All-Atom Structural Investigation of Kinesin–Microtubule Complex Constrained by High-Quality Cryo-Electron-Microscopy Maps

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ABSTRACT: In this study, we have performed a comprehensive structural investigation of three major biochemical states of a kinesin complexed with microtubule under the constraint of high-quality cryo-electron-microscopy (EM) maps. In addition to the ADP and ATP state which were captured by X-ray crystallography, we have also modeled the nucleotide-free or APO state for which no crystal structure is available. We have combined flexible fitting of EM maps with regular molecular dynamics simulations, hydrogen-bond analysis, and free energy calculation. Our APO-state models feature a subdomain rotation involving loop L2 and α6 helix of kinesin, and local structural changes in active site similar to a related motor protein, myosin. We have identified a list of hydrogen bonds involving key residues in the active site and the binding interface between kinesin and microtubule. Some of these hydrogen bonds may play an important role in coupling microtubule binding to ATPase activities in kinesin. We have validated our models by calculating the binding free energy between kinesin and microtubule, which quantitatively accounts for the observation of strong binding in the APO and ATP state and weak binding in the ADP state. This study will offer promising targets for future mutational and functional studies to investigate the mechanism of kinesin motors.

Kinesins, a superfamily of microtubule (MT)-based motor proteins, are involved in numerous cellular functions, ranging from cell division to transportation of cellular cargos.1,2 Kinesin converts the chemical energy from ATP hydrolysis to mechanical work that drives its movement along the MT track,1 which is composed of repeating dimeric subunits formed by α- and β-tubulin monomers.3 A kinesin molecule consists of a catalytic core domain which exhibits MT-stimulated ATPase activity,4 a neck region serving as a mechanical amplifier,5 a stalk domain for dimerization, and a tail domain for cargo binding.

The primary biochemical pathway of a monomeric kinesin in the presence of MT has been outlined by extensive kinetic studies.6–10 Starting from a weak MT-binding ADP state, MT binding to kinesin causes ADP release,11 which leads to a strong MT-binding APO state. Then ATP binding is thought to trigger force generation via the docking of neck linker along the catalytic core domain in the ATP state.12 ATP hydrolysis and subsequent release of inorganic phosphate (Pi) return kinesin to the ADP state. The kinetic cycling through the above three main states (ADP state → APO state → ATP state) underscores the biological functions of kinesin motors.

The structural basis of kinesin’s kinetics remains elusive because detailed structural information is not available for all biochemical states of kinesin. High-resolution crystal structures were only solved for kinesins bound with various nucleotide analogues in the absence of MT.11–13 Although comparisons of distinct kinesin crystal structures have revealed key conformational changes (such as α4-helix movement and neck-linker docking) underlying force generation,16 such conformational changes seem to be decoupled from the chemical and structural changes in the active site.17 This is not unexpected given the general observation that MT binding is required for the activation of ATPase activities in kinesin (including ATP hydrolysis and ADP release).18 How does MT binding impact kinesin structurally and dynamically? To answer this important question, many efforts have been made to model a kinesin–MT complex by fitting a kinesin crystal structure and an MT model19,20 into cryo-electron microscopy (EM) maps of kinesin-decorated MT filaments.12,14,21–23 Thanks to recent improvement in resolution, EM imaging of kinesin–MT complexes24–29 has enabled well-constrained fitting of kinesin structures into EM maps and identification of local structural changes in active site and MT-binding interface. The EM-constrained modeling of kinesins has highlighted the importance of probing those local structural changes29 with high resolution.

Computer modeling techniques, ranging from kinetic modeling30,31 to structure-based coarse-grained32–36 and all-atom simulations, have complemented experimental efforts to probe kinesin motor function. Molecular Dynamics (MD) simulations37 based on all-atom force fields38 provide a powerful tool for investigating protein dynamics in solution for tens of nanoseconds. Previously, MD simulations were performed to investigate the structural dynamics of various kinesins in the...
absence\textsuperscript{39–42} and presence\textsuperscript{43} of MT. In a previous study, we conducted 30-ns MD simulations of kinesin–MT complex in the ADP and ATP state, and then calculated the change in kinesin–MT binding free energy following alanine scanning mutagenesis of conventional kinesin and KIF1A.\textsuperscript{44} Recently, many computational techniques have been developed to incorporate cryo-EM data as a constraint for structural modeling via “flexible fitting” — optimizing the fitting with the EM maps by utilizing the flexibility of a protein structure.\textsuperscript{45–52} For example, the MDFF program flexibly fits a molecule into an EM map within the framework of MD simulation, which preserves the correct stereochemistry and secondary structures of the molecule with each atom being steered toward high-EM-density regions.\textsuperscript{53,54} The MDFF program has been used to model the complex between actins and myosin which is evolutionarily related to kinesin.\textsuperscript{55}

This study aims to investigate how MT binding affects the global and local structures of kinesin in the ADP, ATP, and APO state. To that end, we have combined MDFF,\textsuperscript{53,54} regular MD simulations, MD-based hydrogen-bond analysis, and free energy calculation. Our APO-state models feature a subdomain rotation involving loop L2 and α6 helix, and local structural changes in the active site highly similar to myosin motors. We have identified a list of hydrogen bonds involving key residues in the active site and the binding interface between kinesin and MT. We have validated our models by calculating the binding free energy between kinesin and MT, and comparing it with experimental binding data. This study has offered, to our knowledge, the most comprehensive structural investigation of major biochemical states of kinesin in the presence of MT with atomic details.

\section*{METHODS}

\subsection*{Structural Modeling of Kinesin and Tubulin Dimer.}
We structurally model the following three biochemical states of human conventional kinesin motor domain (referred as kinesin):

\textbf{ADP State.} An ADP-state kinesin model is built based on a crystal structure of ADP-bound kinesin (PDB id: 1BG2).\textsuperscript{56} As found in ref 29, the α4 helix in 1BG2 (with four turns) is too short to fully fit the corresponding EM densities. So we remodeled the α4 helix using the conformation of an extended α4 helix (with six turns) from a crystal structure of ADP-bound Eg5 (PDB id: 1II6).\textsuperscript{57} Such homology modeling is made possible by the high sequence similarity between Eg5 and kinesin (with ∼40\% sequence identity). We modeled the backbone coordinates of residues 247–256 of 1BG2 with that of residues 281–290 of 1II6, and add the side chains using the Swiss Modeler server (http://swissmodel.expasy.org/). Following the extension of the α4 helix, the shortened loop L11 (residues 237–249) is remodeled to maintain the chain connectivity between L11 and α6 helix. For L11 modeling, we keep the coordinates of residues 237–242 from 1BG2, and remodel residues 243–246 with the Swiss Modeler.

\textbf{ATP State.} An ATP-state kinesin model is built based on a crystal structure of ADP-bound kinesin with a docked neck linker (PDB id: 1MKJ).\textsuperscript{17} We use an AMPPNP-bound Eg5 structure (PDB id: 3HQD)\textsuperscript{58} as a template to model several

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Comparison of rigidly fitted and flexibly fitted models of kinesin and tubulin dimer overlaid with EM maps in the following states: (a) ADP state, (b–g) APO state (models APO1–6, see Methods), (h) ATP state. The rigidly fitted models are colored red, and the flexibly fitted models are colored blue. Key secondary structure elements (α2, α3, α4, α6 helices, and L2, L7, L11 loops) and active-site motifs (P-loop and switch 1) of kinesin are labeled.}
\end{figure}
ATP-like structural features missing in 1MKJ, including extended α4 helix, shortened loop L11, and closed switch I. We model the backbone coordinates of residues 190–204 and 237–256 of 1MKJ with that of residues 221–235 and 271–290 of 3HQD, and then add side chains using the Swiss Modeler. All missing residues are added by the Swiss Modeler.

**APO State.** In addition to the ADP-state model built above based on 1BG2, we further construct several alternative kinesin models based on 1BG2 with switch I remodeled to adopt a closed conformation. To sample possible closed conformations of switch I, we scan all crystal structures of the kinesin superfamily available in the Protein Data Bank, and then select those structures that meet the following criteria:

a. Switch I moves closer to the P-loop by in least 1 Å compared with 1BG2 (measured by the distance between the geometric centers of Ca atoms of switch I and P-loop).

b. After being superimposed along the P-loop, the Ca atoms of the N- and C-terminal residues of switch I are within 4 Å from the corresponding residues of 1BG2 (residues 190 and 204).

c. Switch I region has no missing residues.

d. The active site is bound with ADP.

Five kinesin crystal structures meet the above criteria, and their chain ids and PDB ids are as follows: chain A of 2OWM (neurospora crassa kinesin-3), 59 chain B of 3LRE (kinesin-8), 60 chain A of 2ZFM (KIF1A), 15 chain C of 1CZ7 (NCD), 61 and chain A of 2NCD (NCD). 62 We used these five structures as templates to model the backbone coordinates of switch-I residues of kinesin (residues 190–204) with that of corresponding residues in the template structure (residues 539–553 of 1CZ7 and 2NCD, 254–268 of 2OWM, 203–217 of 2ZFM, and 214–228 of 3LRE), and added the side chains using the Swiss Modeler. We used the above six kinesin models as initial models for flexibly fitting of the EM map in the APO state (see below), and the resulting APO-state models are named APO1–APO6, whose switch-I conformation is modeled based on 1BG2, 1CZ7, 2NCD, 2OWM, 2ZFM, and 3LRE, respectively.

Because loop L11 is structurally ordered in the strong-binding states (ATP and APO state), we remodeled α4 helix and L11 following the same procedure as the ATP state (see above).

We modeled MT as a dimer of α- and β- tubulin using the crystal structure of bovine α- and β- tubulin (PDB id: 1JFF) with missing residues added by the Swiss Modeler.

**Flexible Fitting of EM Maps by MDFF.** Following ref 29, we fit kinesin and tubulin dimer separately as two rigid bodies into the EM map using the Chimera program. 53 The ligands (Mg and ADP/ATP) are included when fitting the EM maps in the ADP and ATP state. Similar to ref 29, the overall fitting is good except for several local regions (such as loop L11 and active site, see Figure 1). To improve the fitting and refine the kinesin models, we performed flexible fitting with the MDFF program. 54

Prior to running MDFF, we added hydrogen atoms with the VMD program. 64 All models are immersed into rectangular boxes of water molecules extending up to 10 Å from the proteins in each direction by VMD. To ensure an ionic concentration of 150 mM and zero net charge, Na+ and Cl− ions are added by VMD. Energy minimization for 4000 steps is performed to optimize the initial models.

We performed 2-ns MDFF simulations using the NAMD program, 65 and the CHARMM27 force field 66 with the CMAP correction. 67 Water molecules are described by the TIP3P model. 68 The particle-mesh Ewald summation method 69 is used to evaluate long-range electrostatic forces with a grid size of <1 Å. An integration time step of 1 fs is used in the framework of a multiple time-stepping algorithm following ref 70. A 12-Å cutoff distance is used for nonbonded interactions, and a temperature of 300 K is maintained using the Langevin thermostat, 71 which is coupled to all heavy atoms with a damping coefficient of 5.0 ps−1.

In MDFF, two external potentials (UEM, Uα) are added to the usual MD potential (UMD), where UEM is derived from the gradient of EM density, and Uα enforces harmonic restraints to preserve secondary structure elements such as α-helices and β-strands. Only the protein atoms are subject to the external potentials. The parameters for UEM and Uα are chosen to be ξ = 0.3 kcal mol−2 and kα = 300 kcal mol−2 Å−2, as recommended in ref 53. We have not seen structural distortions which may be caused by high EM-map forces (see Figure 1). To allow local flexibility in loop L11 and active site, we do not enforce secondary structure restraints for residues of L11, switch I, switch II, and P-loop.

**Model Equilibration by MD.** Following the MDFF simulation, we relax and equilibrate all models without the EM-map forces:

First, a 2000-step energy minimization is carried out using the steepest descent method, with harmonic restraints (force constant = 1 kcal/mol/Å2) applied on the backbone atoms of all residues except P-loop, switch I, switch II, and loop L11 (residues 85–92, 190–204, 231–236, 237–249). These restraints maintain the global backbone conformation of kinesin optimized for EM-map fitting while allowing relaxation of all side chain coordinates and key loops in MT-binding interface and active site.

Second, the systems are gradually heated to 300 K over 100 ps, and then subjected to a 2-ns constrained MD simulation performed in NPT ensemble. Three MD simulations are run starting from each MDFF-generated model, resulting in 3 trajectories for ADP/ATP state, and 18 trajectories for APO state. The Nosé–Hoover method 72 is used with temperature T = 300 K and pressure P = 1 atm. Periodic boundary conditions are applied to the systems. A 10-Å switching distance and a 12-Å cutoff distance are used for nonbonded interactions. The bonds involving hydrogen atoms of water are considered rigid, and a time step of 1 fs for MD simulations is used. The snapshots of the systems are saved every 1 ps during MD simulations for later analysis.

The energy minimization and MD simulation are conducted with the NAMD program 65 using the CHARMM27 force field. 66

**Hydrogen-Bond Analysis.** Hydrogen bonds are identified with the CORMAN command of CHARMM program 73 using the following criteria: first, the maximum distance between acceptor and hydrogen is 2.5 Å; second, the minimum angle of donor–hydrogen–acceptor is 90°. 74, 75

We follow a two-step procedure for hydrogen bond analysis:

First, for the last 500 ps of each MD trajectory in a given state (ADP, ATP, or APO state), we calculate the hydrogen-bond occupancy (defined as the percentage of snapshots where a given hydrogen bond is formed) and only consider those hydrogen bonds with occupancy > 0.3.

Second, we sum up the hydrogen-bond occupancy for each residue pair over all trajectories in a given state, and then divide it by the number of trajectories. Since multiple hydrogen bonds may form between two residues, the average occupancy for a residue pair may exceed 1.
Calculation of Kinesin–MT Binding Free Energy. Following our previous paper, we calculate the kinesin–MT binding free energy $\Delta G$ in three states (ADP, ATP, and APO state). We extract 10 snapshots of the last 500 ps of MD trajectories in each state. For each snapshot, we calculate $\Delta G$ using a continuum solvent model (see ref 76), which expresses $\Delta G$ as $\Delta G_{\text{exp}} = \alpha E_{\text{vdw}} + \beta E_{\text{elec}}$. Here the nonpolar contribution $\Delta G_{\text{vdw}} = \alpha E_{\text{vdw}}$ is empirically written as a fraction ($\alpha < 1$) of the van der Waals (vdW) interaction energy $E_{\text{vdw}}$ between kinesin and tubulin dimer, and the electrostatic contribution $\Delta G_{\text{elec}} = \beta E_{\text{elec}}$ is written as a fraction ($\beta < 1$) of the electrostatic energy $E_{\text{elec}}$ from unbound kinesin and MT to kinesin–MT complex. $E_{\text{elec}}$ is calculated using the Poisson–Boltzmann (PB) method, which expresses trajectories in each state. For each snapshot, we calculate $\Delta G$ using mutational data in our previous paper. The average and standard error of $\Delta G$ are calculated for all snapshots collected in each state following ref 44.

Estimation of Kinesin–MT Binding Free Energy from Binding Experiments. Ma and Taylor measured the dissociation constants ($K_D$) of kinesin construct K332 in the presence of ADP ($K_D \sim 16 \mu M$ with 10 mM NaCl buffer), ADP-AlF$_4$ ($K_D \sim 1 \mu M$ with 50 mM NaCl + NaF buffer), and under nucleotide-free conditions ($K_D \sim 0.7 \mu M$ with 100 mM NaCl buffer), which correspond to the ADP state, ATP state, and APO state, respectively. As observed by Ma and Taylor, the dependence of logarithm of $K_D$ on ionic strength is essentially linear up to 100 mM NaCl and the slopes are independent of nucleotide. Therefore, one can estimate the binding affinity of kinesin under the same ionic condition ($10 \text{mM NaCl buffer}$) using $\Delta G_{\text{exp}} = k_BT \ln(K_D)$ (temperature $T = 22 ^\circ C$, $k_B$ is Boltzmann constant) and obtain $-6.5 \text{ kcal/mol}$ in the ADP state, $-9.7 \text{ kcal/mol}$ in the ATP state, and $-8.3 \text{ kcal/mol}$ in the APO state. The differences in $\Delta G$ are essentially independent of ion concentration.

RESULTS AND DISCUSSION

Structural Modeling of ADP, ATP, and APO State of Kinesin. We have structurally modeled the ADP, ATP, and APO state of human conventional kinesin motor domain. For the ADP state, we used a crystal structure of ADP-bound kinesin (PDB id: 1BG2), where the $\alpha 4$ helix is extended by $\sim 2$ turns to improve the fitting of the EM map (see Methods).

For the ATP state, we used another crystal structure of ADP-bound kinesin (PDB id: 1MKJ) where the $\alpha 4$ helix undergoes both rotation and translation relative to 1BG2, allowing the neck linker to dock with kinesin motor domain. However, this structure lacks ATP-like structural features in the active site (such as a closed switch-I conformation). Therefore, we have used an AMPPNP-bound Eg5 structure (PDB id: 3HQD) as a template to remodel switch I, $\alpha 4$ helix, and loop L11 (see Methods).

The modeling of APO state is more challenging for the lack of a nucleotide-free crystal structure of kinesin (kinesin is structurally unstable in the absence of nucleotide and MT, see ref 79). To meet this challenge, we have constructed six kinesin models (see Methods) and used them as initial models to flexibly fit into the EM map of kinesin-decorated MT in the APO state (see below). For five of the six kinesin models, the switch I region is remodeled to adopt a closed conformation, which offers a better fit to the EM map in the APO state featuring a bridge between the densities of switch I and P-loop.

Flexible Fitting of EM Maps in the ADP, ATP, and APO State. In a previous study, Sindelar and Downing performed a rigid fitting of kinesin and tubulin dimer into the EM maps of kinesin-decorated MT. They solved three EM maps in the presence of ADP, ADP-AlF$_4$, and in the absence of nucleotide, which correspond to the ADP, ATP, and APO state, respectively. The unprecedentedly high resolutions (8–9 Å, see ref 29) allow them to not only determine the orientation of kinesin on MT, but also resolve secondary structures like $\alpha$-helices. They found that the kinesin crystal structure does not fit well with the EM densities in some regions, especially in active site and kinesin–MT interface, which was attributed to conformational changes induced by the binding of kinesin with MT.

To model the MT-induced conformational changes in kinesin, we have employed the MDFF program to flexibly fit the kinesin models in three states (ADP, ATP, and APO state) and tubulin dimer into corresponding EM maps of kinesin-decorated MT. Following ref 29, we first fit kinesin and tubulin dimer as two rigid bodies into each EM map using the Chimera program. Then we performed three independent MDFF simulations starting from each rigidly fitted model, and kept the final frame of the simulation that best fits the EM map. We also checked to make sure that the three MDFF runs yield structurally converging models. To evaluate fitting quality, we have calculated the local cross-correlation coefficient (CCC) for EM densities within the molecular envelope of kinesin (see Table S1, Supporting Information).

The MDFF simulations have generated models that fit better with the EM maps. The CCC values increase from 0.636 to 0.721 for ADP-state map, from 0.664 to 0.774 on average for APO-state map, and from 0.704 to 0.742 for ATP-state map. The better fitting for the APO and ATP state than the ADP state may be attributed to the stronger binding between kinesin and MT in the APO and ATP state than the ADP state. Then we visually compare the fitting of EM densities by both rigidly fitted models and MDFF-generated models (see Figure 1):

For the ADP state (see Figure 1a), we have observed extensive structural changes within kinesin after flexible fitting. $\alpha 3$ helix and switch I move upward (away from MT), causing the N-terminal part of switch I to move closer to ADP which remains exposed to solvent. Switch I partially sticks out of EM densities, suggesting its high flexibility. The two-turn helix in switch I is preserved during MDFF simulation. Similar to ref 29, the extended $\alpha 4$ helix fits well into the EM map. However, loop L11 is mostly out of EM densities, which points to its high flexibility in the ADP state consistent with weak kinesin–MT binding in the ADP state. Other conformational changes have been observed in P-loop, $\alpha 2$ helix, $\alpha 6$ helix, loop L2, and loop L7.

For APO state (see Figure 1b–g), we have generated six kinesin models with MDFF after remodeling switch I using different kinesin structures as template (see Methods). Despite large structural variations in switch I, all models share the common feature of switch I and P-loop moving toward each other to fill the bridge density between them. As a result, the active site becomes more closed, although its local conformation cannot be uniquely determined by fitting EM densities. The extended $\alpha 4$ helix fits well into the EM densities after moving downward (toward MT) together with loop L11, which may lead to stronger binding between kinesin and MT in the APO state.

For the ATP state (see Figure 1h), several structural changes within kinesin have been observed after flexible fitting. For example, switch I moves closer to ATP. The first turn of
extended α4 helix is partially unwound to allow better fitting of loop L11 into EM densities.

To assess the conformational stability of MDFF simulations, we have calculated the root mean squared deviation (rmsd) for all backbone atoms relative to the rigidly fitted models (see Figure S1, Supporting Information). For all MDFF runs, the RMSDs are saturated near 1.5−2.0 Å within 2 ns of simulation time.

We have calculated the kinesin−MT contact surface area using the VMD program. The flexible fitting has greatly increased the contact surface area for all three states (from 2151.8 Å² to 2607.4 Å² for ADP state, from 2090.2 Å² to 2741.0 Å² on average for APO state, from 2224.5 Å² to 2652.1 Å² for ATP state). The observation of contact surface area ascending in the order of ADP, ATP, and APO state agrees with the experimental observation of kinesin−MT binding affinity increasing in the same order (see below).

**Analysis of Conformational Changes among the ADP, APO, and ATP State.** Following the flexible fitting, we have compared the kinesin models in three states (ADP, APO, and ATP state), which has revealed significant conformational changes accompanying kinesin−MT binding, ADP release, and ATP binding.

To explore the global and local motions of kinesin relative to MT, we superimpose all the MDFF-generated models along the tubulin dimer.

We first compared the ADP and ATP state. As found previously,29 kinesin undergoes a rotation of ~13° from the ADP to ATP state. This rotation was also observed in other kinesins such as mouse KIF1A,26 *Caenorhabditis elegans* UNC10440 and rat conventional kinesin,81 with the angle varying from 5° to 20°. It remains controversial whether this rotation contributes to the unidirectional movement of kinesin along MT.52,82 The above rotation can be decomposed into two rotations: first, kinesin rotates in the counterclockwise direction as viewed from the top of kinesin (see Figure 2a); second, kinesin tilts like a seesaw in the counterclockwise direction as viewed along the MT axis (see Figure 2b). Sindelar proposed that the seesaw motion controls the allosteric coupling between active site closing and neck linker docking.29 Indeed, the seesaw motion is coupled to an upward movement of the right side of kinesin where neck linker is docked (see Figure 2b). Therefore, it may be induced by a force that pulls the neck linker away from MT. Indeed, an upward load was found to increase the stepping rate of kinesin motor.64 Besides the above rotation of kinesin, we have also found a translation of kinesin to the left side by ~3.3 Å (see Figure 2a), which may allow the control of ADP-ATP exchange by a sideways pulling force.85

Next, we compare the ADP and APO states. Similar to ref 29, we have observed a small rotation (~1.4° on average) from the ADP to APO state, which is accompanied by a small translation (~1.2 Å on average) toward the minus end of MT (see Figure 2a). Such a backward translation of kinesin may allow a backward pulling force to induce the transition from the weak-to strong-binding state. This is consistent with a single-molecule study that found the unbinding force obtained by loading directed toward the minus end of MT was 45% greater than that for plus-end-directed loading.86 Therefore, the strain developed within a kinesin dimer could kinetically coordinate the two heads such that the front head (under backward strain) stays bound to MT while the trailing head can detach.

Besides the global motion of kinesin, we have also observed local changes in the kinesin−MT interface (see Figure 2c). For example, loop L7 moves closer to microtubules in the APO state (relative to ADP state), which may enable strong binding between kinesin and microtubules. Loop L8 moves slightly away from microtubules in the ATP state (relative to ADP and APO state). α4 helix moves slightly downward from the ADP state to the APO and ATP state, which is accompanied by a leftward shift and local restructuring of loop L11. α6 helix moves to a new position in the APO state (relative to ADP and ATP state), which is associated with a subdomain rotation within kinesin (see below).

To explore the tertiary structural changes within kinesin, we superimpose all the MDFF-generated kinesin models with a crystal structure of ADP-bound kinesin (PDB id: 1BG2) along the central β-sheet (residues 8−15, 79−84, 126−138, 205−216, 222−231, 295−302). In addition to previously discovered structural changes between the ADP and ATP state14,17 (such as the rotation and translation of α4 helix, docking of neck linker, etc.), we have found that a subdomain of kinesin (comprising of residues 20−49 and 306−320) rotates by ~3.2°, 6.6°, and 9.0° in the ADP, ATP, and APO state (relative to 1BG2, see Figure 2d). Notably, this subdomain adopts a new conformation in the APO state instead of an intermediate conformation between the ADP and ATP state. The involvement of loop L2 and α6 helix in this subdomain rotation hints at a functional role in modulating kinesin−MT binding.

To explore the nucleotide-dependent structural changes in the active site (consisting of three conserved motifs: P-loop, switch I, and switch II), we superimpose all the MDFF-generated kinesin models with a crystal structure of ADP-bound kinesin (PDB id: 1BG2) along the P-loop, then we examine the structural motions of switch I and switch II relative to ADP and ATP state, which is accompanied by a leftward shift and local restructuring of loop L11. α6 helix moves to a new position in the APO state (relative to ADP and ATP state), which is associated with a subdomain rotation within kinesin (see below).

Figure 2. Conformational changes among the ADP, APO, and ATP states: (a) top view of the global motions of kinesin relative to MT (the rotation from the ADP to ATP state is shown by a red curved arrow, the translation from the ADP to APO/ATP state is shown by a green/red block arrow); (b) side view of the seesaw motion of kinesin relative to MT (shown by curved arrows); (c) bottom view (with MT removed) of the movements of structural elements involved in kinesin−MT binding (including loop L7, L8, L11, and α4, α5, α6 helix); (d) subdomain rotation within kinesin in the ADP, APO, and ATP state (relative to a crystal structure of kinesin colored silver). Models of the ADP, APO, and ATP state are colored blue, green, and red, respectively. Same color scheme applies to rotational axes in panels (a), (b), and (d). The viewing directions of panel (a−c) are shown in the upper-right cartoon.
to the P-loop (see Figure 3a). Since the P-loop forms the most extensive interactions with the bound nucleotide, a P-loop-centric view allows us to probe relative motions between switch I/II and the nucleotide. Among the three active-site motifs, switch I undergoes the largest structural changes as kinesin proceeds from the ADP state to APO state and then to the ATP state in the presence of microtubules (see Figure 3a):

a. In the ADP state, the N-terminal part of switch I clamps downward (toward P-loop) upon binding with microtubules, which is consistent with the EPR finding that the active site is closed after the ADP-bound kinesin binds with MT.  

b. In the APO state, switch I shifts leftward (toward switch II) and its N-terminal part is highly flexible, which may enable ADP to exit and ATP to enter the active site.

c. In the ATP state, switch I shifts rightward to tightly close the active site, which allows ATP hydrolysis to occur.

Our finding echoes the observation of considerable variations in switch I among kinesin crystal structures with bound ADP, particularly in the N-terminal part (loop L9). Switch I also undergoes a change in secondary structure from a two-turn helix flanked by a short loop in the ADP state to an extended loop in the ATP state (see Figure 3a). We have also observed smaller motions of switch II coupled to switch-I motions, which may further fine-tune the opening/closing of the active site (see Figure 3a).

The active-site motifs are highly conserved between kinesin and myosin. For both kinesin and myosin, switch I and switch II are thought to move closer to the nucleotide in the prehydrolysis state, forming interactions with the γ-phosphate of ATP to enable ATP hydrolysis. Indeed, our modeling has revealed intriguingly similar structural changes in switch I and switch II between kinesin and myosin. Similar to kinesin, switch I of myosin shifts leftward upon ADP release and then shifts rightward to close the active site for ATP hydrolysis (see Figure 3b), and switch II of myosin also undergoes similar changes with larger scale.

**Analysis of Hydrogen Bonds in the ADP, APO, and ATP State.** Following the MDFF simulations, we have performed MD simulations to relax and equilibrate the local conformations of active-site motifs (P-loop, switch I, and switch II) and loop L11, while restraining the backbone atoms of the rest of system (see Methods). Three 2 ns-long MD simulations have been conducted starting from each MDFF-generated model, resulting in 3, 3, and 18 MD trajectories for the ADP, ATP, and APO state. The backbone RMSDs achieve equilibrium within 2 ns of simulation time (see Figure S2, Supporting Information).

On the basis of the last 500 ps of MD simulations, we have analyzed the hydrogen bonds (HB) (including salt bridges) formed in the ADP, or ATP or APO state with an average occupancy >0.3 (see Methods). We focus on two groups of HBs: those involving residues of active-site motifs (P-loop, switch I and switch II) and those between kinesin and MT. The results are shown in Table 1 and Figure 4.

**HBs Involving Active-Site Motifs.**

- **a. HBs between Kinesin and Ligand.** In the ADP and ATP state, the residues that form HBs with the ligand are mainly from P-loop (residues 88–93). In the ADP state, an HB forms between ADP and S201 of switch I as a result of switch I closing (see Figure 4a). In the ATP state, one HB forms between N198 of switch I and ATP (see Figure 4c). Two HBs were thought to be important for ATP hydrolysis: one is between S202 of switch I and Pi, and the other is between G234 of switch II and Pi. In our MD simulations in the ATP state, we have observed the first HB (with occupancy ~0.2) but not the second one. We note that the distance between atom N of G234 and Pi oxygen is only ~5 Å, so a small displacement of 1−2 Å will bring them within HB distance.

- **b. HBs within Each Active-Site Motif.** Within switch I, 8 and 7 HBs are formed in the ADP and ATP state, respectively. However, only one remains in the APO state due to high flexibility in switch I. Within P-loop, 1, 2, and 2 HBs are formed in the ADP, APO, and ATP state, respectively. Within switch II, two HBs are formed in the ATP state. Some of these HBs may be required to maintain the closure of the active site for nucleotide binding. For example, an HB forms between R190 and S202 of switch I in the ATP state (see Figure 4c), whose disruption by R190A mutation was found to cause reduced motility.

- **c. HBs between Active-Site Motifs.** Between switch II and P-loop, 1, 4, and 2 HBs are formed in the ADP, APO, and ATP state, respectively. For example, an HB forms between E236 and T87 (see Figure 4a–c), which locks switch II in a closed conformation. The occupancy of this HB is low in the APO state but high in the ADP and ATP state, which is consistent with our observation that switch II further opens in the APO state relative to the ADP and ATP state (see Figure 3a).

Between switch I and II, 1 and 2 HBs are formed in the APO and ATP state, respectively. For example, a salt bridge forms between R203 and E236 in the APO and ATP state (see Figure 4b,c). This salt bridge was thought to be essential to maintain a closed active site for hydrolysis and its breaking is required for Pi release. In agreement with this proposal, a mutation R210A in *Drosophila* kinesin (corresponding to R203 of kinesin) caused defective ATPase, and the mutations E237A and E237D in rat kinesin (corresponding to E236 of kinesin) reduced or abolished MT-dependent ATPase.

- **d. HBs between Active Site and Loop L11.** In the ADP state, loop L11 forms 1 HB with switch II, and 2 HBs with switch I. For example, a salt bridge forms between R203 of switch I and E250 of L11 (on the extended α4 helix) (see Figure 4a), which was proposed to modulate the seesaw motion in ref 29. This salt bridge also forms (with lower occupancy) in the APO state (see Figure 4b) but not in...
In fact, hydrophobic interactions contribute more than electrostatic interactions to kinesin–MT binding in the ATP state. The importance of this salt bridge, a mutation E250A resulted in defective MT-activated ATPase. In KIF1A, it was observed that the correspond- ing salt bridge between R216 and E267 stabilizes a raised α helix. Although L11 is involved in all three states, one HB (between N255 of L11 and M413 of kinesin) that blocked MT stimulation of ADP release as supported by two decoupling mutations N650K and N255S. In agreement with our observation that L8 changes little between the ADP and APO state than the ATP state, which is consistent with our finding, it was found that triple-alanine substitutions in L8 (E157A/K159A/Y164A) only forms in the APO and ATP state (see Figure 4b,c), which may contribute discriminatively to strong-binding states.

4, 5, and 1 HBs form between loop L8 (residues H156, E157, K159, R161) and β-tubulin in the ADP, APO, and ATP state, respectively. L8 is more involved in kinesin–MT binding in the ADP and APO state than the ATP state, which is consistent with our observation that L8 changes little between the ADP and APO state and moves away from MT in the ATP state (see Figure 2c). In agreement with our finding, it was found that triple-alanine substitutions in L8 (E157A/K159A/Y164A) only caused slight reduction to the MT gliding velocity, suggesting that L8 is not essential for MT activation of ATPase activity.

One HB forms between K141 of loop L7 and E159 of β-tubulin in the ATP state (see Figure 4b,c), which may contribute discriminatively to strong-binding states.
the finding that mutation R153A of KIF1A (corresponding to K141 of kinesin) caused impaired MT-activated ATPase.15

One HB forms between K44 of loop L2 and E423 of α-tubulin in the ADP and APO state (see Figure 4a,b), supporting its role in MT binding as found by EM21 and computation.44

2, 3, and 2 HBs form between α6 helix (residues S310, K313, S314, R321) and α-tubulin in the ADP, APO, and ATP state, respectively. For example, an HB forms between R321 of α6 helix and E415 of α-tubulin in all three states (see Figure 4a,b,c). A mutational study of MT identified E415 of α-tubulin as important for coupling MT binding and ADP release,96 and the binding partner of E415 was proposed to be K237 of kinesin. In our models, the binding partner of E415 is R321, while K237 interacts with E414 and E417 of α-tubulin.

The functional importance of R321 was shown in a recent mutational study.97 Many of the above residues were also identified as important to kinesin−MT binding by both experimental and computational mutagenesis studies.44,87

**Calculation of Kinesin−MT Binding Free Energy in the ADP, APO, and ATP state.** Biochemical measurements of kinesin−MT binding/dissociation were conducted under different nucleotide conditions.8,18 It was found that the binding affinity increases in the order of ADP, ATP, APO state, with a difference of 3.2 kcal/mol between the APO and ADP state, and 1.8 kcal/mol between the ATP and ADP state (see Methods). These results impose an energetic constraint on the structural models of the three states.

To validate our models using the binding data, we have calculated the kinesin−MT binding free energy (ΔG) using an MD-based protocol introduced in our previous paper.44 In this protocol, we express ΔG in terms of the van der Waals interaction energy between kinesin and MT, and the electrostatic energy calculated using the Poisson−Boltzmann method.44 Because of high uncertainty in entropy calculation, we do not include the entropic contribution to ΔG, which results in a negative shift of ΔG by 7−15 kcal/mol.44 The calculated ΔG for ADP, APO, and ATP state is −18.98 ± 0.44, −21.91 ± 0.18, and −21.54 ± 0.35 kcal/mol, respectively (see Table S2, Supporting Information). Therefore, our calculation indicates that the binding affinity in the APO and ATP state is higher than the ADP state by 2.9 and 2.5 kcal/mol, respectively. This is in good agreement with experimental results, which supports the validity of our models.

To probe the physical origin of stronger kinesin−MT binding in the APO and ATP state than the ADP state, we have conducted a breakdown of ΔG into polar and nonpolar contributions. The polar or electrostatic contribution to ΔG in the ADP, APO, and ATP state is −5.74, −5.47, and −5.09 kcal/mol, respectively. The nonpolar or van der Waals contribution to ΔG in the ADP, APO, and ATP state is −13.24, −16.43, and −16.45 kcal/mol, respectively. Therefore, stronger nonpolar interactions are responsible for the stronger kinesin−MT binding in the APO and ATP state than the ADP state, while electrostatic interactions are most involved in the weak-binding ADP state.

**Relating Our Modeling to MT-Activated ADP Release.** Our structural modeling of kinesin complexed with MT in the
ADP and APO state has shed new light on the structural mechanism of MT-activated ADP release. Our modeling of the APO state has revealed a structurally flexible switch I, which adopts a closed conformation after a pronounced displacement (relative to the ADP state). Our finding supports the proposal of MT-induced switch-I displacement that perturbs Mg coordination and thereby stimulates the release of MgADP. In addition, our modeling predicts a slight opening of switch II in the APO state, which may further destabilize MgADP coordination by switch II. Unlike switch I and II, the P-loop remains relatively rigid in the APO state although it moves relative to MT.

How does MT binding trigger the movements of switch I and II in the active site? We have identified a number of HBs which may mediate the signaling from MT-binding residues (such as K141 of loop L7, K237 and N255 of loop L11) to active-site residues (such as R203 of switch I, E236 of switch II). Alternatively, an HB between R203 of switch I and E414 of α-tubulin may directly couple MT and the active site. The functional importance of R203, E236, and N255 was demonstrated by mutational studies.

**CONCLUSION**

In conclusion, we have performed all-atom structural modeling of three main biochemical states of a kinesin complexed with MT under the constraint of high-quality cryo-EM maps. In addition to the ADP and ATP state captured by previous crystallographic studies, we have modeled the structurally unknown APO state which features a subdomain rotation involving loop L2 and α6 helix, and local changes in the active site similar to the myosin motor. We have identified a list of hydrogen bonds involving key residues in the active site and the binding interface between kinesin and MT. Some of these hydrogen bonds may mediate the coupling between MT binding and ATPase activities. We have validated our models by calculating the binding free energy between kinesin and MT, which quantitatively accounts for the observation of strong binding in the APO and ATP state and weak binding in the ADP state.

**ASSOCIATED CONTENT**

1. Supporting Information

Table S1: The cross-correlation coefficients for all models built by rigid fitting and flexible fitting.

Table S2: Results of binding free energy calculation between kinesin and MT.

Figure S1: The backbone rmsd for MDFF simulations.

Figure S2: The backbone rmsd for MD simulations.

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**ABBREVIATIONS USED**

ADP, adenosine diphosphate; ATP, adenosine triphosphate; CCC, cross-correlation coefficient; EM, electron microscopy; HB, hydrogen bond; Pi, inorganic phosphate; MT, microtubule; MD, molecular dynamics; PDB, Protein Data Bank; rmsd, root mean squared deviation

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