Docking and Rolling, a Model of How the Mitotic Motor Eg5 Works*

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Steven S. Rosenfeld†1, Jun Xing‡, Geraldine M. Jefferson§, and Peter H. King¶

From the †Department of Neurology, Columbia University College of Physicians and Surgeons, New York, New York 10032, the ‡Department of Biochemistry and Molecular Genetics and the §Department of Neurology, the University of Alabama at Birmingham, Birmingham, and the ¶Neurology Service, Birmingham Veterans Affairs Medical Center, Birmingham, Alabama 02138

Whereas kinesin I is designed to transport cargoes long distances in isolation, a closely related kinesin motor, Eg5, is designed to generate a sustained opposing force necessary for proper mitotic spindle formation. Do the very different roles for these evolutionarily related motors translate into differences in how they generate movement? We have addressed this question by examining when in the ATPase cycle the Eg5 motor domain and neck linker move through the use of a series of novel spectroscopic probes utilizing fluorescence resonance energy transfer, and we have compared our results to kinesin I. Our results are consistent with a model in which movement in Eg5 occurs in two sequential steps, an ATP-dependent docking of the neck linker, followed by a rotation or “rolling” of the entire motor domain on the microtubule surface that occurs with ATP hydrolysis. These two forms of movement are consistent with the functions of a motor designed to generate sustained opposing force, and hence, our findings support the argument that the mechanochemical features of a molecular motor are shaped more by the demands placed on it than by its particular family of origin.

The last decade has witnessed a marked advance in our understanding of how molecular motors generate force and movement (1–6). Studies of both myosins and kinesins have revealed a variety of conserved structural elements that play key roles in mechanochemical transduction. These include switch I, switch II, and the P loop located within the catalytic site (3, 5, 6). Movements of these elements during ATP binding, hydrolysis, and product release lead to a series of conformational changes that are transmitted ultimately to the “business end” of the motor, the mechanical element that produces force and movement. In kinesin I, this mechanical element consists of an extended peptide sequence with variable conformation and flexibility, called the “neck linker” (2, 3). Spectroscopic and kinetic studies have led to a convincing model in which the neck linker assumes a random coil in the absence of nucleotide. ATP binding to the active site causes the neck linker to dock along a hydrophobic surface in the motor (7–10). This process, which is very rapid (>800 s⁻¹) at room temperature, immobilizes the neck linker and effectively “throws” the tethered head of a kinesin dimer forward, toward the next tubulin-docking site (11, 12, 25).

Variable flexibility and ATP-induced docking are features of the kinesin I neck linker that are well suited to the physiologic role of this motor as a transport engine. Variable flexibility in the neck linker of the tethered head allows it to undergo a diffusive search for the next microtubule-docking site, whereas ATP-induced docking of the neck linker of the attached head helps position the tethered head in a forward position and reduces the probability of backward stepping (10, 13–16). Thus, the physiologic requirements placed on a transport motor like kinesin I translate into structural features of its mechanical element that subserve this function. What about other motors that serve other functions? Do they utilize the same mechanism to generate forward stepping?

To address this question, we have turned our attention to another member of the kinesin superfamily, one that serves functions other than vesicle transport. Eg5 is a plus-end directed member of the kinesin 5 subfamily, which localizes to interpolar spindle microtubules and to the spindle poles, and is required for normal spindle function (17–22). Unlike kinesin I, which moves vesicles along microtubule tracks, Eg5 moves the microtubules themselves. It accomplishes this through its formation of bipolar homotetramers that cross-link microtubules and induce microtubule-microtubule sliding and pivoting (17–19).

Despite the fact that kinesin and Eg5 subserve different cellular functions and physiologies, they share several important features. First, they share important structural elements. Their primary structures are 40% identical (23). Like other members of the kinesin superfamily, Eg5 contains several key motor elements, including switch I, switch II, the P loop, and the neck linker. Furthermore, the β sheets that form the core of the motor domains are nearly identical (23, 24).

However, there are also significant differences between these two motors. The most striking of these is in the position of the neck linker in crystallographic models in the presence of ADP. In the ADP-bound state, the kinesin neck linker is disordered and undocked (8–10), whereas in Eg5, it is ordered, and projects perpendicularly from the long axis of the motor core (23). It has been argued that for a motor that functions in ensembles to slide microtubules, flexibility in the neck linker would be counter-productive. Instead, what would be required would be preservation of neck linker structure in the presence of both ATP and ADP, so that Eg5 could act instead like a “ratchet” (23).

In our previous studies on kinesin I, we utilized a set of novel spectroscopic techniques to monitor the timing of neck linker and motor domain movements during the ATPase cycle (11, 12, 25). These studies demonstrated that neck linker docking is rapid, occurs after an ATP-induced conformational change and before hydrolysis, and leads to separation of the two neck linkers of a dimeric kinesin motor. Furthermore, we developed a model in which the timing of these state changes in the neck linker was a key component in the allosteric communication mechanism used by kinesin to maintain its processive movement (11, 12). In this study, we have utilized a similar spectroscopic approach to monitor the timing and direction of neck linker and motor domain movements in Eg5. Our results suggest that the different physiologic roles played by Eg5 and kinesin I translate into differences in the way they generate movement.

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† Supported by National Institutes of Health Grant AR048565. To whom correspondence should be addressed: The Neurological Institute, Rm. 204, Columbia University, 710 West 168th St, New York, NY 10032. E-mail: sr2327@columbia.edu.

‡ Supported by National Institutes of Health Grant AR048565. To whom correspondence should be addressed: The Neurological Institute, Rm. 204, Columbia University, 710 West 168th St, New York, NY 10032. E-mail: sr2327@columbia.edu.
EXPERIMENTAL PROCEDURES

Materials—Oligonucleotide primers were synthesized by Invitrogen. The QuickChange site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). Media components were obtained from Difco. Protease inhibitors, isopropyl β-D-thiogalactopyranoside, and reagents for buffers and agarose gel electrophoresis were obtained from Sigma. Ribonuclease A and deoxyribonuclease I were purchased from Roche Applied Science. Pre-packed Sephadex G-25 columns were purchased from Amersham Biosciences. Nickel-nitrilotriacetic acid-agarose was obtained from Qiagen Inc. (Chatsworth, CA). Fluorescent probes were purchased from Molecular Probes. Synthesis and purification of 2'-deoxy-mant-ATP, 2'-deoxy-mant-ADP, and mant-GTP from N-methylisatoic anhydride and the corresponding nucleotides were carried out as described (26).

Generation of Cysteine-light Eg5 367 Mutants—Our starting material for these studies was a plasmid encoding the amino-terminal 367 residues of human Eg5, followed by a His₆ tag for affinity purification (Eg5-367), kindly provided by Drs. Zoltan Maliga and Timothy Mitchison (Harvard Medical School). This plasmid encodes a construct that contains four cysteine residues at positions 25, 43, 87, and 99 (27). In preliminary studies, we determined that all four residues were reactive with the fluorescent probe 1,5-IAEDANS² (data not shown). Furthermore, for several of the studies described in this work, we wished to perform fluorescence resonance energy transfer (FRET) between microtubule tryptophans and an AEDANS probe on the Eg5 neck linker. We therefore generated a cysteine-light Eg5 construct with a reactive cysteine within the neck linker and with the single tryptophan converted to phenylalanine. This was accomplished by utilizing the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) starting with the Eg5-367 plasmid in the pRSET vector (Invitrogen). The following mutations were generated within the construct: cysteine 25 to valine, cysteine 43 to serine, cysteine 87 to alanine, cysteine 99 to alanine, tryptophan 127 to phenylalanine, and valine 365 to cysteine. The resulting construct, CLM-W127F, was verified by sequence analysis. For several of the experiments described in this study, we also created a revertant, CLM containing the neck linker and with the single tryptophan converted to phenylalanine.

2 The abbreviations used are: 1,5-IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)-naphthalene-1-sulfonic acid; 2'-dM, 2'-deoxy; 3'-mant-ADP; 2'-dM, 2'-deoxy; 3'-mant ATP; FRET, fluorescence resonance energy transfer; Rₐ, Förster critical energy transfer distance; AEDANS, acetylatedioethyl-1-aminonaphthalene-5-sulfonate; mant, 2',3'-bis(O-;N-methylanthranolol); dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; AMPNNP, adenosine 5'-[(β,γ-imino)triphosphate.

RESULTS

Using FRET to Monitor Eg5 Domain Movement—Our approach in this study was to utilize five different FRET donor-acceptor pairs in order to monitor the kinetics of movement of one part of the Eg5 motor relative to another or relative to the microtubule. These pairs utilized a series of probes located on the microtubule (Oregon Green taxol, mant-GDP, and tryptophan) and on the Eg5 motor (tryptophan 127, AEDANS, or Oregon Green on the neck linker, and 2'dM in the catalytic site). Fig. 1 illustrates on the tertiary structure of Eg5 the locations of tryptophan 127 (green), valine 365 (yellow), converted to cysteine in the Eg5 CLM mutants, and ADP (multicolored).

Because all four of the cysteines in wild type Eg5-367 were reactive with sulfhydryl-specific fluorescent probes, it was necessary to mutate each of them to cysteines, and this is described under “Experimental Procedures.” In addition, because we wished to place a fluorescent probe on the neck linker to monitor movements of this domain, we converted valine 365 to a cysteine. Finally, because we also wished to monitor FRET from microtubule tryptophan residues to an AEDANS probe on the neck linker, we also needed to convert Trp-127 to phenylalanine. The resulting construct was called CLM-W127F. We needed to ensure that these modifications had minimal effects on the enzymatic behavior of Eg5, and to do so, we examined the microtubule-activated ATPase activity of wild type and CLM-W127F. The ATPase rate data fit Michaelis-Menten kinetics, with values of Vₐ₅₀ and Kᵢ₅₀,MT of 8.9 ± 0.7 s⁻¹ and 6.8 ± 1.1 μM (where MT is microtubule) for wild type Eg5, and 8.6 ± 0.7 s⁻¹ and 6.3 ± 1.2 μM, respectively, for CLM-W127F. Hence,
microtubule-dependent activation of the ATPase activity is essentially unaffected by these mutations.

FRET Approach 1, Microtubule Tryptophan to Mant Nucleotide in the Motor Catalytic Site Monitors Nucleotide Binding and Motor Dissociation from the Microtubule—In our previous study of kinesin I, we had shown that binding of the fluorescent ATP analogue 2’dmT to a microtubule-kinesin complex could be monitored by FRET from the microtubule tryptophan residues to the mant fluorophor (25). Mixing a kinesin–microtubule complex with 120 μM 2’dmT leads to weak binding and dissociation, the latter of which abolishes the FRET-sensitized emission. Fluorescence transient produced by mixing a complex of CLM-W127F + microtubules with 120 μM 2’dmT. The rising phase, because of 2’dmT binding, is associated with a rate constant of 48.2 s⁻¹, whereas the falling phase, reflecting dissociation of CLM-W127F, occurs with a rate constant of 6.2 s⁻¹. The amplitudes of both phases are nearly equal, as predicted from the model. Inset, plot of rate constant of the fast, rising phase (solid blue circles, solid blue line) and slow, falling phase (open blue circles, dotted blue line) as a function of 2’dmT concentration. The extrapolated maximum rate constants for these two phases are 62 ± 17 and 6.3 ± 0.3 s⁻¹.

TABLE ONE

| Donor | Donor location | Acceptor | Acceptor location | Steps detected |
|-------|----------------|----------|------------------|----------------|
| Tryptophan | Microtubule | AEDANS | Neck linker | Strong to weak transition |
| Tryptophan | Eg5 (position 127) | AEDANS | Neck linker | ATP-induced neck linker docking |
| Tryptophan | Microtubule | Mant nucleotide | Eg5 nucleotide-binding site | ATP-induced neck linker docking |
| Mant-GDP | Microtubule | Oregon Green | Neck linker | Motor dissociation from microtubule Eg5:ADP binding to microtubule |
| Oregon Green taxol | Microtubule | QSY7 | Neck linker | Dissociation of ADP from Eg5 |
| | | | | Strong to weak transition |
| | | | | ATP, ADP-induced neck linker movement motor dissociation |

FIGURE 2. Kinetics of 2’dmT binding to microtubule-CLM-W127F complex. A, schematic of the experimental design. Rigor Eg5, in equilibrium between two neck linker orientations, binds 2’dmT, allowing for FRET between microtubule tryptophan residues and the mant fluorophor (depicted as the magenta rays emanating from the motor domain). Hydrolysis of 2’dmT leads to weak binding and dissociation, the latter of which abolishes the FRET-sensitized emission. B, fluorescence transient produced by mixing a complex of CLM-W127F + microtubules with 120 μM 2’dmT. The rising phase, because of 2’dmT binding, is associated with a rate constant of 48.2 s⁻¹, whereas the falling phase, reflecting dissociation of CLM-W127F, occurs with a rate constant of 6.2 s⁻¹. The amplitudes of both phases are nearly equal, as predicted from the model. Inset, plot of rate constant of the fast, rising phase (solid blue circles, solid blue line) and slow, falling phase (open blue circles, dotted blue line) as a function of 2’dmT concentration. The extrapolated maximum rate constants for these two phases are 62 ± 17 and 6.3 ± 0.3 s⁻¹.
rate constant of $19 \pm 9.6 \text{s}^{-1}$, an apparent dissociation constant of $55 \pm 12 \mu M$, and a maximum rate of $62 \pm 17 \text{s}^{-1}$ (TABLE ONE).

The rate of the falling phase (Fig. 2B, inset, open circles), reflecting dissociation, reached a maximum value of $6.3 \pm 0.3 \text{s}^{-1}$. This corresponds to a dissociation rate constant measured by turbidity of $6.7 \pm 0.6 \text{s}^{-1}$ at a final [ATP] of 1 mM (data not shown) and compares to a value of $7.8 \pm 0.2 \text{s}^{-1}$ reported in a prior study and at a higher temperature ($25^\circ \text{C}$; Ref. 29). Furthermore, the amplitudes of the two phases were identical within 10%, as would have been predicted from the model (Fig. 2A). Qualitatively similar results were seen with the fluorescent ADP analogue 2'dmD, and results will be discussed below.

FRET Approach 2, Microtubule Oregon Green Taxol to Eg5 Neck Linker QSY7 Monitors Neck Linker Docking and Motor Dissociation—

The rate constant for binding of 2’dmT to a microtubule-kinesin I complex is $>1000 \text{s}^{-1}$ at room temperature (25). This $>20$-fold difference suggests that in Eg5, ATP binding occurs only after a slow, rate-limiting
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isomerization that affects the structure of the catalytic site. We wished to determine what this isomerization looks like. To do so, we developed a different FRET approach that would allow us to correlate movements of the neck linker with the kinetics of ATP binding. This consisted of FRET from Oregon Green 488-labeled taxol, bound to the microtubule, to QSY7 maleimide, a nonfluorescent probe, bound to the CLM-W127F neck linker. Because this FRET approach is new, we wished to validate its use by first applying it to kinesin I, because we had previously characterized the kinetics of its neck linker movements (11, 12, 25).

Current models of kinesin I propose that in the absence of ATP, the neck linker is flexible and disordered. Binding of ATP, but not ADP, causes the neck linker to “dock” along a hydrophobic groove on the motor domain, and thereby become ordered and oriented parallel to the long axis of the motor domain and the microtubule protofilament (1–3). Hence, we would predict that FRET between a donor on the microtubule and an acceptor on the kinesin I neck linker might be able to sense this change in state. Fig. 3A illustrates a schematic of the reaction produced by mixing a complex of Oregon Green taxol microtubules plus a QSY7-labeled monomeric kinesin construct (K349) with ATP. In a rigor complex, under conditions where the neck linker is presumed to be disordered, the mean distance between these two probes, determined by FRET, is 37 Å. In the presence of AMPPNP, where the neck linker is docked, it is 57 Å (TABLE THREE). We would therefore predict that mixing a complex of Oregon Green taxol-labeled microtubules + QSY7-labeled K349 should increase the fluorescence emission of the donor in at least two steps, one associated with ATP binding (producing docking) and the second with dissociation from the microtubule.

Fig. 3B illustrates the fluorescence transients produced by mixing with ATP (red) and ADP (green) and confirms this prediction. The transient produced by mixing with ATP consists of two phases of increasing fluorescence, separated by a lag. Maximum rate constants for each of these phases are 686.7 ± 157.8 s⁻¹, 292.2 ± 13.0 s⁻¹ (lag), and 54.8 ± 1.8 s⁻¹ (Fig. 3C, red, blue and green curves). These correspond to previously measured rates of neck linker docking, ATP hydrolysis, and ATP-induced dissociation of ~700–800, 250–300, and 75–85 s⁻¹, respectively (25, 30, 31). Thus, this donor-acceptor FRET pair can detect the processes of neck linker docking and motor dissociation. When this experiment was repeated in the absence of acceptor, no fluorescence change could be detected over a 500-ms observation period (data not shown).

For ADP, the fluorescence increase, reflecting separation of the QSY7 quencher from the Oregon Green taxol donor, is monophasic and presumably reflects the fact that although ADP cannot dock the kinesin I neck linker, it can still dissociate kinesin I from the microtubule. This would separate the Oregon Green taxol from the QSY7 fluorescence quencher and increase the fluorescence. The rate constant for this process, reflecting ADP-induced dissociation, fits a hyperbolic dependence on [ADP], defining a maximum rate of dissociation 110.6 ± 11.8 s⁻¹ (Fig. 3B, inset).

By having established the utility of this FRET pair in kinesin I, we next turned our attention to Eg5. We measured the distances between QSY7 on the Eg5 neck linker and Oregon Green taxol on the microtubule in rigor and in the presence of AMPPNP, and the results are summarized in TABLE THREE. As in the case of the kinesin I construct, these results would predict an increase in FRET emission associated with ATP binding, followed by a second increase as the Eg5 construct dissociates. Fig. 4A illustrates the experimental design. Fig. 4B demonstrates the fluorescence transients produced by mixing Oregon Green taxol-labeled microtubules + QSY7-labeled CLM-W127F with ATP (red) or ADP (green) and confirms our prediction. As with kinesin I, the transient with ATP consists of two phases of increasing fluorescence, although in this case there is no intervening lag. However, unlike kinesin I, two phases are also seen with ADP. Over an ATP concentration range of 10–1000 μM, the rate of the first phase (Fig. 4C, solid red curve) is similar to the rate of the rising phase in the mant-ATP fluorescence transient (Fig. 2A), and it extrapolates to a maximum rate constant of 78.8 ± 6.3 s⁻¹ (TABLE TWO). This similarity suggests that the isomerization that we discussed above, which controls how fast ATP can bind to the catalytic site, is neck linker docking. This conclusion in turn implies that, unlike kinesin I, the structure of the catalytic site of Eg5 is tightly correlated with the orientation of its neck linker. The slower phase in the ATP-induced transient reaches a maximum of 7.5 ± 0.6 s⁻¹ (Fig. 4C, dashed red curve; TABLE TWO), similar to the rate of dissociation measured with the microtubule tryptophan → mant nucleotide FRET approach (Fig. 2).

We repeated these experiments with ADP, and the resulting transient was biphasic. This result implies that the neck linker of Eg5 behaves in at least some manner that is fundamentally different from its kinesin I counterpart, an issue that will be discussed at length below. The rates of both phases of the transient varied with ADP concentration, defining maximum values of 210 ± 60 s⁻¹ (Fig. 4D, closed red circles, solid red curve) and 15.2 ± 3.8 s⁻¹ (Fig. 4D, open red boxes, dashed curve) for the fast and slow phases, respectively.

In order to understand what these two phases may represent, we wished to correlate their kinetics with those for ADP binding and ADP-induced dissociation from the microtubule. To do so, we applied the methods described above under “FRET Approach 1.” We monitored the kinetics of binding of the fluorescent ADP analogue 2’dmD by FRET from microtubule tryptophan residues. As with the case for 2’dmT, the resulting fluorescence transient consisted of rising and falling phases, reflecting 2’dmD binding to the catalytic site and dissociation, respectively (data not shown). Unlike 2’dmT, however, the rate constant of the rising phase varied linearly with 2’dmD concentration, defining an apparent second order rate constant of 0.32 μM⁻¹ s⁻¹ (solid blue curve in Fig. 4D; TABLE TWO). The rate constant of the slower phase also varies with 2’dmD concentration (Fig. 4D, open blue triangles), and extrapolates to a maximum value of 12.2 ± 4.5 s⁻¹.

By comparing the two phases of the transient produced with microtubule tryptophan → 2’dmD FRET to the two phases with the Oregon Green taxol → QSY7 FRET, several points become apparent. First, although the apparent second order rate constants for the faster phase with these two FRET pairs are similar (Fig. 4D, solid red and blue curves), the rate of this phase with the Oregon Green taxol → QSY7 FRET pair reaches a maximum. This implies that the Oregon Green taxol → QSY7 FRET pair monitors a conformational change that requires ADP binding but occurs after this event. Second, the slower phase of the Oregon Green taxol → QSY7 transient shows an ADP concentration dependence that is very similar to that for 2’dmD. This suggests that both FRET pairs are reporting the same event, dissociation induced by ADP or its fluorescent analogue.

FRET Approach 3, Microtubule Tryptophan to Neck Linker AEDANS Monitors an Additional Step That Follows ATP Binding but Precedes Motor Dissociation—We have proposed that the first phase in the transient produced by mixing Oregon Green taxol-labeled microtubules + QSY7-labeled CLM-W127F is because of neck linker docking onto a stationary motor domain. However, we cannot rule out the possibility that this phase in the transient is due instead to movement of both the neck linker and the motor domain together as a rigid unit. In order to distinguish between these possibilities, we needed to develop a way of monitoring the position of a FRET acceptor on the Eg5 neck linker.
relative to donors on both the microtubule and the Eg5 motor domain. The approach we utilized is described in this section and below and is based on an approach developed in our prior studies of the kinesin neck linker (25).

Of the eight tryptophan residues in αβ tubulin, four are within 20 Å of the surface of the microtubule lattice and are therefore able to serve as donors to an AEDANS probe on the neck linker. We found that when we mixed an AEDANS-labeled kinesin I + microtubule complex with ATP in the stopped flow, and excited the AEDANS fluorescence by FRET from the microtubule tryptophans, we noticed a fluorescence decrease that occurred in two phases, an initial rapid drop in FRET (∼800 s⁻¹ at 20 °C), which we showed was because of neck linker docking, followed by a slower further drop, because of dissociation of kinesin I from the microtubule (25).

We were expecting to see similar results with Eg5, e.g. mixing AEDANS-labeled CLM-W127F + microtubules with ATP should produce a biphasic decrease in FRET emission, with rate constants of ∼65 s⁻¹ (neck linker docking) and ∼7 s⁻¹ (Eg5 dissociation). However, we observed instead that the fluorescence decrease consisted of a single phase, and its maximum rate constant was faster than that for dissociation. Fig. 5A shows a schematic of the experimental results with ATP, where the red circles represent the four microtubule tryptophan residues...
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| Reaction | MT Trp → Eg5 mant nucleotide in catalytic site | MT Trp → Eg5 neck linker AEDANS | Eg5 Trp-127 → Eg5 neck linker AEDANS | MT Oregon Green taxol→ Eg5 neck linker QSY7 | MT mant GDP → Eg5 neck linker Oregon Green |
|----------|-----------------------------------------------|---------------------------------|--------------------------------------|---------------------------------------------|---------------------------------------------|
| Neck linker docking ($k_1$) | $62 \pm 17 \text{ s}^{-1}$ | $62.4 \pm 6.5 \text{ s}^{-1}$ | $78.8 \pm 6.3 \text{ s}^{-1}$ | | |
| ATP binding to Eg5:MT ($k_2$) | $1.1 \pm 0.1 \mu\text{M}^{-1} \text{s}^{-1}$ | | | | |
| ATP dissociation from Eg5:MT ($k_{-1}$) | $19 \pm 9.6 \text{ s}^{-1}$ | | | | |
| Strong → weak transition with ATP ($k_s$) | $10.3 \pm 0.3 \text{ s}^{-1}$ | $9.9 \pm 0.6 \text{ s}^{-1}$ | | $11.6 \pm 0.7 \text{ s}^{-1}$ | |
| ATP-induced dissociation ($k_6$) | $6.3 \pm 0.3 \text{ s}^{-1}$ | | | $7.5 \pm 0.6 \text{ s}^{-1}$ | |
| Isomerization of Eg5:ADP neck linker ($k_0 + k_{00}$) | $28.2 \pm 8.1 \text{ s}^{-1}$ | | | $33.1 \pm 3.2 \text{ s}^{-1}$ | |
| Eg5:ADP binding to MT ($k_0$) | $17.3 \pm 1.7 \mu\text{M}^{-1} \text{s}^{-1}$ | $20.1 \pm 3.8 \mu\text{M}^{-1} \text{s}^{-1}$ | | $19.2 \pm 3.1 \mu\text{M}^{-1} \text{s}^{-1}$ | |
| Dissociation of Eg5:ADP from MT ($k_{-1}$) | $46.6 \pm 10.9 \text{ s}^{-1}$ | | | $51.1 \pm 18.4 \text{ s}^{-1}$ | |
| Equilibration of weak and strong Eg5:ADP states ($k_0 + k_{00}$) | $254 \pm 46 \text{ s}^{-1}$ | | | $220 \pm 35 \text{ s}^{-1}$ | |
| ADP dissociation from Eg5:MT ($k_{-1}$) | $76 \pm 15 \text{ s}^{-1}$ | | | | |
| ADP binding to Eg5:MT ($k_0$) | $0.3 \pm 0.1 \mu\text{M}^{-1} \text{s}^{-1}$ | | | | |

* MT indicates microtubule.

Dyes that can act as donors in this experiment (25, 32), the blue circles represent the AEDANS probe, and the blue rays represent the sensitized FRET emission. Fig. 5C demonstrates a transient (green) produced by mixing with ATP. The transient could be readily fit to a single exponential decay. Direct excitation of the dansyl probe produced no fluorescence change (data not shown), establishing that the fluorescence decay depicted in Fig. 5C is not due simply to changes in acceptor quantum yield. The inset in Fig. 5C depicts a plot of rate versus nucleotide concentration and reveals a maximum rate of $10.7 \pm 0.3 \text{ s}^{-1}$ (Fig. 5C, inset, red). By contrast, when we mixed AEDANS-labeled CLM-W127F + microtubules with 2 mM ATP in the stopped flow and monitored turbidity, we observed a single exponential decay with rate constant of $6.9 \pm 0.7 \text{ s}^{-1}$ (data not shown).

The process that we do monitor with the microtubule tryptophan → neck linker AEDANS donor acceptor pair, at $10.7 \pm 0.3 \text{ s}^{-1}$, is over 60% faster than the rate at which CLM-W127F dissociates from the microtubule and is similar to the rate of ATP hydrolysis (29). What is happening during this step? Because we do observe a change in FRET that precedes dissociation, we can say that this step produces a further reorientation of the neck linker relative to the microtubule. However, we cannot tell whether the neck linker in this step is moving by itself, as it would during docking, or whether it moves in concert with the rest of the motor domain. To do so requires a FRET approach that can monitor the distance between a probe on the neck linker and one on the Eg5 motor domain. This is described below.

FRET Approach 4, Eg5 Tryptophan 127 to Eg5 Neck Linker AEDANS Monitors Both Neck Linker Docking and Motor Rolling—We modified the tryptophan-to-AEDANS FRET approach by repeating these experiments with a CLM construct in which position 127 in the mutant was converted back to tryptophan (referred to as CLM-W127). Fig. 5B illustrates the schematic of the experimental approach, in which the AEDANS acceptor now can be excited by FRET from both microtubule tryptophans and Eg5 tryptophan 127. Fig. 5C demonstrates the transient resulting from mixing AEDANS-labeled CLM-W127 + microtubules with ATP (red transient). This time, there is an additional, faster component (arrow). Furthermore, the amplitude of the slower phase with CLM-W127 is similar to the amplitude of the transient for the CLM-W127F construct (Fig. 5C, green transient). We interpret this to mean that the first phase is due to a movement of the neck linker relative to tryptophan 127 and the motor domain to which it is attached, whereas the second phase reflects movement of the motor domain + the neck linker together, as a unit, relative to the microtubule. Fitting to a double exponential decay (Fig. 5C, inset) reveals that the faster phase has a maximum rate constant of $62.4 \pm 6.5 \text{ s}^{-1}$ (solid blue curve) and the slower phase has a maximum rate constant essentially identical to what is seen in the CLM-W127F mutant (TABLE TWO) and ~50% faster than the rate of Eg5-microtubule dissociation.

By combining these last two FRET approaches, we arrive at two conclusions. First, ATP binding changes the orientation of the neck linker relative to both the microtubule and the motor domain. This is similar to what has been referred to as “docking” in the case of kinesin I (10), although note that the rate of this process in Eg5 is over 10-fold slower. Second, there is an additional reorientation of the neck linker that precedes dissociation, occurs with ATP hydrolysis and formation of a weak binding state, and in which the neck linker and motor domain rotate as a unit relative to the microtubule. We refer to this process, which has not been described for kinesin I, as "rolling.

FRET Approach 5, Microtubule Mant-GDP to Eg5 Neck Linker Oregon Green 488 Also Monitors Motor Rolling—Our conclusion that Eg5 undergoes a second reorientation with formation of a weak binding state is based so far on one donor-acceptor system, utilizing microtubule tryptophan → neck linker AEDANS FRET. In order to support our conclusions, we utilized one additional FRET donor-acceptor pair. In this approach, the donor is mant-GDP in the exchangeable nucleotide-binding site of the microtubule, and the acceptor is Oregon Green 488 maleimide attached to the CLM-W127F neck linker. We formed a complex of mant-GDP-labeled microtubules + Oregon Green 488-labeled CLM-W127F and mixed it in the stopped flow with varying concentrations of ATP. The resulting transient consists of a monophasic decrease in fluorescence that fits a single exponential decay (data not shown). A plot of rate constant versus ATP concentration is illustrated in Fig. 6 (solid magenta boxes), and the plot shows that the data for this FRET pair is nearly superimposable on that for the microtubule tryptophan → AEDANS-labeled CLM-W127F FRET pair (open green boxes), as well as the slower phase in the transient for experiments with CLM-W127 (solid green boxes). Fitting to a hyperbola reveals a maximum rate of $11.6 \pm 0.7 \text{ s}^{-1}$ (TABLE TWO).

Fig. 6 also reproduces, for comparison sake, the plots of rate versus [ATP] for the slower phases in the transients illustrated in Figs. 2 and 4. As noted above, we propose that these phases in the transients using the
Using FRET to Monitor Eg5:ADP Rebinding to the Microtubule and ADP Release—In the previous sections, we developed a series of FRET probes to monitor the timing of neck linker and motor domain movements in relation to ATP binding, hydrolysis, and motor dissociation. We next wanted to use this same approach to examine what happens during the second half of the Eg5 mecanochanical cycle, when Eg5:ADP rebinds to the microtubule and releases ADP.

We first monitored the kinetics of the interaction of Eg5:dmD with the microtubule by using FRET from microtubule tryptophan to ADP. We performed these studies so we could correlate the kinetics of the tryptophan-to-AEDANS FRET changes produced by mixing ATP with either microtubules + AEDANS-labeled CLM-W127F or microtubules + AEDANS-labeled CLM-W127. A schematic of the reaction with CLM-W127F. Energy transfer efficiency between the four vicinal microtubule tryptophan residues (symbolized as the red circle in the tubulin heterodimers) and the AEDANS probe (blue circle) on the Eg5 neck linker is presumed to be the same in the two orientations that we propose the neck linker assumes in rigor. Binding of ATP drives this equilibrium distribution to favor neck linker docking and is followed by hydrolysis. The formation of a weak binding conformation alters the orientation of the neck linker so that energy transfer is no longer detectable. Energy transfer can now occur between both the microtubule and Eg5 tryptophan residues (red circles). Furthermore, energy transfer between Eg5 tryptophan 127 and the AEDANS probe can only occur in the undocked orientation. ATP-induced neck linker docking would then reduce overall energy transfer efficiency by separating the AEDANS probe from tryptophan 127 by a distance of 2R_0 (~36–40 Å). Energy transfer between microtubule tryptophan residues and the AEDANS probe, however, would continue until the motor assumes the weak binding conformation. C, fluorescence transients produced by mixing microtubules + AEDANS-labeled CLM-W127F (green) or microtubules + AEDANS labeled CLM-W127 (red) with 800 μM ATP. The transient with CLM-W127F is monoexponential. By contrast, the transient with the CLM-W127 has an additional faster component (arrow). Inset, plot of rate constant versus ATP concentration with the two constructs. The data with CLM-W127F (open red circles) fit a hyperbola (solid red curve) with maximum rate constant of 10.3 ± 0.3 s⁻¹. The rate constant of the slower phase of the transient with the CLM-W127 shows an essentially identical ATP concentration dependence, with a maximum rate constant of 9.9 ± 0.6 s⁻¹. The rate constant of the faster initial phase seen with CLM-W127 also varied with [ATP] (solid blue circles), with a maximum rate of 62.4 ± 6.5 s⁻¹.
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FIGURE 6. Plots of rate versus [ATP] or (2’dM) for FRET pairs monitoring dissociation and strong — weak transition (rolling). Oregon Green maleimide-labeled CLM-W127F + mant-GDP-labeled microtubules were mixed in the stopped flow with ATP. The resulting fluorescence decay was monoexponential (data not shown) and its rate constant varied hyperbolically with ATP concentration (magenta boxes, solid magenta curve), extrapolating to a maximum rate of 11.6 ± 0.7 s⁻¹. This rate dependence is nearly superimposable on the corresponding data with CLM-W127F (open green boxes) and CLM-W127 (solid green boxes). By contrast, the rate constant for slower phase of the transient produced by mixing Oregon Green taxol-labeled microtubules + QSY7-labeled CLM-W127F (solid red circles, curve) extrapolates to a maximum rate that is nearly 60% smaller (7.5 ± 0.6 s⁻¹). Likewise, the rate constant for the falling phase in the transient produced by mixing microtubules + CLM-W127F with 2’ dmD (open red circles, dotted red line) extrapolates to a maximum rate that is nearly 80% smaller (6.3 ± 0.3 s⁻¹).

of the fluorescence changes that we would observe by using our other FRET pairs with those for microtubule binding and product release. Experiments in this section were performed in two ways, by using either a large excess of Eg5 or a large excess of microtubules. Both methods gave identical results (data not shown). The transient resulting from mixing a complex of CLM-W127F:2’ dmD with microtubules + 2 mM ATP resembled that in Fig. 2A, with both a rising and falling phase, reflecting Eg5:2’ dmD binding to the microtubule and 2’ dmD dissociation, respectively (data not shown). The rates of both phases are plotted in Fig. 7, B and C (solid and open green circles), under conditions of Eg5 excess. The rate constant of the rising phase (Fig. 7, B and C, solid green circles), which measures how rapidly Eg5:ADP forms a weak bond with the microtubule, varied linearly with Eg5 concentration, defining an apparent second order rate constant of 17.3 ± 1.7 μM⁻¹ s⁻¹ and an apparent dissociation rate constant of 46.6 ± 10.9 s⁻¹. This rate constant for the falling phase, reflecting microtubule activated 2’ dmD release, extrapolated to a maximum rate of 76 ± 15 s⁻¹ (TABLE TWO).

Current models of kinesin I propose that the neck linker is disordered when the motor is in rigor or in ADP (1–3). If the Eg5 neck linker behaved similarly, its orientation relative to probes on the microtubule would remain random and would therefore not change after binding to the microtubule. Hence, we would predict that mixing QSY7-labeled CLM-W127F with Oregon Green taxol-labeled microtubules or AEDANS-labeled CLM-W127F with unlabeled microtubules should both produce a FRET signal that consists of a single phase.

Fig. 7A depicts the fluorescence transient resulting from mixing an excess of QSY7-labeled CLM-W127F:ADP with Oregon Green taxol-labeled microtubules, and the inset shows the fluorescence transient produced by mixing an excess of AEDANS-labeled CLM-W127F + ADP with microtubules. The transients are in opposite directions, as expected from the nature of the donor-acceptor pairs. In both cases, however, they consist of two well separated phases, contrary to what would have been predicted if the Eg5 neck linker behaved identically to that for kinesin I. For the Oregon Green taxol → QSY7 FRET pair, the rate constants for both phases vary hyperbolically with CLM-W127F concentration (Fig. 7B, solid red circles), extrapolating to a maxima of 229 ± 35 s⁻¹ (solid red curve) and 33.1 ± 3.3 s⁻¹ (dotted red curve). The corresponding phases for the microtubule tryptophan → AEDANS FRET pair also varied hyperbolically with CLM-W127F concentration with maximum rates of 254 ± 46 s⁻¹ (Fig. 7C, solid blue curve) and 28.2 ± 8.1 s⁻¹ (Fig. 7C, dashed blue curve).

**DISCUSSION**

The FRET Strategy—We have used the FRET approach detailed in this study to develop a model of how and when Eg5 generates force and movement, which is discussed below. TABLE ONE summarizes the donor-acceptor pairs used in this study and the steps in the Eg5 mechanochemical cycle that we propose they can detect. TABLE TWO summarizes the rate constants for the various reactions monitored by the FRET donor-acceptor pairs.

There are several advantages to utilizing FRET in kinetic studies. First, FRET signals have a high signal-to-noise, an essential feature for rapid kinetic studies. Second, it is possible to design a variety of different FRET approaches that, by providing multiple constraints, can be used to test the validity of the mechanochemical models. This is evident in TABLE TWO, where six of the rate constants were measured by at least two different donor-acceptor pairs, with good concordance between results. Finally, the high sensitivity of FRET efficiency changes in interprobe distance provides the potential to monitor inter-domain movements in real time. FRET efficiency depends on the interprobe distance, R, and the Förster critical energy transfer distance, R0. The value of the latter is affected by the orientation factor κ², which cannot be precisely determined for the donor-acceptor pairs utilized in this study (33, 34). This means that the fluorescence changes seen in Figs. 3–7 could theoretically be because of changes in orientation, rather than changes in distance between the donor and acceptor probes. However, in a separate study3 we have found that the limiting anisotropies for probes on the Eg5 neck linker are <0.25, which effectively reduces the error in determining κ² to <10% (35).

ATP Binding Leads to Docking and Rolling—Our model of the Eg5 mechanochemical cycle is summarized in Fig. 8, and it was designed to satisfy the multiple constraints provided by using five donor-acceptor pairs. We enter the ATPase cycle with a rigor complex, which is an equilibrium mixture of two neck linker orientations with equilibrium constant K1. One of these is equivalent to that seen in ATP, and we will call this the “docked” orientation. The other, represented by the leftmost portion of Fig. 8, we will call the “rigor” orientation. We do not know the precise nature of this latter orientation, but several lines of evidence suggest that it is not far from the docked position. First, in contrast to kinesin I, cryo-electron microscopic reconstructions of Eg5-decorated microtubules indicate rather subtle differences in the location of the tethered head in rigor versus AMPPNP. Furthermore, in rigor, there appears to be some evidence that the tethered head (and presumably, the neck linker to which it is attached) can occupy two different orientations located toward the plus end of the microtubule (40). Second, our FRET distance measurements demonstrate that there is less than a 10% change in mean distance between a probe on the Eg5 neck linker and one on the microtubule between rigor and AMPPNP. By contrast, kinesin I shows a nearly 54% difference in the distance between these two states (TABLE THREE). Although we were not able to measure a value of K1, our kinetic results suggest that this equilibrium markedly favors the rigor orientation. Our kinetic results also argue that there is tight coupling between the position of the neck linker and the structure of the catalytic site, such that ATP cannot bind to Eg5 unless its neck linker is docked.

Following neck linker docking and ATP binding, there is a second conformational change with rate constant k3, which can be monitored.

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3 J. Xing and S. Rosenfeld, manuscript in preparation.
by both the microtubule tryptophan → neck linker AEDANS and microtubule mant-GDP→ neck linker Oregon Green FRET pairs. It consists of a single transition that precedes dissociation. Although the rate constant for this process is less than 2-fold faster than that for dissociation (Fig. 6), we see it consistently using two different FRET pairs. In a previous study, the rate of ATP hydrolysis was measured for a similarly sized Eg5 construct to be 13.6 s⁻¹ at 25 °C (29). Given the lower temperature of our study (20 °C), we argue that the change in FRET efficiency measured by these donor-acceptor pairs is because of a second reorientation of the neck linker, one that occurs with ATP hydrolysis and formation of a weak binding state. Furthermore, our results with the tryptophan 127 → neck linker AEDANS FRET (Fig. 5) lead us to conclude that weak binding leads to a rolling of the entire motor complex on the microtubule. A similar orientation change upon formation of the weak binding state has been proposed for Kif1A (36). Motor rolling is then followed by dissociation from the microtubule, which we measured with both the microtubule tryptophan→ mant and Oregon Green taxol→ QSY7 FRET pairs. Our measured rates of 6.3−7.5 s⁻¹ are similar to what had been reported previously (29) with a wild type monomeric construct.

From Motor Dissociation to Product Release—In the docked orientation, the neck linker lies in a groove on the motor core, parallel to the long axis of the motor (37). However, the crystal structure of Eg5:ADP in the absence of microtubules demonstrates that the neck linker is perpendicular to the long axis of the motor (Protein Data Bank code 1ll6; see Ref. 23). This implies that once dissociated from the microtubule, Eg5:ADP,P must move its neck linker into a new position, which is illustrated in Fig. 8 by the reorientation of the magenta bar to a perpendicular position relative to the green motor domain. Although we have no direct measure of k₆ + k₅, the kinetics of Eg5:ADP rebinding to the microtubule, discussed below (Fig. 7; TABLE TWO), provide us with an indirect measure of 28–33 s⁻¹.

Binding of Eg5:ADP to the microtubule was monitored by three FRET approaches (Fig. 7). Our results suggest that the microtubule tryptophan → 2'dmD approach senses Eg5:ADP binding, that the microtubule tryptophan → AEDANS approach senses the weak → strong transition, and that the Oregon Green taxol → QSY7 approach senses neck linker movement (TABLE ONE). Consequently, we conclude that after initial weak binding of Eg5:ADP to the microtubule, the motor undergoes two conformational changes that occur in a concerted manner, formation of a strong bond with the microtubule and rotation of the neck linker from a perpendicular orientation to the rigor orientation (Fig. 8). This is then followed by ADP release, to complete the cycle.

Although this discussion explains most of our findings, it does not explain why binding of our Eg5:ADP construct produces two phases in the fluorescence transients with the microtubule tryptophan → AEDANS and Oregon Green taxol → QSY7 approaches. For both donor acceptor pairs, the plots of rate constant versus CLM-W127F concentration are similar (Fig. 7, B and C, dotted red and blue curves), with hyperbolic dependence on construct concentration. We propose what is depicted in Fig. 2 (data not shown). The rate constant for the rising phase, representing initial binding to the microtubule (solid green circles), varied linearly with [CLM-W127F], defining an apparent second order rate constant of 17.3 ± 1.7 μM⁻¹ s⁻¹ and an apparent dissociation rate constant of 50.3 ± 4.9 s⁻¹. The falling phase, reflecting 2'dmD dissociation, extrapolated to a maximum value of 76 ± 15 s⁻¹. The two phases of the transient produced by mixing QSY7-labeled CLM-W127F with Oregon Green taxol-labeled microtubules (red closed and open circles) showed a hyperbolic dependence of rate on CLM-W127F concentration, and the results are summarized in TABLE TWO. C, corresponding plot for the two phases of the transient produced by mixing AEDANS-labeled CLM-W127F with microtubules (solid, open blue circles). The data with microtubule tryptophan → 2’dmD (green) are reproduced here for comparison.

![Figure 7](http://www.jbc.org/content/280/42/35693/F7.large.jpg)

**FIGURE 7.** Kinetics of Eg5:ADP rebinding to microtubule and of ADP release. A, transient produced by mixing 5 μM QSY7 maleimide-labeled CLM-W127F with 1.5 mM Oregon Green taxol-labeled microtubules. The fluorescence change consists of two phases of decreasing fluorescence, implying that the QSY7 probe is approaching and progressively sensitized by the tryptophan donors. B, plots of rate constants versus CLM-W127F concentration for microtubule tryptophan → 2’dmD (green) and Oregon Green taxol → QSY7 (red). CLM-W127F labeled with 2’dmD was mixed in the stopped flow with microtubules + 2 mM ATP. The resulting fluorescence transient consisted of a rising and falling phase, similar to the microtubule tryptophan → neck linker AEDANS and reverse fluorescence transient consisting of two phases of increasing fluorescence, implying that the AEDANS probe is approaching and becoming progressively sensitized by the tryptophan donors.
that these results are most consistent with the assumption that the step characterized by the equilibrium constant $K_5$ is relatively slow compared with the next step, that both orientations of the neck linker of dissociated Eg5:ADP are populated (Fig. 8), and that $k_5/k_{11002}$ is $28–33$ s$^{-1}$.

Comparison with Kinesin I—We note that there are some important similarities between the mechanochemical cycles of Eg5 and ATP. For example, like kinesin I, ATP binding leads to neck linker docking, in a process that precedes hydrolysis. Likewise, as was pointed out previously (29), rebinding of monomeric Eg5:ADP to the microtubule is quite rapid. However, there are a number of striking differences between these two motors as well, most of which center around the nature and orientation of the neck linker and the motor domain-microtubule interface in the two motors.

The first difference that we note is that a rate-limiting isomerization precedes ATP binding and that there is tight coupling between nucleotide affinity and neck linker orientation. If this coupling did not exist, rapid ATP binding, followed by hydrolysis, could occur in a population of Eg5 motors before neck linker docking could occur. This would enhance the probability that a motor could dissociate from the microtubule before it had docked its neck linker and thrown its tethered partner motor toward the next microtubule-binding site. This degree of safety may be necessary for a motor generating sustained tension, because loss of even a few cross-bridges could lead to spindle collapse.

We also find evidence for a second class of movement, which we refer to as rolling, which occurs with formation of a weak binding state. We have not been able to detect similar movements with our prior studies of kinesin I. However, there is crystallographic evidence for a similar orientational change between weak and strong binding states in Kif1A (36). What function would rolling serve? In a motor like Eg5, which is

### TABLE THREE

**FRET-determined distances between microtubule-bound taxol and neck linker for monomeric kinesin I and Eg5 constructs**

| Construct        | Condition | $R_{0}$ | $R$ | Half-width |
|------------------|-----------|---------|-----|------------|
|                  |           | Å       | Å   | Å          |
| K349             | Rigor     | 37.7    | 37.3| 3.6        |
| K349             | AMPPNP    | 37.7    | 56.9| 1.2        |
| CLM-W127F        | Rigor     | 58.9    | 60.2| 9.1        |
| CLM-W127F        | AMPPNP    | 58.9    | 65.2| 4.9        |

FIGURE 8. **Model of the Eg5 mechanochemical cycle.** The Eg5 motor domain is depicted as the green ellipse or box, the neck linker as the magenta line, and the microtubule as the linear array of $\alpha$ and $\beta$ subunits. Strong binding states are depicted as green ellipses, whereas weak binding ones are depicted as green rectangles. Starting with rigor-bound Eg5 (extreme left), we propose that the neck linker remains in an equilibrium distribution between two discrete orientations, parallel to the long axis of the motor (docked) or oriented at a slight angle (rigor). Tight coupling between the conformations of the catalytic site and the neck linker ensures that ATP can bind only to Eg5 with its neck linker in the docked position. This is followed by hydrolysis and formation of a weak binding state, which produces a rolling of both the motor domain and the neck linker on the microtubule lattice. Dissociation and phosphate release follow, and this allows the neck linker of Eg5:ADP to rapidly assume its favored orientation, perpendicular to the motor long axis. Rebinding to the microtubule, with the neck linker still perpendicular to the long axis of the motor is rapidly followed by conversion to a strong binding conformation, reorientation of the neck linker to a rigor position, and release of ADP. The rate constants for each of the measurable steps are listed in TABLE TWO.
designed to generate sustained opposing force, it may be necessary to keep the neck linker relatively rigid in all states, so tension is not dissipated by neck linker compliance. This would explain why the crystallographic model of Eg5:ADP has a defined structure for the neck linker (23). However, it has been argued that neck linker flexibility is an essential feature of a processive kinesin, because it would allow the tethered head to undergo a diffusional search for the next microtubule-binding site (10–16), and this could pose a potential problem if Eg5 were processive. Thus, rolling may provide a type of compromise, allowing the Eg5 motor, and the neck linker to which it is attached, to generate enough flexibility at the right time in the mechanochemical cycle so the tethered head can still undergo a diffusive search for the next binding site on the microtubule lattice.

In addition, there appears to be a fundamental difference in how the neck linker in Eg5 behaves when compared with kinesin I. As has already been discussed, the kinesin I neck linker is proposed to be flexible and disordered when the motor is dissociated from the microtubule, and when it is bound to the microtubule in rigor or in the presence of ADP (10). This variable flexibility appears to be central to how this transport motor remains processive (11). However, our results indicate that Eg5 cannot use a similar mechanism. First, the distance between an ADP-bound state to the rigor state, consistent with the proposal that the neck linker in rigor is still at least somewhat ordered. Consequently, rolling may provide a type of compromise, allowing the head to undergo a diffusional search for the next microtubule lattice.

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