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Multiple Conformations of the Nucleotide Site of Kinesin-Family Motors in the Triphosphate State

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Abstract

Identifying conformational changes in kinesin-family motors associated with nucleotide and microtubule binding is essential to determining an atomic-level model for force production and motion by the motors. Using the mobility of nucleotide-analog spin probes bound at the active sites of kinesin-family motors to monitor conformational changes, we previously demonstrated that in the ADP state the open nucleotide site closes upon microtubule (MT) binding¹. We now extend these studies to kinesin-1 (K) and ncd motors in ATP and ATP-analog states. Our results reveal structural differences between several triphosphate and transition-state analogs bound to both kinesin and ncd in solution. The spectra of kinesin/ncd in the presence of SLADP•AlFx/BeFx and kinesin with the mutation E236A (K-E236A, does not hydrolyze ATP) bound to ATP show an open conformation of the nucleotide pocket similar to that seen in the kinesin/ncd•ADP states. In contrast, the triphosphate/analog K•SLAMPPNP and K-E236A•SLAMPPNP induce a more immobilized component of the EPR spectrum, implying a closing of the nucleotide site. The MT-bound states of all of the triphosphate-analogs reveal two novel spectral components. The equilibrium between these two components is only weakly dependent on temperature. Both components have lower mobility than those observed in MT-bound diphosphate states. Thus the closing of the nucleotide pocket when the diphosphate state binds to MTs is amplified in the triphosphate state, perhaps promoting accelerated ATP hydrolysis. Consistent with this idea, molecular dynamics simulations show a good correlation between our spectroscopic data, X-ray crystallography, and electron microscopy of MT-bound triphosphate analog states.

Keywords

kinesin; ncd; electron paramagnetic resonance spectroscopy; spin probes; Switch 1

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Introduction

Kinesin-family motors use the free energy from ATP hydrolysis to generate mechanical forces for unidirectional movement along microtubules. Identifying the conformational changes in the motors associated with the conversion of chemical free energy into mechanical work remains a crucial step in understanding how these motors function. The original X-ray structures of conventional kinesin² and *ncd*³ motors showed significant structural homology, especially at the nucleotide site, despite their opposite directionality of movement. The nucleotide site of kinesin-family motors shares the P-loop, Switch 1 and Switch 2 motifs with myosin-family proteins and with the G-proteins, suggesting evolution from a common ancestor protein⁴. While subsequent studies suggested differences in the function of the peripheral elements associated with stepping⁵⁻⁷, X-ray crystallography of kinesin-family motors continued to suggest structural homology at the nucleotide site⁸⁻¹⁵. The nucleotide site is where hydrolysis must be coordinated with any conformational changes at the periphery that result in translocation. Therefore, identification of nucleotide-site conformational changes is essential to understanding how kinesin-family motors function.

In the X-ray structures of kinesin² and *ncd*³, the bound nucleotide was seen to rest in a very open, trough-like nucleotide pocket. Conversely, in the initial X-ray structures of myosin with bound nucleotide, the nucleotide phosphates were tightly enclosed in a “phosphate tube”¹⁶ comprised of the two switch motifs and the P-loop¹⁷⁻¹⁹. Comparison of these structures showed the opening of the nucleotide-pocket to result from a displacement of Switch 1 away from the phosphates in the kinesin-family structures relative to that seen in the myosin structures. This led us to show using molecular dynamics simulations that the kinesin-family motors could have a more closed-Switch 1 conformation not seen in the X-ray structures with either a diphosphate or triphosphate substrate at the nucleotide site²⁰. Subsequent electron paramagnetic resonance (EPR) spectroscopy of a series of diphosphate nucleotide-analog spin probes bound at the active site of kinesin-family motors consistently reported a decrease in probe mobility when these motors bound to microtubules (MTs)^{1,21}. An EPR probe placed directly on Switch 1 likewise reported conformational changes in Switch 1 when kinesin bound to microtubules²². Taken together, the above results imply the involvement of Switch 1 in the conformational changes associated with the closing of the nucleotide pocket. Subsequent cryo-electron microscopy studies of the kinesin•microtubule complex²³ and the X-ray structure of Eg5•AMPPNP also found closed-Switch 1 conformations identical to the closed conformation originally demonstrated by molecular dynamics simulations²⁰. Figure 1 shows the relationship between the open-Switch 1 domain in the X-ray structure of kinesin (ref.², PDB ID 1BG2) and the closed phosphate-tube domain of Minehardt and co-workers (ref.²⁰, PDB ID 1SZ4). There is considerable structural homology at the nucleotide site except for Switch 1, which undergoes a loop to α -helix–loop transition to open the nucleotide pocket.

X-ray structures of kinesin-family motors have been solved with bound ADP, ADP•AlFx and AMPPNP^{8-10,15,24-26}. Of particular relevancy, structures of the kinesin-family motor, kif1a, show a more open nucleotide pocket in the AMPPNP and ADP•AlFx triphosphate/analog states¹⁵. The X-ray structure of the kif1a•AMPPCP also showed a slightly more

closed, but still very open Switch 1 domain¹¹. Figure 2 further demonstrates the considerable spread of the Switch 1 domain in the existing structures.

With such extensive variability in these structures, the questions remain as to what is the structure of the triphosphate state in kinesin-family motors, and what is its dependence on the presence or absence of microtubules? Structural studies are confounded by several issues. X-ray diffraction provides high resolution, but only of a single structure fixed in space. Additionally, the structure of the microtubule-bound states continues to elude investigators. Electron microscopy can resolve the nucleotide-site of a microtubule-bound state, but not the microtubule-free state for control comparison. EPR spectroscopy provides lower resolution information. However in the present context, EPR spectroscopy has significant advantages in that it can be used under a wide variety of conditions, both in the presence and absence of microtubules. EPR spectroscopy can also resolve multiple protein conformations in equilibrium.

Here we extend previous EPR studies to investigate conformational changes at the nucleotide site of kinesin and *ncd* with triphosphate/analog spin probes at the nucleotide site. We show that in the absence of microtubules, EPR probe mobility is independent of whether the bound nucleotide is in the SLADP state, or in the presence of the SLADP•BeFx or SLADP•AlFx triphosphate-analog states. Probe mobility in the kinesin (K) K•SLAMPPNP state decreases, showing that this analog promotes a more closed nucleotide site, as observed for the Eg5•AMPPNP crystal structure²⁷. In the MT•motor•triphosphate state, there is again a closing of the nucleotide pocket as measured by a decrease in EPR probe mobility. However, the probes are more immobilized than in the MT•motor•diphosphate state, again suggesting a further closing of the nucleotide pocket in the triphosphate state. Molecular dynamics simulations demonstrate that the EPR spectra correlate with existing X-ray structures, bridging the gap between the two distinct data sets. In particular, we are able to connect conformational states observed by spectroscopy with specific high-resolution structures.

Results

The phosphate analogs AlFx and BeFx do not alter the conformation of the nucleotide site of kinesin-family motors in the absence of microtubules

ATP and SLATP are hydrolyzed by kinesin-family motors¹, which has led to the use of the diphosphate species bound in conjunction with aluminum and beryllium metallofluorine complexes as stable analogs of the triphosphate state. However, AlFx (an analog of the hydrolysis transition state intermediate) and BeFx (with AMPPNP, an analog of the ATP state with tetrahedral coordination) represent different conformations of the γ -phosphate moiety. Fig. 3A shows the EPR spectra obtained from the K•2',3'-SLADP state. Fig. 3B shows the triphosphate-analog K•2',3'-SLADP•AlFx state. In Fig. 3, the three central peaks (P2-P4) are primarily signal from free probe tumbling rapidly in solution. When the nucleotide-analog spin probe binds at the active site, probe mobility becomes restricted by the adjacent surface of the protein. This more restricted mobility of the probe bound at the nucleotide site results in a broadening of the spectrum and the presence of the additional P1 and P5 components of the spectrum. The spectrum in the K•SLADP•AlFx state is virtually

identical to that of the $K \bullet SLADP$ state. Using EPR probe mobility as our reporter, the observation of no significant change in the mobility of the probe between the diphosphate and triphosphate-analog state implies no conformational change at the nucleotide site in the transition to this triphosphate-analog state. The concentration of metallofluoride complex used in these experiments (2mM) is twice that used by Nitta et al.¹⁵ for the solution of the X-ray structure of the kinesin-family motor $kif1a \bullet ADP \bullet AlF_3$. In order to test for saturation, the concentration was raised to 4 mM. Identical spectra were obtained (data not shown). The spectrum of the $K \bullet 2',3'-SLADP \bullet BeFx$ state was virtually identical to that of the $K \bullet 2',3'-SLADP \bullet AlFx$ state, again arguing for no conformational change in the transition to this triphosphate analog state (data not shown, also see Table 1). Similar conclusions were reached comparing $ncd \bullet 2',3'-SLADP$ and $ncd \bullet 2',3'-SLADP \bullet BeFx/AlFx$, see Figure 4 and Table 1. The results were independent of whether the $AlFx/BeFx$ species were added to a buffer containing $2',3'-SLADP$ or $2',3'-SLATP$. Additionally, the analog $2'-SLADP$ gave similar results for both kinesin and *ncd*, showing that our conclusions are not dependent on the specific EPR probe used.

Modeling probe mobility

EPR probe mobility can be modeled as motion in a cone of revolution^{28,29} with the vertex angle of the cone determined by the magnitude of the splitting between the P1 and P5 components of a spectrum. The smaller is the splitting, the larger is the cone angle of mobility. Although the restricting protein surface is most certainly not a true cone of revolution (Figs. 1, 2), the analysis can still serve as a useful approximation to relate the change in the magnetic field variable into a quantitative physical representation of the magnitude of the conformational change we are observing. The splitting observed here for kinesin (~ 4.3 mT) corresponds to motion in a cone with a vertex angle of $\sim 134^\circ$, with a similar result for *ncd* (~ 4.8 mT, $\sim 117^\circ$). Thus the spectra imply more open nucleotide pockets for both motors in both the diphosphate and the triphosphate-analog states. Spectral splittings and cone angles for all observations are summarized in Table 1. For brevity, subsequent values in the text will be given as splitting (mT), cone angle ($^\circ$).

Comparison of the results with $ADP \bullet AlFx/BeFx$ with a true ATP state

$ADP \bullet AlFx$ and $ADP \bullet BeFx$ are ATP analogs. Additional confidence in our results can be obtained using other triphosphate-state species. Kinesin with the mutation E236A (*K-E236A*) binds ATP but cannot hydrolyze ATP⁶. Thus this mutant allows us to observe the nucleotide-pocket conformation of ATP-bound kinesin. Fig. 3 compares the spectra of the $K \bullet 2',3'-SLADP$ and $K \bullet 2',3'-SLADP \bullet AlFx$ states with the spectrum from *K-E236A* $\bullet 2',3'-SLATP$ (Fig. 3C). All three of these states show an open nucleotide site, with similar low-field to high-field splittings (Table 1).

The nucleotide pocket of kinesin in solution closes upon binding the triphosphate analog AMPPNP

AMPPNP is an analog of the tetrahedral coordination of the oxygens at the γ -phosphate of ATP and has been used extensively to stabilize a kinesin $\bullet$ triphosphate state. It is hydrolyzed by kinesin-family motors³⁰, but much too slowly to impact any of our conclusions. The spectra of the ATP-analog states *K-E236A* $\bullet 2',3'-SLAMPPNP$ (Fig. 3E) and $K \bullet 2',3'-$

SLAMPPNP (Fig. 3F) both show components with an increased splitting (Table 1) relative to the ADP•AlFx/BeFx states. Thus in these triphosphate states, there is a significant (60°-70°) decrease in the effective cone angle of mobility, or equivalently a closing of the nucleotide site. The spectrum of K-E236A•2',3'-SLAMPPNP also contains a more mobile component, which is partly obscured by the P2 and P4 components that arise from free probe in solution, and has a splitting comparable to that obtained from the diphosphate state. The difference in splittings between this more mobile component (4.88 mT) and the mobile component in the spectrum of K•2',3'-SLADP (4.31 mT) indicates the mobile component in the spectrum of K-E236A•2',3'-SLAMPPNP is not from contamination of more tightly binding SLADP in the SLAMPPNP. AMPPNP binds only very weakly to kinesin. Thus the P2-P4 components of Fig. 3F corresponding to free probe are much larger than in the other panels and we cannot rule out the possibility that there is also a more mobile component in the spectrum of K•2',3'-SLAMPPNP that is hidden by the P2 and P4 components. The binding of 2',3'-SLAMPPNP to ncd was too weak to obtain any measurable signal from any ncd•2',3'-SLAMPPNP state.

Binding to microtubules induces an enhanced closing of the nucleotide pocket in the triphosphate analog states

We have previously shown that in the diphosphate state, the nucleotide sites of both kinesin and ncd are more closed upon binding to microtubules¹. Here we show that in the triphosphate state, the nucleotide site assumes multiple conformations, some of which are more closed than the diphosphate state. Fig. 4 shows that upon binding to microtubules, the spectra obtained from MT•ncd•2',3'-SLADP (Fig. 4B) contains two bound spectral components, both with greater P1-P5 splitting than seen for the single bound spectral component seen in the ncd•2',3'-SLADP state (Fig. 4A) in the absence of microtubules. This increase in splitting, the high affinity of the ncd•2',3'-SLADP state for microtubules, and the high concentration of microtubules in our pellet sample (see Methods), eliminates the possibility that the more mobile component is from ncd•2',3'-SLADP motors that are not bound to microtubules^{1,31}. We have previously reported a closing of the ncd nucleotide site in the diphosphate state upon binding to microtubules^{1,22}. However, in that study only one probe mobility in the MT•ncd•2',3'-SLADP state was observed because the more mobile of the two spectral components was obscured under the much larger P2 and P4 spectral components from unbound probe in solution. In the present study, access to a more sensitive EPR spectrometer and improved methods for eliminating the unbound probe in experimental buffers now allow for resolution of the additional more mobile component.

The spectra for the triphosphate-analog states, MT•ncd•2',3'-SLADP•AlFx (Fig. 4C), MT•ncd•2',3'-SLADP•BeFx (Fig. 4D), and MT•ncd•2',3'-SLAMPPNP (Fig. 4E) also show an equilibrium between two bound spectral components. This is seen in the two peaks (dips) in the P1 (P5) components of all spectra. The magnitude of the splittings for the two components in the MT•ncd•2',3'-SLADP•AlFx, MT•ncd•2',3'-SLADP•BeFx, and MT•ncd•2',3'-SLAMPPNP states (Table 1) show there is a further closing of the nucleotide site in the triphosphate states relative to the microtubule-bound diphosphate states. However, one of the two states still remains more open. The other is significantly more closed (an approximately 45° decrease in cone angle). The fractions of motors in the more open and

more closed states do not change significantly with temperature. This suggests that there is little change in the thermodynamic parameters of the interaction with temperature. Stabilization constants between multiple states are given in Table 1. The use of three different analogs (ADP•BeFx/AlFx, AMPPNP) allows us to ensure that our conclusions regarding the equilibrium of conformations in the triphosphate-state nucleotide-binding site are not dependent upon the analog employed. Although the P1-P5 splittings are virtually identical for the triphosphate-analog states, it is also clear from the relative peak heights in the P1 and P5 components of the spectra that the fractions of motors in the two spectral components varies. The MT•ncd•2',3'-SLADP•AlFx state has the largest fraction of the more mobile component. The two analogs with the tetrahedral arrangement of the γ -phosphate position have a much smaller fraction, approximately 20% of the more mobile component. The equilibrium between the two conformations is again only weakly dependent on temperature in the range 2°C-30°C (data not shown). When K-E236A•2',3'-SLATP binds to microtubules, the nucleotide site is also more closed than in the absence of microtubules (Fig. 3C).

Fig. 5 shows that similar conclusions are reached concerning the conformation of the nucleotide pocket of kinesin. There is a single component in the spectrum of K•2',3'-SLADP (Fig. 5A). In the MT•K•2',3'-SLADP state (Fig. 5B) there is a closing of the nucleotide binding site as monitored by the EPR probe due to the decrease in P1-P5 splitting. Just as was the case for ncd triphosphate-analog states, there are now two bound components to the spectra for the MT•K•2',3'-SLADP•AlFx (Fig. 5C) and MT•K•2',3'-SLADP•BeFx (Fig. 5D) states. The splitting is greater in both cases relative to the splitting in the MT•K•2',3'-SLADP state. Thus the triphosphate state again shows a further closing of the nucleotide-binding site. A difference is that in all the microtubule-bound, kinesin triphosphate spectra, the more immobilized component of the spectrum is much smaller in relative magnitude than that seen with ncd. In the spectrum of MT•kinesin•2',3'-SLADP•BeFx, the more immobilized of the two components represents only ~37% of the bound probes (determined from double integration of spectral components). Fig. S1 shows that similar conclusions are reached with 2'-SLADP bound at the nucleotide site. Thus we can eliminate the potential explanation that the two enantiomer species of 2',3'-SLADP are responsible for the presence of multiple states in the microtubule-bound spectra. Splittings and effective cone angles of mobility are summarized in Table 1.

Discussion

The triphosphate-moiety binding site of kinesin-family motors shares the P-loop, Switch 1 and Switch 2 motifs with G-proteins and myosin. Current hypotheses for kinesin motility posit that conformational changes at the nucleotide site associated with nucleotide hydrolysis are amplified and/or rectified by peripheral elements resulting in directed movement, reviewed in^{11,32-34}. Thus understanding the relationships between conformational changes at the nucleotide site of kinesin-family motors and the underlying biochemical, hydrolysis cycle is essential to understanding the mechanisms of motility for these motors. A confounding observation is that different structural approaches have suggested a large number of possible conformations of the active site. However, each experimental approach has its own strengths and weaknesses. Here we combine EPR

spectroscopy with other high-resolution structural approaches and atomic-level computational modeling to understand better the conformational changes at the nucleotide site associated with the triphosphate state.

The nucleotide pocket conformation can assume both open and closed conformations in solution

In the absence of microtubules, we have observed two possible conformations of the nucleotide pocket in the triphosphate/analog state. One is more open with probe mobility comparable to that observed in the diphosphate state. This conformation is seen in the SLADP•BeFx/AlFx states. The cone angle of mobility for the SLADP and SLADP•BeFx/AlFx states vary between 117° and 134°. These are very wide-open nucleotide pockets. The concentration of AlFx/BeFx used in these experiments was varied up to 4 mM. The 4 mM maximum value is four times the 1 mM concentration used in the crystallization liquor for the X-ray structure of kif1a•ADP•AlFx. That X-ray structure showed AlF₃ bound at the nucleotide site¹¹. However, we have no other independent verification that AlFx/BeFx are at the nucleotide site of the different proteins, kinesin and ncd. This motivated the additional experiment of examining the spectrum of K-E236A•SLATP. Here we are guaranteed a triphosphate state at the nucleotide site⁶. The spectra again show very wide-open nucleotide sites with cone angles of mobility of 129° (122°) for K-E236A•2',3'-SLATP (K-E236A•2'-SLATP). The virtually identical cone angles of mobility again confirm that our result is not dependent upon a specific EPR spin probe. We conclude that spectroscopy reports a more open nucleotide-pocket conformation for kinesin-family motors in the triphosphate state, consistent with the X-ray database as shown in Fig. 2.

On the other hand, SLAMPPNP shows a very tightly closed nucleotide-site conformation when bound to either kinesin (cone angle of mobility of 60°) or K-E236A (cone angle of mobility of 58°) in solution. A thermodynamically stable, closed-Switch 1 domain had previously been demonstrated for ncd•ATP²⁰. The only X-ray structure with a closed nucleotide-binding domain is the structure of Eg5•AMPPNP²⁷. AMPPNP has a P-NH-P moiety at the β,γ-AMPPNP position³⁵. MD simulations of the Eg5•AMPPNP structure show that the NH moiety will have all hydrogen-bonding possibilities satisfied modulo a reversal of the ND2/OD1 X-ray structure assignment of N229 at the nucleotide site. The additional lone pair on the side-chain NH nitrogen hydrogen bonds to the backbone NH of G108 in the P-loop. Thus a number of lines of structural evidence point to the closed nucleotide-site structure observed in the absence of microtubules. Additional computational support is provided below. However, not all crystal structures of kinesin-family motors with AMPPNP are as tightly closed. The X-ray structures of kif1a•AMPPNP¹¹ and the non-motile members, nod•AMPPNP¹² and kif2c•AMPPNP³⁶ all have open-Switch 1 domains. We also see an additional mobile component in the spectrum of K-E236A•2',3'-SLAMPPNP. Thus the aggregate data imply both open and closed nucleotide-site conformations.

MT-binding induces further closing of the triphosphate state relative to the diphosphate state

When kinesin or *ncd* binds to microtubules, there is an additional closing of the nucleotide site. In the MT•motor•diphosphate state, there is a single more immobilized component to the spectrum for kinesin corresponding to a decrease in the cone angle of mobility of 10°-20°. For *ncd* in the MT-diphosphate state, there are two components in the spectrum. One has probe mobility comparable to *ncd* in the absence of microtubules. The other represents a 15° decrease in the cone angle of mobility. In the MT-triphosphate state, two components are seen in the spectra for both kinesin and *ncd*. These imply there is a further closing of the nucleotide-binding site in the triphosphate state relative to the diphosphate state (cone angle of mobility decreases by an additional 20°-40°). We have also previously shown that Eg5 has a more mobile and a more immobilized EPR spectral component from bound nucleotide in the Eg5•2',3'-SLADP, Eg5•2',3'-SLADP•AIFx and their microtubule-bound states²¹. However, Eg5 has a very long Loop-5 (L5, 17 amino acids) projecting from the α 2-helix flanking the P-loop and adjacent to the nucleotide binding site that may contribute to the more immobilized component via interaction with the nucleotide.

High-resolution structural studies, EPR spectroscopy, and modeling all indicate there are multiple Switch 1 conformations

There are now a large number of X-ray structures of kinesin-family motors and EM reconstructions of the motor•microtubule complexes with different nucleotides or analogs at the active site. The nucleotide-analog EPR probes we have used allow a reporter to be placed specifically at the active site. Although the X-ray and EM structures show conformational changes at a number of locations in the motors, the focus here is the conformational changes at the active site that can produce the large changes in EPR probe mobility that we observe. In this regard, Switch 1 is the only element at the nucleotide site for which there is any X-ray or EM evidence for sufficiently large motions to generate the effects we are seeing. In addition, Switch 1 is in a position such that its various conformations will influence probe mobility (Fig. 2), while e.g. Switch 2 is far from the EPR probe moiety and undergoes more subtle conformational changes that are not expected to significantly influence probe mobility. EPR spectroscopy of a probe attached to the Switch 1 domain of kinesin also supports large-scale conformational changes in the Switch 1 domain²² upon binding to microtubules.

An overlay of structures showing representative observed Switch 1 conformations is given in Fig. 2. 2'-SLADP is docked at the nucleotide site. To facilitate display of the location of Switch 1, only limited fragments of the C α -backbone of the Switch 1 domain are shown at the most open point of the nucleotide site. For most structures, this is in the loop immediately adjacent to the α 3-helix. The most open conformation available in the Protein Data Bank is in the original kinesin•ADP structure (ref. ², PDB ID 1BG2), with the Switch 1 domain located ~20Å from the nucleotide (Fig. 2). Compared to the structure of kinesin•ADP, the structures of kif1a•ADP (ref. ¹¹, 1I5S), kif1a•AMPPNP (ref. ¹⁵, 1VFV), *ncd*•ADP (ref. ³, 2NCD), and kif1a•ADP•AIFx (ref. ¹⁵, 1VFX) show a progressive closing of the nucleotide site. However, all these structures remain quite open, with a minimum 13.3Å distance to the spin moiety. Although not shown in the figure, the comparable

distance for the structure of the triphosphate-analog kif1a•AMPPCP state is 14.8 Å (ref ¹¹, 1I6I). None of these crystal structures show a nucleotide site sufficiently closed to be expected to severely restrict probe mobility. Conversely, the structures of Eg5•AMPPNP (ref. ²⁷, PDB ID 3HQD) and ncd•2'-SLADP (ref. ²⁰, PDB ID 1SZ4) show a tightly closed nucleotide pocket, with the Switch 1 domain directly adjacent to the spin moiety closing the phosphate-tube. The ncd•2'-SLADP structure is approximately 1 Å more closed than the Eg5•AMPPNP structure. The homology modeling approach ²⁰ that was used to generate the structure of ncd•2'-SLADP found that both open- and closed-Switch 1 conformations of ncd•ADP and ncd•ATP were also stable conformations of the Switch 1 domain. Thus the Switch 1 motifs of structures from the Protein Data Bank and homology modeling partition into only very open and tightly closed conformations. There are no intermediate conformations lying between the two groups shown in Fig. 2. Our goal becomes to combine the structural and spectroscopic data into a more coherent picture of the structure of the nucleotide site as a function of nucleotide and microtubule binding.

Upon binding of the motor•SLADP to microtubules, EPR probe mobility shows that the nucleotide pocket becomes more closed. There is a single more immobilized component to the spectrum for MT•K•SLADP corresponding to a decrease in the cone angle of mobility of 10°-20° (Fig. 5). For the MT•ncd•SLADP state, EPR spectroscopy shows that there are two components in equilibrium in the spectrum. One has probe mobility slightly less than ncd in the absence of microtubules. The other represents a 15° decrease in the cone angle of mobility. Addition of phosphate analogs to the MT•motor•SLADP complex induces a more closed conformation than seen in the MT•motor•SLADP state (cone angle of mobility decreases by an additional 20°-40°). Crystal structures of this complex are not available, but an EM reconstruction of MT•kinesin•ADP•AlFx also shows only a single conformation with a closing of the nucleotide site in agreement with the probe mobility result ²³. Thus X-ray crystallography, spectroscopy and electron microscopy all agree on multiple conformations.

Probe mobility modeled by molecular dynamics simulations

Fig. 2 shows that almost a continuum of open conformations of the Switch 1 domain (K•ADP, kif1a•AMPPNP, kif1a•ADP, ncd•ADP, kif1a•ADP•AlFx) have been observed. A question becomes whether the spectroscopic observations correlate with existing structural observations? Cone angles provide only an approximation to the relationship between changes in spectral splitting and protein structure. It is additionally not clear how to translate an irregular protein surface such as those shown in Fig. 1 and Fig. 2 into a quantitative value for a symmetric cone angle of mobility for a spin probe. We have instead used molecular dynamics (MD) simulation to determine more quantitatively the degree to which X-ray structures correlate with the open and closed nucleotide-site conformations observed by EPR spectroscopy. We consider the two extremes of Switch 1 seen in Fig. 2.

2'-SLAMPPNP was docked “in silico” at the nucleotide site of the structure of ncd with a closed-Switch 1 domain (PDB ID 1SZ4²⁰) and 2'-SLADP was docked at the open nucleotide site of the kinesin•ADP structure (PDB ID 1BG2²). 2'-SLAMPPNP was docked into the closed ncd structure instead of kinesin since there is no closed kinesin structure in any database. Assuming rapid probe motion, the experimentally observed EPR spectrum is a

function of the time-dependent angular deviation, θ , of the EPR probe from a fixed axis in the laboratory reference frame (see Fig. S2). MD simulations were used to model probe mobility as a function of time, and from this the angular deviation of the probe as a function of time could be determined. Fig. 6 shows the angular deviation as a function of time for the two 15 ns MD simulations and Fig. 7 shows the accumulated angular probability distribution, $\rho(\theta)$, over the entire simulations. For the simulation of ncd•2'-SLAMPPNP, $\rho(\theta)$ is bunched at low values of θ , as would be expected for a tightly closed nucleotide site. The simulation of K•2'-SLADP shows a much broader distribution as would again be expected. The probability distribution for K•2'-SLADP shows three distinct modes. This can also be seen in Fig. 6B, with the maximum residence time between transitions only a few nanoseconds. The shutter speed of the spectrometer for X-band data collection is on the order of 100 ns, so these would be averaged out in the experimental spectrum. The low, but non-zero, probability near 90° results from steric interference between the spin moiety and the 3-position of the adenine ring. Bimodal distance distributions have likewise been seen in MD simulations modeling FRET spectroscopy observations³⁷.

Having determined $\rho(\theta)$, the advantage of our approach over traditional cone angle analysis is that we can now *quantitatively* correlate the orientational distributions obtained from the MD simulations for the EPR probes docked into the X-ray structures with the experimentally observed EPR spectra. Using Eq. (1) (Methods) with a simulated $\rho(\theta)$, an order parameter, S_m , can be determined. For the simulation of ncd•2'-SLAMPPNP, $S_m = 0.84$ and for K•2'-SLADP, $S_m = 0.34$. Then via Eq. (2) (Methods), these order parameters would equate to standard cone angles of mobility of 55.0° for ncd•2'-SLAMPPNP and 124.4° for K•2'-SLADP. These are in excellent agreement with the actual experimentally determined values of 59.8° for K•2',3'-SLAMPPNP and 124.2° for K•2'-SLADP. Thus the more open X-ray structure of K•2'-SLADP is in excellent agreement with its EPR spectrum. Combining EPR spectroscopy, crystallography and molecular modeling indicates that the structural state reporting the most immobile EPR probes we observe can be correlated with the tightly closed Switch 1 motif seen in the nucleotide site of ncd•ADP²⁰, Eg5•AMPPNP²⁷, or a very similar structure such as the closed nucleotide site seen in the cryo-EM reconstructions of the MT•kinesin•ADP•AlFx state²³. The important observation is that via MD simulation, we can connect the EPR spectra with specific open and closed atomic-level structures and bypass the ambiguities of a cone angle analysis.

We have been able to computationally consider only the two extremes of the location of Switch 1 in Fig. 2 since EPR spectra from kif1a are not available. Fig. 2 shows irregular Switch 1 domains partitioning into open and closed conformations, with variability particularly in the location of an open Switch 1, varying between 13.3Å and 20Å from the nucleotide. To examine the effect of Switch 1 variability on our results, more limited MD simulations in which the Switch 1 domain of K•2'-SLADP was “in silico” mechanically deformed into a slightly more open or closed conformation indicated that only a minor change in the location of Switch 1 (1-3Å) could generate a 15°-30° change in the MD-simulated cone angle. Additionally, the spin-probe moiety (Fig. S3) is very hydrophobic due to the saturated carbon atoms, particularly the methyl groups. “In silico” introduction of more/less hydrophobic residues from either ncd or kinesin at the active site while

maintaining a fixed location of the Switch 1 backbone atoms also results in changes of the MD-simulated mobility of the probe. Considering the above additional factors that can modulate probe mobility, the differences in experimentally observed probe mobility for the more mobile probes bound to ncd and kinesin would appear to be consistent with the inherent variability in the location and conformation of the Switch 1 domains in Fig. 2. We note that the computational approach we have employed also provides significant advantages over mobility analyses via cone angles of mobility. Although a highly useful approximation, the irregular nature of the nucleotide pocket and the influences of hydrophobic and other intermolecular forces on probe motion are such that the latter cannot unambiguously be correlated with specific atomic-level structures.

Summary

Taken *in toto*, the spectroscopic, X-ray and EM structural, and the modeling results support our original hypothesis that kinesin-family motors can alternate between open-Switch 1 structures seen in the original kinesin-family crystal structures, and closed structures resembling the structure seen in the original myosin structure²⁰. In the absence of microtubules, the more open conformation of the nucleotide site is greatly favored in all of the nucleotide states except those with bound AMPPNP. This observation also extends to a true ATP state, K-E236A•ATP. The open site would be expected to result in a strong inhibition of nucleotide hydrolysis, as is observed from enzyme kinetics, since the open structure would not be able to control any catalytic water(s)²⁰. However, the fact that a closed site can be achieved in the absence of microtubules and in the presence of AMPPNP shows that under some conditions the site can close, which could account for the slow nucleotide hydrolysis that does occur in kinesin-family motors in the absence of microtubules. It also suggests that AMPPNP populates a state different from that populated by any other nucleotides or nucleotide analogs, including native ATP.

Closed states are favored by the binding of the motor to microtubules, with a nucleotide site that resembles the closed-Switch 1 structure seen in myosin. A comparison of the mobility of water molecules in previous MD simulations of open- and closed-Switch 1 structures led to the original hypothesis that a closed-Switch 1 domain was necessary for the rapid microtubule-activated hydrolysis rate of kinesin-family motors²⁰. The X-ray structure of Eg5•AMPPNP provides further support²⁷. However, in all but one of the microtubule-bound states there are two conformations of the nucleotide site, again showing the dynamic nature of this state.

It is finally of interest to compare nucleotide-site conformational changes in myosin and kinesin-family motors. X-ray diffraction shows both open and closed conformations of the myosin nucleotide pocket, reviewed in^{38,39}. Electron microscopy⁴⁰ and spectroscopy⁴¹⁻⁴⁵ show a closed nucleotide site when myosin is dissociated from actin. The nucleotide pocket opens when myosin binds strongly to actin facilitating ADP release, with the open conformation also in equilibrium with a closed conformation. However, in myosin, ATP-binding is associated with dissociation of the actomyosin complex and nucleotide hydrolysis follows on the detached myosin head, reviewed in^{46,47}. In kinesin, ATP-binding is associated with rapid ATP hydrolysis in the microtubule-bound complex, reviewed in⁴⁸.

Thus the structural and biochemical observations of hydrolysis steps that are 180° out of phase are consistent with myosin hydrolysis when free from actin in a closed phosphate tube structure and kinesin hydrolysis in a closed phosphate tube structure when bound to microtubules.

Materials and Methods

Proteins

Monomeric *Drosophila melanogaster* ncd (aa. 335-700), and “cys-lite” monomeric human kinesin-1, (K, a.a. 1-349), containing the additional mutation, E220C were bacterially expressed, purified, and stored at -80°C as described previously¹. K-E220C had MgATPase and MT sliding velocity similar to wild type¹. The K-E220C construct was employed due to its wild-type function and very high expression rate. We also used K-V333C or K-S188C for some experiments with no difference in the results. The K-E236A construct was expressed and purified as described in⁶. ncd (100 μM) was dialyzed into a rigor buffer containing 25 mM MOPS, 100 mM KOAc, 2 mM Mg(OAc)₂, 1 mM EGTA, 5% sucrose, 30 μM ADP, pH 7.0, 4°C. The high ionic strength was necessary to maintain solubility of ncd, but was used for K as well to standardize conditions. The spin probe nucleotide analog (90 μM) was added. Kinesin was also labeled using the above procedures. The labeled protein was then run through a Micro Bio-Spin column P-30 (Bio-Rad) to eliminate free probe. Tubulin was prepared from porcine brain tissue. Initial purification steps followed the protocol described in⁴⁹. Removal of contaminating MAPs was performed as described in⁵⁰. Briefly, polymerized tubulin was centrifuged through a 1 M PIPES cushion. After cold depolymerization, all remaining MAPs were removed by phospho-cellulose chromatography. Purified tubulin was stored in a buffer containing 100 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, 1 mM Na₂GTP, pH=6.8, -80°C. For microtubule polymerization, thawed tubulin was spun at 100,000×g for 20 min., 4°C, to remove any aggregated material and 1 mM Na₂GTP, 10% DMSO and 2 mM MgCl₂ were then added to the supernatant. Polymerization was induced by 30 min. incubation at 37°C. Paclitaxel (20 μM) was added and the MTs were spun down at 100,000×g for 25 min., 25°C over a 60% v/v glycerol cushion. The pellet was re-suspended in a buffer containing 25 mM NaCl, 25 mM PIPES, 1 mM EGTA, 2 mM MgCl₂, 20 μM paclitaxel, pH 7.0, 25°C. The molecular weight of the dimer was taken to be 110,000. The final concentration of the tubulin αβ-dimer was 200-250 μM.

EPR Spectroscopy

For experiments in the absence of microtubules, the labeled kinesin or ncd sample was introduced into a 25 μl capillary, sealed at one end using Critoseal (Oxford Labware), and placed in the EPR cavity. For experiments involving the motor•MT complex, the labeled kinesin or ncd was then added to polymerized microtubules at a stoichiometry of approximately 1:3 protein/MT dimer in the above buffer. This mixture was centrifuged at 70,000×g for 20 min. and the pellet containing the kinesin•MT complex was scraped with a spatula onto a quartz flat cell. The pellet was covered with a glass cover slip, sealed with vacuum grease to prevent dehydration and placed in the EPR cavity. Experiments performed

in the presence of phosphate analogs were done in the rigor buffer with 10 mM NaF and 2 mM BeCl₂ or AlCl₃ added, producing 2 mM of the fluoride complex.

EPR spectra were recorded using a Bruker EMX EPR spectrometer (Billerica, MA). First derivative, X-band spectra were recorded in a high-sensitivity microwave cavity using 50 s, 10 mT-wide magnetic-field sweeps. The instrument settings were as follows: microwave power, 25 mW; time constant, 164 ms; frequency, 9.83 GHz; modulation, 0.1 mT at a frequency of 100 kHz. Each spectrum used in data analysis was an average of 5–50 sweeps from an individual experimental preparation. Temperature was controlled by blowing dry air (warm or cool) into the cavity and monitored using a thermistor placed close to the experimental sample.

Effective cone angles of mobility were approximated using the order parameter $S_e = (T_{||}' - T_0)/(T_{||} - T_0)$ as a measure of probe mobility. Here $2T_{||}'$ is the observed splitting, $2T_{||}$ is the splitting for an immobilized probe, and $2T_0$ is the isotropic hyperfine splitting for freely tumbling EPR probe in solution. Then the full angular width of the vertex of the cone angle of mobility, $C_\angle$, is then given by $C_\angle = 2\arccos[-0.5 + 0.5(1 + 8S_e)^{1/2}]$. Additional details are in ^{28,29}.

The spin-labeled nucleotides contain a pyrrolidine spin ring moiety bridged by a carbonyl linkage to the ribose ring. For 2',3'-SLATP, there are two enantiomers with the 2'-(3'-) position of the ribose attached to the spin moiety and the 3'-(2'-) position is a hydroxyl. For 2'-SLATP the 2'-ribose position is attached to the spin moiety and the hydroxyl on the 3'-position is replaced by a hydrogen. Structures are shown in Figure S3. All analogs were synthesized using slight modifications of previously published procedures ⁵¹, except that 2', 3'-SLAMPPNP used AMPPNP as the starting material. Both 2',3'-SLATP and 2'-SLATP are hydrolyzed by kinesin and ncd ¹ indicating that their spectra are from the diphosphate species.

Molecular dynamics simulations

The atomic-level protein structures for the ncd•2'-SLADP (PDB ID 1SZ4) and the original X-ray structure of K•ADP (PDB ID 1BG2) were the starting point for the MD simulations. 2'-SLAMPPNP was docked at the nucleotide site of the ncd•2'-SLADP structure and 2'-SLATP was docked at the nucleotide site of the K•ADP structure following the protocols described in ²⁰. This resulted in the spin moiety pointing out of the nucleotide site toward the solvent phase. Simulations were done using the Amber10 suite of codes ⁵². Hydrogen atoms were added to the structure using the tleap module. Charges for 2'-SLATP and 2'-SLAMPPNP derived by first performing a single-point energy calculation using Gaussian03 ⁵³ to obtain electrostatic charges. These are then fit to the molecules by using the RESP procedure ⁵⁴. Equilibrium bond lengths and angles for the spin-ring moiety were averages of the values in ^{55,56}. The nitroxide bond was modeled as having sp² hybridization. Van der Waals parameters for the Mg²⁺ that cofactors the nucleotide are taken from ⁵⁷ and adjusted as described in ²⁰. The generalized Born approach was used for the MD simulation (reviewed in ⁵⁸⁻⁶⁰). The structures were first energy minimized using 1500 steps of the steepest descent method, followed by 1500 steps of conjugate gradient minimization. The RMS deviation of the structures over the last 500 steps was less than 0.03Å. The structures

were then equilibrated to 298K over a 50 ps time period with all atoms restrained by a weak harmonic constraint (0.5 Kcal/mol/Å²). For the MD simulations of probe mobility, atoms greater than 23 Å from the 2'-ribose ring oxygen were constrained as above. Atoms closer were unrestrained. Temperature was maintained via coupling to an external bath using the Berendsen algorithm⁶¹. The SHAKE algorithm⁶² was employed for numerical integration. The MD simulated motions of the protein and spin probe moiety were followed for 15 ns using a 2 fs time step. LaConte and coworkers⁶³ have shown this is sufficient simulation time for a spin probe to sample the available space via thermal motion. A limited number of longer simulations confirmed this observation. The EPR spectrum is dependent on the angular deviation of the p-orbital of the nitroxide bond, which is perpendicular to the plane of the C-N-C bond of spin moiety ring at the nitroxide nitrogen, from a fixed direction in the laboratory reference frame (see Figure S2)²⁸. This directional vector was output every 4 fs for mobility simulation analysis.

The angles of the directional vector were binned in 1° increments to approximate the probability density, $\rho(\theta)$, as a function of angular deviation, θ , from the mode vector of the angular distribution. The order parameter for the modeled mobility from the simulation is then given by

$$\langle \cos^2 \beta \rangle = \frac{\int_0^\pi \rho(\theta) \cos^2(\theta) \sin(\theta) d\theta}{\int_0^\pi \rho(\theta) \sin(\theta) d\theta},$$

and the spin-label order parameter is given by

$$S_m = 1.5 \langle \cos^2 \beta \rangle - 0.5. \quad (1)$$

Thus a spin probe undergoing rapid, uniformly distributed motion within a cone with vertex angle

$$C_L = 2 \arccos \left[-0.5 + 0.5(1 + 8S_m)^{1/2} \right] \quad (2)$$

would have the same order parameter, S_e , based on the experimentally observed spectrum. This allows direct comparison between cone angles of mobility determined experimentally from EPR spectra and the order parameter determined from the MD simulations. Additional details are in^{28,63}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ncd	nonclaret disjunctional protein
K	kinesin
MT	microtubule
SLADP	spin-labeled ADP
SLATP	spin-labeled ATP
SLAMPPNP	spin-labeled AMPPNP
EPR	electron paramagnetic resonance

Figure 1.

A ribbon diagram is shown of a superposition of the structures of K•ADP (ref. ², PDB ID 1BG2, turquoise) and ncd•2'-SLADP (ref. ²⁰, PDB ID 1SZ4, coral). Structures were superimposed via a P-loop least-squares distance minimization. As seen in the figure, this procedure results in a superposition of the core β -sheet platforms of the structures and superposition of the α 3-helix domains. The rms deviation of the ADP moieties of the nucleotides is 0.33Å, showing no significant difference in the locations of the nucleotide in the binding sites. Only 2'-SLADP is shown at the active site. 2'-SLADP is colored by standard atom colors, except for the spin moiety, which is colored purple. The Switch 1 domain is immediately adjacent to the spin moiety in the closed (coral) ncd•2'-SLADP structure, but pulled well away in the open (turquoise) K•ADP structure.

Figure 2.

The relative locations of Switch 1 in different kinesin-family X-ray structures are shown. The different structures were overlaid via a least-squares distance minimization of the respective P-loops. To facilitate display, only the C α -backbone atoms are shown for a short segment of the Switch 1 domain adjacent to the α 3-helix, but closest to the bound nucleotide. This is the portion of the Switch 1 domain that will interact with the spin-probe moiety. X-ray structures shown as a function of increasing distance from the nucleotide are ncd•2'-SLADP (ref. ²⁰, PDB ID 1SZ4, black), Eg5•AMPPNP (ref. ²⁷, 3HQD, coral), kif1a•ADP•AIFx (ref. ¹⁵, 1VFX, cyan), ncd•ADP (ref. ³, 2NCD, red), kif1a•AMPPNP (ref. ¹⁵, 1VFV, magenta), kif1a•ADP (ref. ¹¹, 1I5S, green), and the original kinesin•ADP structure (ref. ², PDB ID 1BG2, blue). The first two represent tightly closed nucleotide pockets. Although there is considerable spread in the location of Switch 1 in the remainder of the structures, they all represent very wide-open nucleotide pockets. The nucleotide analog, 2'-SLADP, is colored in gray at the left, except for the spin moiety, which is in blue with the nitroxide bond orange.

Figure 3.

EPR spectra from (A, red) K•2',3'-SLADP and from the triphosphate (analog) species (B, black) K•2',3'-SLADP•AlFx, (C, cyan) K-E236A•2',3'-SLATP, (D, Ochre) K-E236A•2',3'-SLATP•MT, (E, blue) K-E236A•2',3'-SLAMPPNP, and (F, green) K•SLAMPPNP. Components P2, P3, P4 are from unbound EPR probe in solution. The more immobilized components, P1 and P5, are from probe bound to kinesin. The sharp spikes in spectrum E, of particular note on the low-field side of component P2, are termed satellite peaks. They arise from interaction of the unpaired electron in the spin moiety with ^{13}C (methyl groups) isotopes 64 and not from motor-bound analog. Additional confirmation of this identification comes from the fact that the spectral shift between the satellite component and the P1 peak arising from ^{13}C is 0.47 mT, the same as observed for 2',3'-SLADP in solution in the absence of protein. The spectra we observe from kinesin-family motors are a summation of spectra from unbound EPR probe and spectra from probes bound to multiple conformations of the motor. The observation of satellite peaks is dependent upon the relative contributions of each component to a given spectrum. The satellite peaks are contributed primarily by unbound probe. Thus satellite peaks are not evident in all spectra.

Figure 4.

EPR spectra from (A, red) $\text{ncd} \cdot 2',3'\text{-SLADP}$, (B, blue) $\text{MT} \cdot \text{ncd} \cdot 2',3'\text{-SLADP}$, (C, green) $\text{MT} \cdot \text{ncd} \cdot 2',3'\text{-SLADP} \cdot \text{AlFx}$ (D, cyan), $\text{MT} \cdot \text{ncd} \cdot 2',3'\text{-SLADP} \cdot \text{BeFx}$ and (E, ochre) $\text{MT} \cdot \text{ncd} \cdot 2',3'\text{-SLAMPPNP}$ states are shown. Multiple conformations of the nucleotide site are reported in the microtubule-bound states. There is a closing of the nucleotide site in the $\text{MT} \cdot \text{ncd} \cdot 2',3'\text{-SLADP}$ state relative to the $\text{ncd} \cdot 2',3'\text{-SLADP}$ state. There is a further closing in the triphosphate-analog states.

Figure 5.

EPR spectra from (A, red) $\text{K}\cdot 2',3'\text{-SLADP}$, (B, blue) $\text{MT}\cdot\text{K}\cdot 2',3'\text{-SLADP}$, (C, green) $\text{MT}\cdot\text{K}\cdot 2',3'\text{-SLADP}\cdot\text{AlFx}$ (D, cyan), and $\text{MT}\cdot\text{K}\cdot 2',3'\text{-SLADP}\cdot\text{BeFx}$ states are shown. As was the case for *ncd*, the microtubule-bound states report a more closed nucleotide-binding pocket, with the ATP-analog states more closed than the diphosphate state.

Figure 6. MD-simulated orientational trajectory of the spin probe with the angle θ plotted as a function of time during the MD simulation for (A) the closed structure of ncd•2',3'-SLADP and (B) the open structure of K•2'-SLADP. The individual data points from the simulation are separated by 0.015ns in time.

Figure 7.
Probability distribution, $\rho(\theta)$, for the simulated MD trajectories.

Table 1
Table of Splittings for Ncd and Kinesin

The low-field to high-field splittings obtained from the EPR spectra, along with effective cone angles of mobility are given. Splittings are the mean of 2-6 different observations. Standard errors of the EPR splittings were ± 0.02 mT. The last column gives the ratio of probe in the less mobile state to more mobile state and the energetic stabilization of the less mobile component in units of $k_B T$ for spectra with two components from spin probe bound at the nucleotide site.

Complex	Splitting (mT)	Cone Angle ($^{\circ}$)	Component Stabilization ($k_B T$)
ncd•2',3'-SLADP	4.78	117.4	
ncd•2',3'-SLADP•AlFx	4.78	117.4	
ncd•2',3'-SLADP•BeFx	4.77	117.8	
ncd•2',3'-SLADP•MT	4.81, 5.33	116.4, 99.2	2.4, -0.87
ncd•2',3'-SLADP•AlFx•MT	5.09, 5.94	107.1, 78.1	2.3, -0.83
ncd•2',3'-SLADP•BeFx•MT	5.06, 6.01	108.1, 75.6	3.2, -1.16
ncd•2',3'-SLAMPPNP•MT	5.22, 6.12	102.8, 71.5	4.6, -1.52
kinesin•2',3'-SLADP	4.31	133.6	
kinesin•2',3'-SLADP•AlFx	4.30	133.9	
kinesin•2',3'-SLADP•BeFx	4.30	133.9	
kinesin•2',3'-SLAMPPNP	6.41	59.8	
kinesin•2',3'-SLADP•MT	4.96	111.4	
kinesin•2',3'-SLADP•AlFx•MT	5.17, 6.30	104.5, 64.4	1.4, -0.34
kinesin•2',3'-SLADP•BeFx•MT	5.06, 6.37	108.1, 61.5	0.6, 0.51
kinesin•2',3'-SLAMPPNP•MT	5.07, 6.37	108.6, 62.3	0.9, 0.11
kinesin-E236A•2'-SLATP	4.64	122.1	
kinesin-E236A•2',3'-SLAMPPNP	4.88, 6.45	114.1, 58.1	2.0, -0.69
kinesin-E236A•2',3'-SLATP	4.44	129.0	
kinesin-E236A•2',3'-SLATP•MT	4.76	118.2	
kinesin•2'-SLADP	4.58	124.2	
kinesin•2'-SLADP•MT	4.68, 5.40	120.8, 96.8	1.5, -0.40
kinesin•2'-SLADP•AlFx•MT	4.86, 5.71	114.8, 86.3	2.1, -0.74
kinesin•2'-SLADP•BeFx•MT	4.87, 5.70	114.4, 86.6	2.1, -0.74