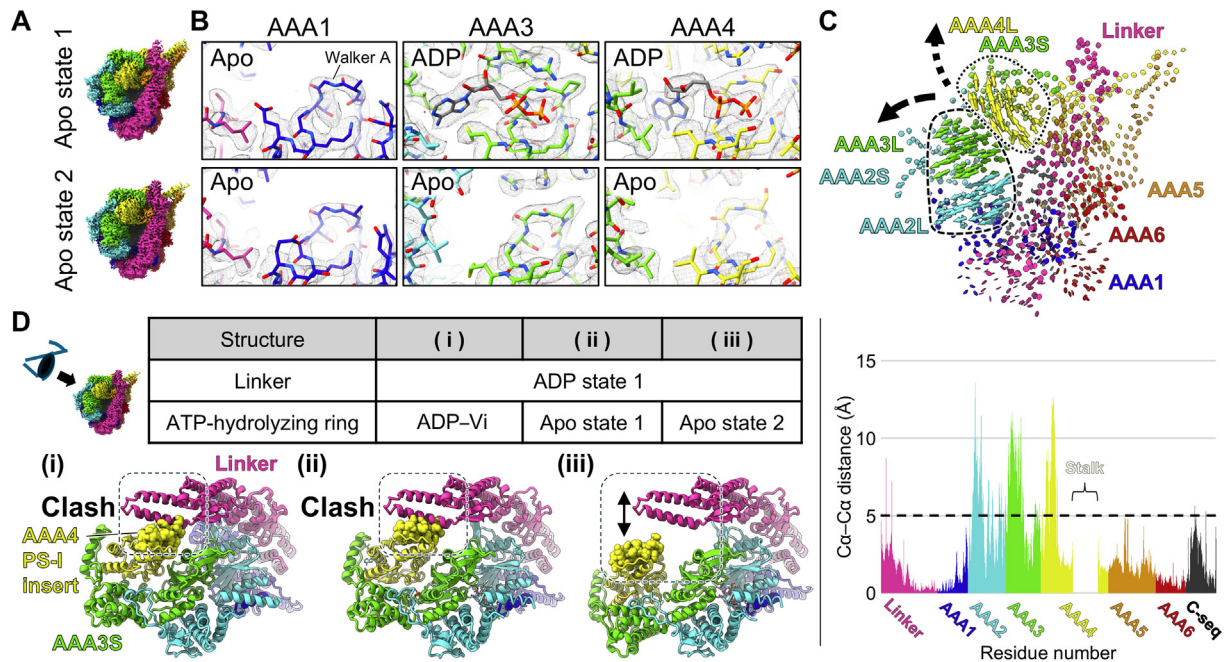


Figure 4. The Apo state 2 ring architecture displaces the AAA4 PS-I insert and disfavors recovery-stroke initiation. (A) Cryo-EM reconstructions of Apo state 1 and Apo state 2. (B) Close-up views of the AAA1, AAA3, and AAA4 ATPase sites. Apo state 1 shows no detectable nucleotide density at the AAA1 ATPase site but has resolved ADP density at the AAA3 and AAA4 ATPase sites, whereas Apo state 2 shows no detectable nucleotide density at any of the three sites. ADP and surrounding residues are shown as sticks. (C) Structural comparison of Apo state 1 and Apo state 2 after alignment on AAA1L. $C\alpha$ - $C\alpha$ displacement vectors, sampled at every third residue, point from Apo state 1 to Apo state 2. Dashed and dotted outlines highlight local rearrangements in the AAA2L-AAA3L and AAA3S-AAA4L submodules, respectively. The displacement profile is shown below; dashed lines represent 5 Å. (D) Composite models assessing the relative positions of the AAA4 Pre-Sensor-I (PS-I) insert and the straight post-force-generating linker during recovery-stroke initiation. In the ring conformations based on ADP-Vi and Apo state 1, the AAA4 PS-I insert forms a predicted steric clash with the linker, whereas in the Apo state 2-based ring conformation, the PS-I insert is displaced from the linker. The AAA4 PS-I insert is shown as yellow spheres.

ATPase sites has little impact on structural rearrangement of the motor domain beyond these local regions.

AAA3/AAA4 nucleotide-free states may structurally constrain the recovery stroke and microtubule release

We next asked how structural changes associated with ADP binding to and release from the AAA3 and AAA4 ATPase sites regulate dynein motor activity. Based on the structural features of Apo state 1 and Apo state 2 observed in this study, we hypothesize that these states may directly modulate the recovery stroke, a critical conformational transition underlying the chemomechanical cycle of dynein. According to the widely accepted model for dynein force generation [1,5], upon ATP binding to the primary ATPase site in the AAA1 module, dynein undergoes a recovery stroke, during which its linker structure transitions from a straight conformation to a bent conformation, thereby priming the motor for the subsequent power stroke (see Figure 3 in [24]). This

linker transition is thought to be driven by the Pre-Sensor-I (PS-I) insert, a segment that protrudes from the AAA4 module of the ring toward the linker. The PS-I insert has been proposed to physically collide with the linker, thereby initiating the recovery stroke. In agreement with this model, deletion of the AAA4 PS-I insert prevents the recovery stroke, even in the presence of ATP, thereby locking the linker in the post-force-generating straight conformation [24].

We examined the role of ADP binding to and release from the AAA3 and AAA4 ATPase sites in the recovery stroke by analyzing the pre-force-generating ADP-Vi structure obtained in this study, along with the two apo structures, Apo state 1 and Apo state 2. Our structural comparison indicated that, when ADP is bound at both the AAA3 and AAA4 ATPase sites, a substantial steric clash—essential for initiating the recovery stroke—between the AAA4 PS-I insert and the linker would be expected to occur during the structural transition of the motor domain to the pre-force-generating state (Figure 4D(i) and (ii)). In contrast, when the AAA3 and AAA4 ATPase

sites are nucleotide-free, outward tilting of the corresponding L submodules from the ring displaces the AAA4 PS-I insert away from the linker. In this configuration, the PS-I insert is positioned too far from the linker and would thus be unlikely to trigger the recovery stroke (Figure 4D(iii)).

Moreover, our data suggest that localized structural changes in the AAA3 and AAA4 modules accompanying ADP release from their ATPase sites may also inhibit another essential motor action immediately preceding the recovery stroke, namely, the transition from a high- to a low-affinity microtubule-binding state. When ATP binds to the primary ATPase site in the AAA1 module, the dynein ring adopts a closed conformation and induces a large-scale structural rearrangement in the upper ring region comprising the AAA4–AAA6 modules. This rearrangement is transmitted to the microtubule-binding site at the tip of the stalk, remodels its structure, and thereby promotes dynein dissociation from microtubules [22,24,30–33,37,73]. Although this transition in the microtubule-binding state is primarily governed by the primary ATPase site in the AAA1 module [13,17,24], the regulatory ATPase sites in the AAA3 and AAA4 modules have also been implicated as important modulators [13,14,18–21,25,72,74]. Indeed, biochemical and biophysical studies have demonstrated that point mutations that prevent nucleotide binding to the AAA3 or AAA4 ATPase site severely disrupt dynein dissociation from microtubules, resulting in dynein remaining tightly bound to microtubules [13,18–20,72]. However, the structural basis for this regulation has remained largely unknown.

Based on our structural analyses, we propose the following model to explain how the AAA3 and AAA4 modules regulate dynein motor activity: (i) in the nucleotide-free state, the L submodules of the AAA3 and AAA4 modules tilt outward from the ring, thereby disfavoring the recovery stroke of the linker; the linker would consequently remain in a post-force-generating conformation in which its N-terminal tip docks tightly onto AAA5 of the ATP-hydrolyzing ring; (ii) this tight linker–ring interaction would in turn constrain and suppress ATP-induced structural rearrangements in the upper ring region, comprising the AAA4–AAA6 modules; (iii) consequently, the transmission of these ATP-induced upper-ring rearrangements through the stalk–buttress (strut) element to the microtubule-binding site at the stalk tip would be inhibited, disfavoring the conformational changes required for dynein dissociation from microtubules and thereby maintaining dynein in a strongly microtubule-bound state; and (iv) through this mechanism, the two functionally critical elements for dynein motility—the force-generating linker and the microtubule-binding stalk—would be conformationally constrained, thereby suppressing

motor activity (Figure 5C). This model provides a structural basis for the prevailing view that the AAA3 and AAA4 modules regulate dynein–microtubule interactions via allosteric modulation of the primary ATPase site in the AAA1 module [12,20,74]. Specifically, nucleotide-dependent conformational changes associated with the regulatory ATPase sites in the AAA3 and AAA4 modules remodel the ATP-hydrolyzing ring to drive this allosteric regulation.

In summary, we focused on the ADP-bound and ADP-release steps that are key to the force-generation and regulatory mechanisms of cytoplasmic dynein and characterized the corresponding structures in detail. First, for the primary ATPase site in the AAA1 module, our combined cryo-EM analyses and MD simulations suggest that the chemomechanical cycle involves a dynamic equilibrium between two distinct ADP-bound states. We propose that these two states collectively serve as the structural basis for dynein's tension-sensing gating mechanism. Furthermore, for the two regulatory ATPase sites in the AAA3 and AAA4 modules, we elucidated, at near-atomic resolution, the structural transition from a partially nucleotide-free state, with ADP retained at the AAA3 and AAA4 ATPase sites, to a fully nucleotide-free state. Based on this transition, we propose a new model detailing how localized structural rearrangements in the AAA3 and AAA4 modules physically disfavor the recovery stroke, thereby allosterically regulating motor activity. Collectively, these findings provide important mechanistic insights into the chemomechanical cycle of cytoplasmic dynein. However, further structural and functional investigations are required to fully elucidate the entire cycle. In particular, the structures of several transient but functionally essential intermediates are not yet well resolved. For example, the conformational pathway by which dynein dissociates from microtubules after ATP binding, and the sequence of structural events by which ADP-bound dynein rebinds to microtubules to generate force, remain largely unknown. Time-resolved cryo-EM with millisecond temporal resolution is likely to be a key approach for resolving these questions [75–78]. The experimental and analytical framework established in this work should provide a solid technical basis for such future studies.

Materials and Methods

Protein expression and purification

We used the 380-kDa motor domain (Val1388–Ile4730) of the cytoplasmic dynein heavy chain from the *D. discoideum* Ax2 strain for all experiments. The recombinant dynein motor domain, referred to as HF380 [23], comprises the 380-kDa motor domain preceded by a His₆–FLAG

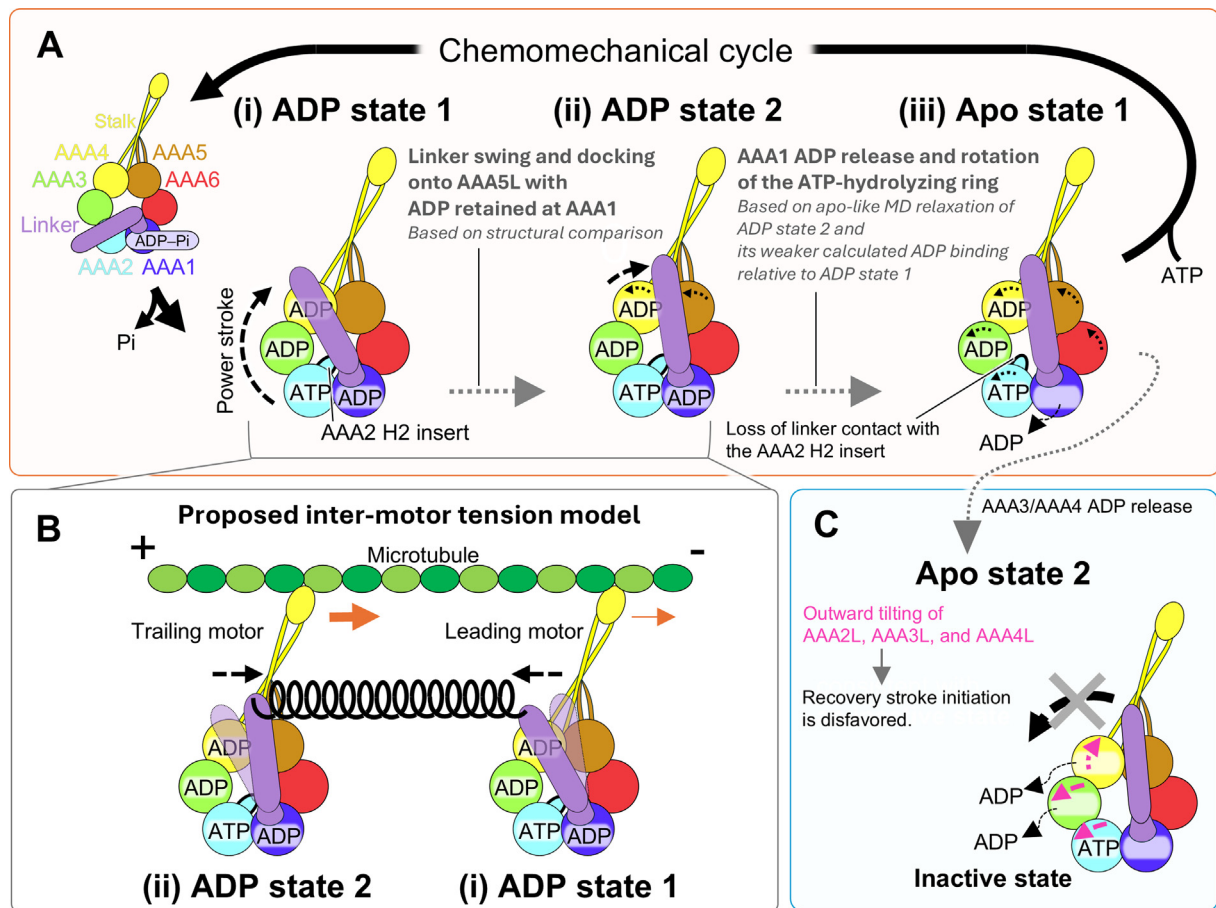


Figure 5. Proposed model for ADP-to-apo regulation of chemomechanical transitions in cytoplasmic dynein. (A) Structural model for the two-step post-force-generating transition. Schematic representation of a possible ordering among ADP state 1 (i), ADP state 2 (ii), and Apo state 1 (iii). In this model, following Pi release and the associated power stroke, dynein adopts the ADP state 1 conformation. While ADP remains bound at the AAA1 ATPase site, the linker swings clockwise and docks onto AAA5L, yielding ADP state 2. Subsequent ADP release from the AAA1 ATPase site and rotation of the ATP-hydrolyzing ring lead to Apo state 1. Linker contacts with the AAA2 H2 insert are retained in ADP state 1 and ADP state 2 but are lost in Apo state 1. **(B) Inter-motor tension model.** Inter-motor tension generated between separated motor domains could bias the leading and trailing motors toward ADP state 1 and ADP state 2, respectively. The ADP state 2 bias in the trailing motor may facilitate ADP release from the AAA1 ATPase site, promoting the motor's transition first to the apo state and subsequently, upon ATP binding, to the ATP-bound states, thereby favoring forward stepping. This model has not been tested under load and is presented as a hypothesis. **(C) Regulation of the recovery stroke by the AAA3 and AAA4 ATPase sites.** A model in which ADP release from the AAA3 and AAA4 ATPase sites promotes the transition from Apo state 1 to Apo state 2, displacing the AAA4 PS-I insert away from the linker and thereby potentially disfavoring the recovery stroke and the stalk-registry transition.

tag; it lacks the entire N-terminal tail region, including the dimerization domain, and contains no glutathione S-transferase tag, coiled-coil or leucine zipper module, and DNA linker or other artificial dimerization device. HF380 protein was expressed and purified as described previously [23,35,42]. Briefly, the procedure was as follows. HF380 was expressed in MB35-integrated *D. discoideum* Ax2 cells transformed with the extrachromosomal MB38 expression vector and selected and maintained in the presence of 10 $\mu\text{g/mL}$ G418, 10 $\mu\text{g/mL}$ blasticidin S, and 12 $\mu\text{g/mL}$ tetracycline. Cells

were cultured in HL5 medium at 22°C with shaking at 150 rpm. When the culture reached approximately 1×10^7 cells/mL, the cells were transferred to HL5 medium containing 10 $\mu\text{g/mL}$ G418 but lacking tetracycline and blasticidin S, and HF380 expression was induced for approximately 24 h before harvesting. Cells were lysed in buffer containing 100 mM PIPES, 4 mM MgCl_2 , 0.1 mM EGTA, 0.9 M glycerol, and 10 mM imidazole, pH 7.0, freshly supplemented with 10 $\mu\text{g/mL}$ each of chymostatin and pepstatin A, 50 $\mu\text{g/mL}$ leupeptin, 0.5 mM PMSF, 1 mM 2-mercaptoethanol, and

100 μ M ATP. The lysate was clarified by centrifugation at 24,000*g* for 20 min, followed by centrifugation of the resulting supernatant at 187,000*g* for 60 min. The clarified supernatant was first subjected to Ni-NTA affinity purification, and the Ni-NTA eluate was subsequently incubated with anti-FLAG M2 affinity resin. The resin was washed with PMEGS buffer containing 100 mM PIPES, 4 mM MgCl_2 , 5 mM EGTA, 0.1 mM EDTA, 0.9 M glycerol, 100 μ M ATP, and 150 mM NaCl, pH 7.0, followed by PMEG30 buffer containing 30 mM PIPES, 4 mM MgCl_2 , 5 mM EGTA, 0.1 mM EDTA, 0.9 M glycerol, and 100 μ M ATP, pH 7.0. Bound protein was eluted with PMEG30 buffer containing 0.2 mg/mL FLAG peptide. No chemical cross-linking was performed at any stage. To obtain nucleotide-free preparations, 100 μ M ATP was omitted from the final elution buffer during anti-FLAG affinity purification. The purified protein was divided into 5- μ l aliquots, flash-frozen in liquid nitrogen, and stored in liquid nitrogen until use.

Cryo-EM data collection

Protein aliquots were rapidly thawed immediately before grid preparation and kept on ice. For the ADP-Vi condition, HF380 at 10.5 μ M was prepared in dynein assay buffer (10 mM K-PIPES, 4 mM MgSO_4 , and 1 mM EGTA, pH 7.1) supplemented with 1 mM ATP, 200 μ M sodium orthovanadate, and 0.1% (v/v) Tween-20. For the ADP condition, HF380 (10.5 μ M) was prepared in the same buffer supplemented with 50 μ M ADP and 0.1% (v/v) Tween-20. For the nucleotide-free condition, HF380 (10.5 μ M) was prepared in the same buffer alone with 0.1% (v/v) Tween-20.

Quantifoil R1.2/1.3 Cu 200-mesh grids were glow-discharged using a JEC-3000FC sputter coater (JEOL, Japan) at 20 mA for 20 s. A 2.8 μ l aliquot of sample was applied to each grid. The grids were blotted (blot force: 2, blot time: 3 s) using a Vitrobot Mark IV device (Thermo Fisher Scientific, USA) operated at 4°C and 100% relative humidity. The grids were vitrified by plunge-freezing into liquid ethane before storing in liquid nitrogen until imaging.

Cryo-EM data were collected on a JEM-Z300FSC instrument (CRYO ARM™ 300, JEOL) operated at 300 kV and equipped with a K3 direct electron detector (Gatan, Inc., USA) in correlated double sampling mode. An in-column, Ω -type energy filter with a slit width of 20 eV was used. Images were recorded at a nominal magnification, 60,000 $\times$, corresponding to a calibrated pixel size of 0.873 Å. Automated data collection was performed using SerialEM (version 3.8 or 4.050) [79] together with yoneo-Locr (version 1.051) [80]. Movies were collected with defocus values ranging from $-0.7 \mu\text{m}$ to $-2.2 \mu\text{m}$. Each movie consisted of a 40-frame stack and was recorded with a total electron dose of 80 $\text{e}^-/\text{Å}^2$.

Cryo-EM image processing

A gain reference was generated from 300 movies using the `reliion_estimate_gain` command in RELION v3.1 [81]. Subsequent image processing was performed using cryoSPARC (version 4.4.1) [82]. Movies were motion-corrected, contrast transfer function (CTF) parameters were estimated, and micrographs with CTF-estimated maximum resolutions better than 6 Å were retained for further processing.

For the dimeric motor-domain reconstruction from the ADP-Vi dataset, 32,531 movies were imported, and 29,118 micrographs passed the CTF-resolution criterion. Initial particles were picked using a blob picker and cleaned by two-dimensional (2D) classification. The selected particles were used to generate an ab initio reconstruction with C1 symmetry, which served as the initial reference for template-based particle picking and subsequent refinement. After template-based picking and cleanup by 2D classification, 1,755,826 particles were re-extracted from the original micrographs with a 600-pixel box. The re-extracted particles were subjected to 3D classification into 10 classes; particles from nine of the ten classes were then combined for further refinement, yielding 1,378,380 particles. The combined particle set was refined through iterative non-uniform refinement with C2 symmetry imposed, interleaved with global and local CTF refinement. This procedure yielded a final consensus map at 2.20 Å resolution by the Fourier shell correlation (FSC) = 0.143 criterion. The workflow is shown in [Supplementary Figure 3A](#).

The same ADP-Vi dataset was processed separately to obtain a monomeric motor-domain reconstruction. Monomeric particles were picked from Topaz-denoised, low-pass-filtered micrographs using templates generated from an ADP state 1 reconstruction low-pass filtered to 20 Å. After 2D classification and ab initio reconstruction into four classes, particles from one selected class were re-extracted from the original non-denoised micrographs. A total of 217,910 particles were refined by non-uniform refinement with C1 symmetry, interleaved with global and local CTF refinement, yielding a 2.87 Å reconstruction by the FSC = 0.143 criterion. Because of its low abundance and conformational heterogeneity, this monomeric population was not used for further structural interpretation. The workflow is shown in [Supplementary Figure 3B](#).

For the ADP dataset, 16,865 movies were imported, and 14,383 micrographs passed the CTF-resolution criterion. Initial particles were picked using a blob picker, cleaned by 2D classification, and used to generate an ab initio reconstruction, which served as the initial reference for template-based particle picking. Template-picked particles were extracted with a

300-pixel box and cleaned by 2D classification. After ab initio reconstruction and non-uniform refinement, 4,072,620 particles were retained. This particle set was subjected to three-dimensional variability analysis (3DVA) followed by 3D Variability Display in cluster mode with a 7 Å filter, yielding 20 conformational clusters. Three major states were defined based on the linker angle and nucleotide density at the AAA1 ATPase site: ADP state 1, with an approximately 18° linker angle and clear AAA1 ADP density; ADP state 2, with an approximately 6° linker angle and clear AAA1 ADP density; and Apo state 1, with an approximately 6° linker angle and no detectable AAA1 nucleotide density. The particle subsets corresponding to these states were further refined by non-uniform refinement, interleaved with global and local CTF refinement, yielding final maps at 2.61 Å, 3.20 Å, and 2.76 Å resolution for ADP state 1, ADP state 2, and Apo state 1, respectively, by the FSC = 0.143 criterion. The corresponding particle numbers were 2,128,136, 596,990, and 1,347,494, respectively. No cluster corresponding to an approximately 18° linker angle in the absence of nucleotide density at the AAA1 ATPase site was detected. To measure linker angles, linker coordinates were flexibly fitted to the corresponding cryo-EM maps using ISOLDE (version 1.7) [83,84] in ChimeraX (version 1.8) [85], and angles were then measured in PyMOL (version 3.0.3). The workflow is shown in [Supplementary Figures 5 and 6](#).

For the Apo state 2 dataset, 20,673 movies were imported, and 15,783 micrographs passed the CTF-resolution criterion. Initial particles were picked using a blob picker and cleaned by 2D classification. The selected particles were used to generate an ab initio reconstruction, which served as the initial reference for template-based particle picking. Template-picked particles were extracted with a 300-pixel box and further cleaned by 2D classification, yielding 1,108,632 particles. These particles were subjected to 3D classification into five classes; particles from four selected classes were combined for final refinement, yielding 910,656 particles. The combined particle set was refined through iterative non-uniform refinement, interleaved with global and local CTF refinement. This procedure yielded a final Apo state 2 map at 2.66 Å resolution by the FSC = 0.143 criterion. The workflow is shown in [Supplementary Figure 15](#).

Local-resolution maps for all five reconstructions were calculated using the Local Resolution Estimation job in cryoSPARC (version 4.4.1) from the corresponding final-refinement half-maps and were visualized in ChimeraX using a common color scale of 2.2–4.7 Å. For multiple-contour comparisons, the AAA1, AAA3, and AAA4 ATPase sites in Apo state 1 and Apo state 2 were

displayed at contour levels of 0.20, 0.40, and 0.60, with the AAA1 ATPase site of ADP state 1 used as an ADP-bound reference. Reference ADP positions at sites lacking detectable nucleotide density were generated by aligning 21-residue segments encompassing the Walker A motifs of ADP state 1 with the corresponding regions of the Apo-state structures; residues 1970–1990, 2670–2690, and 3012–3032 were used for AAA1, AAA3, and AAA4, respectively ([Supplementary Figures 7 and 8](#)).

Model building and refinement

Atomic models of the HF380 monomer were generated using the crystal structure of the *D. discoideum* dynein motor domain (PDB: 3VKG, chain A) [23] as the initial model. Each model was manually docked into the corresponding cryo-EM density map using UCSF ChimeraX (version 1.7 or 1.8) [85] and subsequently adjusted in Coot (version 0.9.8.92) [86]. Real-space refinement was performed using PHENIX (version 1.21) [87]. Model validation was performed using MolProbity software (version 4.5.2) [88]. Iterative cycles of manual rebuilding and real-space refinement were performed until convergence was achieved. Cryo-EM data collection, image processing, model building, refinement, and validation statistics are summarized in [Supplementary Table 1](#).

Measurements

Linker angles, module rotations, and inter-module distances were measured using the `angle_between_domains` command in PyMOL (version 3.0.3 or v3.1.6; Schrödinger, LLC; <https://www.pymol.org/pymol>). ADP–protein interface areas were calculated using the PISA server [89], and solvent-accessible surface areas were calculated using CCP4i (version 8.0.015) [90]. Hydrogen-bonding and hydrophobic interactions were analyzed using LigPlot⁺ (version 2.2.8) [91]. Structural figures were prepared using UCSF ChimeraX (version 1.9 or 1.10) [85]. The interpolation shown in [Supplementary Movie 1](#) was generated from the independently refined atomic models using the `morph` command in UCSF ChimeraX with default settings; it is presented solely as a visualization aid and is not used as evidence for the directionality or the kinetics of any transition.

C α –C α displacement vector maps were generated using UCSF ChimeraX after sequence alignment of the compared structures with Clustal Omega. For each aligned residue pair, vectors were drawn between the corresponding C α atoms in two structures. Cylinders and cones were generated using the ChimeraX `shape` command with radii of 0.7 and 1.5 Å, respectively, to represent the shaft and arrowhead of each vector.

MD simulations

All-atom MD simulations were performed for ADP state 1, ADP state 2, and Apo state 1. Missing loop regions in the initial structures were automatically built using the “Fit Loop by Rama Search” routine in Coot (version 0.9.8.92) [86] before the MD simulations. In ADP state 1, residues 1443–1448, 2457–2486, and 4443–4458 were modeled. In ADP state 2, residues 2458–2487 and 4446–4457 were modeled. In Apo state 1, residues 2080–2085, 2458–2487, and 4438–4458 were modeled. In contrast, the missing residues in the stalk region were left unmodeled in all three structures (residues 3308–3552 in ADP state 1, residues 3311–3551 in ADP state 2, and residues 3315–3542 in Apo state 1).

For each system, the cryo-EM structure was solvated in a cubic box with an edge length of 22.1 nm with TIP3P (CHARMM) water molecules and 0.05 M KCl. ADP and ATP were modeled using the CHARMM36m force field [92]. Long-range electrostatic interactions were evaluated using the particle mesh Ewald method (1.0 nm real-space cutoff), and the motion equations were integrated with a time step of 2 fs. All covalent bonds involving hydrogen atoms were constrained using the LINCS algorithm. Initially, the systems underwent energy minimization, followed by a heating phase from 50 K to 300 K in 1 ns with a V-rescale thermostat [93]. During this stage, all heavy atoms in the proteins and ADP/ATP molecules were restrained with a force constant of 1000 kJ/(mol·nm²). Subsequently, the systems were equilibrated for 10 ns in an isothermal–isobaric ensemble at 300 K and 1 bar using a V-rescale thermostat and Berendsen barostat while maintaining the same positional restraints. The final snapshots from the equilibration were used as starting points for production runs, performed in an isothermal–isobaric ensemble at 300 K and 1 bar using the Nosé–Hoover thermostat [94,95] and Parrinello–Rahman barostat [96]. Positional restraints were maintained throughout the production runs on the C α atoms of residues 3307 and 3553 (ADP state 1), 3301 and 3552 (ADP state 2), and 3314 and 3543 (Apo state 1) using each cryo-EM structure as the reference, with a force constant of 1000 kJ/(mol·nm²) at disconnected stalk tips. These restraints were introduced to prevent nonphysical relative drift between the two truncated stalk ends flanking the unresolved region. Previous structural and functional studies suggest that the stalk coiled-coil registry does not change among post-force-generating states; therefore, fixing the two truncated stalk ends was considered a reasonable boundary condition for following the ring and linker rearrangements examined here. All other modeled regions, including the linker and the ATP-hydrolyzing ring, were left unrestrained during the production simulations, and the restrained C α atoms are located far from the linker and from the nucleotide-binding pockets of AAA1, AAA3, and

AAA4. However, because alternative restraint schemes were not examined, we cannot exclude the possibility that these restraints affected the absolute amplitude of the ring and linker motions.

Five independent MD simulations were conducted for 1 μ s in each system. All the MD simulations, including those performed using the PaCS-MD procedure described below, were performed using GROMACS (version 2024.6) [97] on a Fugaku supercomputer. For each state, the last 0.5 μ s of the five MD trajectories (2.5 μ s total) were merged and categorized into 10 clusters using the k-means clustering algorithm based on the RMSD of protein heavy atoms. The snapshot closest to the center of the most populated cluster was selected as the representative structure for each state.

Binding free energy calculation

To estimate the binding affinity of ADP at the AAA1 ATPase site in both ADP state 1 and 2, we simulated ADP dissociation from the AAA1 ATPase site using dissociation parallel cascade selection molecular dynamics (dPaCS-MD) [53–55]. PaCS-MD is an enhanced conformational sampling method involving multiple cycles of short MD simulations conducted in parallel (replicas) in which the initial structures for the subsequent cycle are selected based on their proximity to a target state along a predefined selection feature [52].

The inter-center-of-mass (COM) distance, d , between the ADP molecule and surrounding AAA1 binding-pocket residues (residues whose minimum distance of heavy atoms was ≤ 0.35 nm from the center of mass of ADP in each cryo-EM structure) was calculated. The residues shown in [Supplementary Figure 13](#) were employed as selection features to accelerate the dissociation process. For each system, 10 independent dPaCS-MD trials were initiated using snapshots extracted at 0.95 and 1 μ s from the five MD simulations. Each trial consisted of 30 replicates, with each replica simulated for 0.1 ns. The dPaCS-MD cycles were repeated until ADP fully dissociated from the dynein motor domain ($d > 2.0$ nm).

Subsequently, all trajectories from the dPaCS-MD trials were integrated to construct the Markov state model (MSM) using pyEMMA [98]. Microstates were generated via k-means clustering with 100 centers using the k-means++ scheme. After carefully evaluating the implied timescales, a lag time of 50 ps was selected to build MSMs for both ADP states. The obtained MSM provided stationary probabilities of the microstates as a function of COM distance, d , which could be converted into the potential of the mean force (PMF). The standard binding free energy was calculated as the sum of the difference of PMF between the bound ($d \leq 0.48$ and 0.24 nm for ADP state 1 and 2, respectively) and unbound states ($d > 1.4$ nm),

together with a volume-correction term [99,100] to account for the standard state concentration.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the authors used ChatGPT (OpenAI) and Claude (Anthropic) to assist with translating an initial Japanese draft of the manuscript into English and improving the clarity and readability of the English text. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

CRedit authorship contribution statement

Hiroshi Imai: Writing – review & editing, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Riko Kanazawa:** Writing – review & editing, Investigation. **Toshihisa Maeshima:** Writing – review & editing, Visualization, Investigation, Data curation. **Duy Phuoc Tran:** Writing – review & editing, Investigation, Funding acquisition, Formal analysis, Data curation. **Rieko Shimo-Kon:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **Hisaya Tokita:** Writing – review & editing, Investigation. **Tomoko Miyata:** Writing – review & editing, Methodology, Investigation. **Fumiaki Makino:** Writing – review & editing, Software, Methodology. **Junso Fujita:** Writing – review & editing, Methodology. **Takayuki Kato:** Writing – review & editing, Resources. **Akio Kitao:** Writing – review & editing, Methodology, Funding acquisition. **Keiichi Namba:** Writing – review & editing, Resources, Methodology. **Takahide Kon:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

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driven next-generation medicine and drug discovery based on “Fugaku”, JPMXP1020 230120) to D.P.T.

DATA AVAILABILITY

Cryo-EM maps are available from the EMDB under accession codes EMD-81330 (ADP-Vi), EMD-81335 (ADP state 1), EMD-81336 (ADP state 2), EMD-81337 (Apo state 1), EMD-81338 (Apo state 2). Coordinates are available from the RCSB Protein Data Bank under accession codes PDB 27PT (ADP-Vi), PDB 27PY (ADP state 1), PDB 27PZ (ADP state 2), PDB 27QA (Apo state 1), PDB 27QB (Apo state 2). All other data supporting the conclusions of this article are available from the corresponding author upon reasonable request.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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