“Snapshots” of Ispinesib-induced Conformational Changes in the Mitotic Kinesin Eg5*

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Background: Ispinesib is a small molecule inhibitor of Eg5 and induces neck linker docking.

Results: Ispinesib binding to Eg5 induces subsequent movement of switch II.

Conclusion: Conformational changes in L5 can alter the orientation of the neck linker regardless of the state of the catalytic site.

Significance: L5 is a key regulator of both the catalytic site and switch II.

Kinesins comprise a superfamily of molecular motors that drive a wide variety of cellular physiologies, from cytoplasmic transport to formation of the bipolar spindle in mitosis. These differing roles are reflected in corresponding polymorphisms in key kinesin structural elements. One of these is a unique loop and stem motif found in all kinesins and referred to as loop 5 (L5). This loop is longest in the mitotic kinesin Eg5 and is the target for a number of small molecule inhibitors, including ispinesib, which is being used in clinical trials in patients with cancer. In this study, we have used x-ray crystallography to identify a new structure of an Eg5-ispinesib complex and have combined this with transient state kinetics to identify a plausible sequence of conformational changes that occur in response to ispinesib binding. Our results demonstrate that ispinesib-induced structural changes in L5 from Eg5 lead to subsequent changes in the conformation of the switch II loop and helix and in the neck linker. We conclude that L5 in Eg5 simultaneously regulates the structure of both the ATP binding site and the motor’s mechanical elements that generate force.

Kinesins motors serve a wide variety of physiologies, and these differences in function are reflected in corresponding differences in key structural domains (1). Among these is an unusual motif consisting of an α-helix (α2) interrupted by a loop (L5).3 The mitotic kinesin Eg5 has the longest L5 (18 residues), whereas kinesin-1 and CENP-E have the shortest (7–10 residues) (supplemental Fig. S1A) (2). Several lines of evidence indicate that in Eg5, L5 functions to relay information on the conformational state of the catalytic site to the motor mechanical element, the neck linker (NL). First, L5 is located in close proximity to the P-loop (phosphate-binding loop, Walker A motif) and switch I (2) (supplemental Fig. S1B), which coordinate the β- and γ-phosphates, respectively, of bound ATP. Second, point mutations in L5 affect ATP and microtubule (MT) affinity (3, 4), and molecular dynamics simulations of these mutations reveal that L5 modulates the degree of flexibility of switch I and the P-loop. Finally, deleting L5 enhances the mobility of spin-labeled ADP in the active site and uncouples nucleotide binding from NL docking (5).

A number of small molecules specifically bind to this domain in Eg5 and inhibit this motor, in some cases by trapping it in a strong ADP-binding, weak MT-binding conformation (6). Monastrol was the first of these to be extensively characterized (6–10). It binds to Eg5 by forming a predominantly hydrophobic pocket, bounded by L5 and α3 (11). Crystal structures of monastrol bound to Eg5 reveal that drug binding induces a conformation in L5 that is remarkably similar to what is seen in crystallographic models of Eg5-AMPPNP, corresponding to the “ATP-like” state. Furthermore, monastrol, like ATP, induces forward “docking” of the Eg5 NL (11). A recent cryo-EM study of Eg5-decorated MTs (12) has shown that the conformation of L5 in an Eg5-MT complex in the presence of AMPPNP resembles that for monastrol-bound Eg5, determined crystallographically in the absence of MTs. This implies that monastrol binding enforces a physiologically relevant series of conformational changes that lead to a folding over of L5 and docking of the NL. A corollary of this is that studying the structures of drug-Eg5 complexes can yield insights into how changes in L5 structure, induced by drug or by nucleotide binding, are transmitted to the rest of the molecule.

In a previous study, we examined how structural changes in L5 induced by monastrol binding were transmitted to the NL by employing a stopped flow-fluorescence strategy utilizing fluorescence resonance energy transfer (FRET) from a mant-ADP donor in the active site to an Oregon Green 488 acceptor attached to the NL (13). Adding monastrol to a mant-ADP, Oregon Green-labeled Eg5 monomeric construct reduced interprobe distance by 5 Å, consistent with drug-induced NL docking. We found that although binding of monastrol to L5...
was quite rapid ($>500 \text{ s}^{-1}$), drug-induced NL docking was nearly 3 orders of magnitude slower ($\sim 0.7 \text{ s}^{-1}$). We consequently proposed that structural changes produced by drug or ATP binding occur first at L5, leading ultimately to NL docking. This implies that 1) L5 acts as a “relay,” communicating conformational information from the catalytic site to the NL, and 2) during its normal ATPase cycle, there must be at least one structural intermediate, with L5 in an “AMPNNP” (or drug-bound) conformation but with the NL undocked.

Although this work suggested the existence of several Eg5-monomastrol and, by extension, Eg5-nucleotide conformations, only one monastrol-Eg5-ADP structure has been determined, and this presumably represents the “end state” structure with a docked NL. However, higher affinity small molecule inhibitors of Eg5 have been developed, and one of these, ispinesib (supplemental Fig. S2), has been used in multiple phase I and II clinical trials in patients with solid malignancies (14, 15). Ispinesib has been shown to bind to this kinesin not only in the ADP state but also in the presence of AMPNNP and in rigor, in both the absence and presence of MTs (16).

In this study, we combine crystallographic structure determination with transient state kinetics to generate a structural model that explains the sequence of events that occur with and following the conformational changes in L5 produced by ispinesib. These results highlight the importance of L5 as a key relay in the transmission of structural information in kinesin motors.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, Purification, and Labeling of Eg5 Constructs**—A plasmid encoding for a cysteine-light human Eg5 motor construct (residues 1–367) with a single cysteine mutation in the NL (Eg5$_{V365C}$) was generated in pET-21a, and the corresponding protein construct was expressed and purified using a C-terminal His$_6$ tag as described previously (17). The $k_{cat}$ for the MT-activated ATPase for this construct (8.9 ± 0.7 s$^{-1}$) is in the general range of values reported for wild type monomeric Eg5 constructs (5.5–8.9 s$^{-1}$) (3, 18). We labeled the V356C construct with the fluorescent donor 2'-deoxy-3'-mant-ADP (2’dmD) and fluorescent acceptor Oregon Green 488 maleimide (Molecular Probes) for FRET measurements as described previously (13).

We also generated two additional cysteine-containing Eg5(1–367) mutants by means of direct chemical synthesis of the corresponding inserts (GenScript, Edison, NJ) and ligation into pET-21a vectors for bacterial expression. The first of these contains a single cysteine in L5 (Eg5$_{W127C}$). Residue Trp$^{127}$ makes a β-stacking aromatic interaction with a tyrosine in α3 (Tyr$^{211}$) in the AMPNNP and monastrol + ADP crystal structures (11, 19). Like tryptophan, the cysteine-reactive fluorophore mBBr is also composed of two rings. We therefore reasoned that labeling the Eg5$_{W127C}$ construct with mBBr might regenerate a β-stacking interaction with Tyr$^{211}$ and, in the process, generate an Eg5 monomer with an environmentally sensitive fluorophore that might be useful for reporting on conformational changes in L5. Labeling was accomplished by incubating Eg5$_{W127C}$ with a 10-fold excess of mBBr at 4 °C for 24 h, followed by removal of excess probe by gel filtration on Sephadex G25 (PD10, GE Healthcare). This yielded an mBBr/Eg5$_{W127C}$ stoichiometry of 0.6–0.8. The mBBr-labeled Eg5$_{W127C}$ retained an MT-activated ATPase $k_{cat}$ at 6.2 ± 0.2 s$^{-1}$ (correcting for a stoichiometry of 0.76 mol of mBBr/mol of MD).

The second construct contains two cysteines, one located at the C terminus of the NL and the other within the β core of the motor (Eg5$_{V256C,V365C}$). As described in our recent report (12), we have labeled Eg5$_{V256C,V365C}$ with luminescent probes to measure nucleotide-induced distance changes between the central β-sheet core of the motor and the NL. These probes include the FRET donor-acceptor pair AEDANS/DDPM as well as tetramethyl rhodamine (TMR), the latter for measuring distance changes through reversible formation of non-fluorescent rhodamine dimer. As indicated in that report, we typically labeled this construct with a 1:5 to 1:10 AEDANS/DDPM molar ratio in order to enhance the likelihood that the donor (AEDANS) would be attached to the same motor domain (MD) as the acceptor (DDPM). Labeling with a 10-fold molar excess of TMR was carried out at 4 °C for 24 h, followed by removal of excess probe by gel filtration on Sephadex G25 (PD10, GE Healthcare), yielding a labeling stoichiometry of 1.7–1.9 (12) and a preparation with a MT-activated $k_{cat}$ of 5.1 ± 1.6 s$^{-1}$.

Ispinesib has been shown to bind to this kinesin not only in the ADP state but also in the presence of MTs (16).

**Kinetic Methodologies**—Pre-steady-state measurements of binding of ispinesib to the MD were performed on a KinTek SF-2004 stopped flow with an instrument dead time of 1.2 ms. All kinetic studies were performed at 20 °C in 100 mM KCl, 25 mM HEPES, pH 7.5, 2 mM MgCl$_2$, 1 mM EGTA. Tryptophan fluorescence was monitored by excitation at 295 nm, and the emission was monitored at 90° to the incident beam through a 333-nm long pass filter. FRET from MD-bound 2’dmD to the Oregon Green 488 fluorophore on the NL of the Eg5$_{V365C}$ construct was monitored by exciting the mant fluorophore at 350 nm and observing the emission of the Oregon Green 488 through a 500-nm broadband pass filter. For experiments using the AEDANS/DDPM donor acceptor pair, the donor (AEDANS) was excited at 332 nm, and a 500-nm broadband pass filter was used to monitor changes in AEDANS fluorescence emission. We had previously reported (21) that the limiting anisotropies for fluorescent probes on the Eg5 NL are <0.25, which implies that any error in determining $k^2$ is <10% (22). For experiments monitoring changes in TMR dimer formation, the fluorophore was excited at 520 nm, and a 590-nm long pass filter was used to monitor the TMR emission.

Rigor MD-MT complexes were formed prior to stopped flow experiments by removing unbound nucleotide through gel filtration of the MD (PD10, GE Healthcare), followed by the addition of 0.5 units/ml Type VII apyrase (Sigma-Alrich) for 20 min at room temperature. For experiments using AEDANS/DDPM FRET or TMR dimer formation, MD/MT stoichiometries were typically 1.5–7, using a [MT] of 5–20 μM.

**Crystallization of the Eg5-Ispinesib Complex**—Purified Eg5(1–368) was incubated with 1 mM ispinesib and 1 mM Mg$^{2+}$-ATP for 2 h at 4 °C. Before setting up crystal trays, the sample was centrifuged at 14,000 × g for 5 min at 4 °C to pellet
undissolved inhibitor. Imperfect crystals of Eg5 with ispinesib appeared after 2 days in hanging drops by mixing 1 μl of protein-inhibitor complex (13 mg/ml; pH 6.8) with 1 μl of reservoir solution (18% PEG 3350, 0.19 M NH₄H₂PO₄ without the addition of any buffer) in Limbro 24-well plates (Hampton Research) at 4 °C. The crystals were streak-seeded onto drops containing a 1:1 ratio of protein-inhibitor complex to well solution (14% PEG 3350, 0.18 M NH₄H₂PO₄, 0.04% agarose). Crystals were grown by vapor diffusion at 4 °C in hanging drops. Rectangular plate crystals appeared overnight and were allowed to grow for several days before immersing in cryoprotectant solution (24% PEG 3350, 0.26 M NH₄H₂PO₄, 0.06 M KCl, 20% erythritol) and flash-frozen in liquid nitrogen.

Crystallographic Data Collection and Processing—Diffraction data were recorded at the Diamond Light Source on station I03. Data were processed using IMOSdiff (23) and SCALa from the CCP4 suite of programs (24). The structure of the Eg5-ispinesib complex was solved by molecular replacement (using MOLREP) using one molecule of Eg5 (PDB entry 1X88) as a search model. Three subunits were positioned and refined with REFMAC5 (24). Simulated annealing refinement was performed using PHENIX (25). The calculation of R_free used 5% of data. Electron density and difference density maps, all σＡ weighted, were inspected, and the models were improved using Coot (26). The coordinates and the cif dictionary for ispinesib were calculated using the Dundee PRODRG server (27) and PHENIX, respectively. Figures were prepared using PyMOL (28).

RESULTS

Kinetics of Ispinesib Binding to L5—Eg5 contains a single tryptophan residue at position 127, at the apex of L5. The fluorescence of this tryptophan is quenched by the binding of small molecule inhibitors of Eg5 that have a strong near UV absorbance (18), and a previous study has used this effect to measure the kinetics of ispinesib binding to L5 (16). We repeated this experiment with our Cys-light, Eg5V365C mutant to confirm that the Cys-light mutations have not appreciably altered the kinetics of binding. Results are depicted in supplemental Fig. S3. Mixing an excess of ispinesib with 5 μM Eg5V365C + 2 mM ADP in the stopped flow produces a monoeXponential decay in fluorescence (supplemental Fig. S3A), final [ispinesib] = 200 μM). The rate constant for this transient varies linearly with [ispinesib] (supplemental Fig. S3B), defining an apparent second order rate constant of 5.9 ± 0.4 μM⁻¹ s⁻¹ (Table 1). This compares with a previously reported value of 9.4 ± 0.2 μM⁻¹ s⁻¹ at a lower ionic strength and higher temperature (16).

Lad et al. (16) noted that they could still detect a small reduction in tryptophan fluorescence when ispinesib was mixed with a 1:1 Eg5-MT complex, although the αβ tubulin heterodimer has 8 tryptophan residues. However, for our studies, we have typically used a much lower Eg5/MT molar ratio (1:5–1:7) to minimize any confounding effect of motor crowding on the MT or of unbound MD. At these stoichiometries, we were unable to detect any reliable change in tryptophan fluorescence. For this reason, we generated a construct containing a single reactive cysteine at position 127 (Eg5W127C) so that we could attach an environmentally sensitive fluorophore (mBBr) at that position. We measured values for the MT-activated kcat and Km,s,MT of mBBr-labeled Eg5W127C of 5.1 ± 0.5 s⁻¹ and 0.26 ± 0.12 μM, which are very similar to the corresponding values for a monomeric wild type Eg5 construct (18).

Mixing mBBr-labeled Eg5W127C with ispinesib in the stopped flow produced a biphase fluorescence transient. The first phase consisted of a rapid, 15–20% increase in fluorescence intensity (Fig. 1A) and was followed by a very slow (<0.2 s⁻¹) fluorescence decay (data not shown). Mixing mBBr-labeled Eg5W127C with buffer produced only the slow decay component with a rate constant of <0.2 s⁻¹, and we therefore attribute this slow phase to photo-oxidation of the mBBr probe. When this experiment was performed in the presence of either 2 mM ADP or 2 mM AMPPNP, the rate constant for the rapid, rising phase varied linearly with [ispinesib] (Fig. 1B), defining apparent second order rate constants (kapp) of 1.6 ± 0.1 μM⁻¹ s⁻¹ with ADP and 1.9 ± 0.1 μM⁻¹ s⁻¹ with AMPPNP (Table 1). In both cases, the apparent dissociation rate constant, defined as the y intercept of this plot, was within 1 S.D. value of the origin. We note that the values of kapp are ~4-fold lower than that for our cysteine-light Eg5 with tryptophan at position 127. However, the linear dependence of rate on [ispinesib] is similar to what we observe with our cysteine-light Trp127 construct (supplemental Fig. S3), implying that mBBr labeling at position 127 can provide us with a reasonably reliable measure of the kinetics of L5 conformational changes produced by drug binding, particularly in the presence of a large excess of MTs. Although mixing nucleotide-free mBBr-labeled Eg5W127C with ispinesib produced a qualitatively similar transient (Fig. 1A, solid magenta

| Reaction | Method of monitoring | Ligand state | k_app[μM⁻¹ s⁻¹] | k_max[μM⁻¹ s⁻¹] |
|----------|----------------------|--------------|----------------|----------------|
| Ispinesib binding to L5 without MTs | Quenching of W127 | ADP | 5.9 ± 0.4 | 19.6 ± 0.1 |
| Ispinesib binding to L5 with MTs | mBBr fluorescence | ADP | 1.6 ± 0.1 | 32.2 ± 2.4 |
| Ispinesib-induced NL docking without MTs | mBBr fluorescence | Rigor | 316 ± 56 | 316 ± 56 |
| Ispinesib-induced NL docking with MTs | ADP | Rigor | 1.0 ± 0.1 | 1.0 ± 0.1 |
| | mBBr fluorescence | Rigor | 1.5 ± 0.2 | 1.5 ± 0.2 |

* Ligand entries are as follows. ADP, in the presence of 2 mM ADP; AMPPNP, in the presence of 2 mM AMPPNP; rigor, treated with apyrase to render nucleotide-free.
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**FIGURE 1.** Kinetics of ispinesib-induced fluorescence change in mBBr-labeled Eg5W127C. A, mixing mBBr-labeled Eg5W127C in rigor (magenta) in the presence of either 2 mM AMPPNP (blue) or 2 mM ADP (red) with 400 μM ispinesib (containing the same concentration of nucleotide) in the stopped flow produces a rise in fluorescence that is complete within the first 20 ms after mixing. A second slow phase (<0.1 s⁻¹) of declining fluorescence was also observed but was found to occur as well when samples were mixed with buffer, and it is attributed to photo-oxidation of the mBBr probe (data not shown). The rapid transients of rising fluorescence intensity could be adequately fit to a single exponential process (solid line). B, rate constants for the transients produced by mixing with ADP (red) and AMPPNP (blue) varied linearly with [ispinesib], defining apparent second order rate constants of 1.6 ± 0.1 μM⁻¹ s⁻¹ for ADP and 1.9 ± 0.1 μM⁻¹ s⁻¹ for AMPPNP. By contrast, the corresponding rate constants for the transients produced by mixing ispinesib with rigor MD (solid magenta circles and curve) varied hyperbolically with [ispinesib], defining a maximum rate constant and an apparent dissociation constant that are summarized in Table 1. Each point and bar represent the mean of 4–5 independent measurements ± S.D. Conditions were as follows: 100 mM KCl, 25 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, pH 7.5, 20 °C.

We also examined the effect of MT binding on the kinetics of ispinesib-induced L5 conformational changes by mixing a rigor complex of mBBr-labeled Eg5W127C + MTs with ispinesib in the stopped flow. The resulting fluorescence transient produced by mixing with 400 μM ispinesib is depicted in Fig. 2A (magenta transient). The initial component of this transient consists of a rapid fluorescence increase and appears analogous to the corresponding transient seen in the absence of MTs (Fig. 1A, magenta). The rate constant for this phase (Fig. 2B, closed magenta circles and solid line) likewise varies hyperbolically with [ispinesib], defining a maximum forward rate constant of 316 ± 56 s⁻¹ and an apparent dissociation constant of 63 ± 27 μM (Table 1). This initial rise is then followed by a falling phase. Approximately 80% of the amplitude corresponds to a process with a rate constant that averages 1.8 ± 0.2 s⁻¹ over a range of ispinesib concentrations (Fig. 2B, dashed magenta line and open circles). The remaining 20% of the falling phase amplitude is characterized by a rate constant of ~0.2 s⁻¹. This slow phase is also seen when mBBr-labeled Eg5W127C + MTs is mixed with buffer (Fig. 2A, green transient) and presumably reflects photo-oxidation. When we repeated this experiment in the presence of 2 mM AMPPNP, the resulting fluorescence transient consisted only of a rising phase, with a rate constant that averaged little with [ispinesib] (Fig. 2B, open blue circles) and averaged...
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2.1 ± 0.3 s⁻¹. The slower component was ~0.2 s⁻¹, consistent with this phase reflecting photo-oxidation of the probe.

These results suggest that ispinesib binding generates two conformational changes in sequence in L5 that are detected by the mBBr probe. The first is an initial rapid phase, seen in the absence of MTs in AMPMPP, ADP, and rigor and in the presence of MTs in rigor. The second is a slow phase that is present in an MD-MT complex in rigor and AMPNP. In order to identify what the slower phase may represent, we next examined the kinetics of NL movement produced by ispinesib.

Kinetics of Ispinesib-induced NL Movement—In order to study ispinesib-induced NL movement in Eg5-ADP, we applied the same approach used in our prior study of monastrol-induced conformational changes (13). Eg5V365C was double-labeled with 2’dmD in the catalytic site and Oregon Green 488 maleimide on the NL and mixed in the stopped flow with ispinesib, and FRET from the 2’dmD to the Oregon Green acceptor was measured. Fig. 3A illustrates a transient produced by mixing with 400 µM ispinesib. As we had observed with monastrol (13), ispinesib binding induces a rise in fluorescence, implying a shortening of interprobe distance. This fluorescence rise follows a lag of ~100 ms (corresponding to a rate constant of ~10 s⁻¹), implying that NL movement occurs after a faster conformational transition not detected by this FRET pair. By contrast, adding ispinesib to a complex of Eg5V365C-2’dmD in the absence of the Oregon Green 488 acceptor did not change the mBBr probe. The resulting transient consists of an increase in fluorescence, implying a reduction in interprobe distance, following a lag of ~100 ms. The transient could be adequately fit to a double exponential process (small decay followed by a large increase; solid black line), defining an apparent lag phase rate constant in the range of 10–20 s⁻¹. Inset, fluorescence emission spectra (λex = 350 nm) for 2’dmD bound to 20 µM Eg5V365C in the absence (red) and presence (blue) of 100 µM ispinesib, demonstrating essentially no effect of ispinesib on the quantum yield of the 2’dmD fluorophore. B, plot of rate constant for the rising phase versus [ispinesib], which shows that the rate of drug-induced NL docking varies little with [ispinesib] and averages 1.0 ± 0.1 s⁻¹. Conditions were as follows: 100 mM KCl, 25 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, pH 7.5, 20 °C.

Although this FRET approach gives us robust signal changes for monastrol and ispinesib, it cannot be used in the presence of MTs, because this would lead to dissociation of the 2’dmD donor. In order to measure the kinetics of NL movement induced by ispinesib binding to a rigor MT-MD complex, we utilized a different spectroscopic approach. We generated a double cysteine Eg5 construct (Eg5V256C,V365C) with a cysteine at the C terminus of the NL (V365C) and the other within the β-sheet core (V256C, strand β7). In our earlier study (12), we showed that labeling this construct either with an AEDANS/DDPM FRET pair or with TMR produced robust fluorescence signals that could detect NL docking after mixing an MT-Eg5V256C,V365C complex with nucleotide, the former through changes in FRET efficiency and the latter through changes in the content of TMR dimer. We also noted that labeling with either set of probes had little effect on the steady state ATPase parameters (12). We generated MT complexes with AEDANS/DDPM or TMR-labeled Eg5V256C,V365C and mixed this complex in the stopped flow with ispinesib. The resulting transient produced by mixing with 400 µM ispinesib is depicted in Fig. 4A for AEDANS/DDPM (green transient) and TMR (red transient). In both cases, the fluorescence decay was a single exponential process following either a small amplitude rising phase (TMR) or a lag (AEDANS/DDPM FRET). In either case, the apparent rate constant for this initial phase was ~10 s⁻¹. The rate constant for the falling phase showed little depend-

FIGURE 3. Kinetics of ispinesib-induced NL movement in Eg5V365C labeled with 2’dmD in the catalytic site and Oregon Green 488 maleimide in the NL. A, the Oregon Green fluorophore was excited by FRET from the 2’dmD donor. The resulting transient consists of an increase in fluorescence, implying a reduction in interprobe distance, following a lag of ~100 ms. The transient could be adequately fit to a double exponential process (small decay followed by a large increase; solid black line), defining an apparent lag phase rate constant in the range of 10–20 s⁻¹. Inset, fluorescence emission spectra {λex = 350 nm} for 2’dmD bound to 20 µM Eg5V365C in the absence (red) and presence (blue) of 100 µM ispinesib, demonstrating essentially no effect of ispinesib on the quantum yield of the 2’dmD fluorophore. B, plot of rate constant for the rising phase versus [ispinesib], which shows that the rate of drug-induced NL docking varies little with [ispinesib] and averages 1.0 ± 0.1 s⁻¹. Conditions were as follows: 100 mM KCl, 25 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, pH 7.5, 20 °C.
initial L5 conformational change and the final NL docking steps, there may be other structural changes occurring elsewhere in the MD that are not detected by these fluorescent probes. The picture that emerges is that ispinesib binding to L5 induces a series of sequential conformational changes that are transmitted from L5, along the MD, and ultimately lead to the NL docking. These findings in turn imply that there is at least one conformational intermediate produced by ispinesib, with L5 in a drug-bound conformation but with the NL undocked. However, our fluorescence data do not allow us to determine precisely what this intermediate looks like. To address this issue, we therefore examined the crystallographic structures of Eg5-ispinesib complexes.

**Crystal Structure of a Novel Eg5-Ispinesib Complex**—The crystal structure of the Eg5-ispinesib complex was solved to a resolution of 2.45 Å. Data collection and refinement statistics are shown in Table 2. The crystals of the Eg5 motor domain in complex with ispinesib belong to the orthorhombic space group P2₁2₁2₁. The Eg5-ispinesib complex crystallizes with three molecules (subsequently named A, B, and C) in the asymmetric unit. The Eg5-ispinesib complex crystallizes with three molecules (subsequently named A, B, and C) in the asymmetric unit. The final model comprises residues Lys¹⁵–Asn²⁷¹ and Asn²⁸³–Pro³⁶³ for molecule A; Lys¹⁷–Glu⁴⁷, Glu²₅⁴–Asn²⁷⁰, and Asn²⁸⁷–Pro³⁶³ for molecule B; and Lys¹⁵–Asn²⁷¹ and Ala²⁷⁹–Glu³⁶⁴ for molecule C. 96.11% are in the favored region of the Ramachandran plot, 2.97% are in the generously allowed region, and 0.92% of residues are outliers. In molecule B, residues Leu⁶₀–Lys⁶⁰, covering the region of helix α0 and the greater part of the small three-stranded antiparallel β-sheet, are not well defined, probably due to the absence of stabilizing crystal contacts. In this region, which displays high B-factors, the majority of side chains are only tentatively built. Additionally, we removed part of loop L1₀ in molecule B, for which we did not observe electron density. Loop L1₁ is missing, as in most other kinesin structures. All three molecules contain one molecule of ADP bound in the nucleotide-binding pocket. In molecule C, we observed one magnesium ion coordinated with ADP and two structural water molecules. However, we did not observe density for a magnesium ion in either molecule A or B. The inhibitor-binding pocket, consisting of helix α2, L5, helix α₃, and strand β₄, is occupied by ispinesib (supplemental Fig. S₄A), which has a very well defined electron density (supplemental Fig. S₄B). When the three molecules of the Eg5-ispinesib structure were overlaid, the root mean square deviation was quite high (0.84 Å). The reason is that there are large differences in loops L1, L2, L1₀, and L1₃, which could be a consequence of the crystal packing. Apart from these differences in the surface regions of the protein, all three molecules are almost identical, particularly with respect to the core and the conformations of the secondary structure elements known to be pivotal for force generation, including the NL.

**Interactions in the Inhibitor-binding Pocket**—The conformation of ispinesib and the interactions between inhibitor and Eg5 are virtually identical in all three molecules. By comparing the native structure of Eg5 in the absence of inhibitor with that in the presence of ispinesib, we observed that an induced fit pocket is formed by an outward displacement of the Arg¹¹⁹ and Tyr²¹¹ side chains and a downward displacement of the Trp¹²⁷ indole ring. Ispinesib rings A, B, C, and D (supplemental Fig. S₂), which represent the hydrophobic part of the drug, are buried in the allosteric site and display several hydrophobic interactions with residues of the inhibitor binding pocket. The coupled quinazoline rings C and D of ispinesib are stabilized on one side by a salt bridge formed between side chains of Glu¹¹⁶ and...
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Arg<sup>221</sup> (blue broken lines in Fig. 5) and on the other side by a C-H-π interaction with Leu<sup>214</sup>. Ring A stacks onto the Pro<sup>137</sup> ring and is also involved in an edge-face interaction with the phenyl ring of Trp<sup>127</sup>. The isopropyl group of ispinesib is involved in C-H-π interactions with the phenyl ring of Tyr<sup>211</sup>. We also observed two hydrogen bonds (black broken lines in Fig. 5) between the primary amine nitrogen (NAD) of ispinesib and the side-chain oxygen (OE1) of Glu<sup>116</sup> and between the oxygen (OAE) of the inhibitor and a structural water molecule.

Our kinetic results imply the existence of an “intermediate” Eg5-ADP-ispinesib state, one with L5 in the drug-bound conformation but with switch II in the “down” position and the NL undocked. The conformations of switch II and the NL in the Eg5-ADP-ispinesib structure described in this study are consistent with the features predicted for this “intermediate” structure. If this is correct, we would also predict that the conformation of ispinesib and the residues that coordinate it in our current and previously published (PDB entry 4AP0) structures of Eg5-ADP-ispinesib should be very similar. Indeed, we found that the conformation of ispinesib and the residues that coordinate its binding remain essentially unchanged between these two structures, although the solution of the previously published Eg5-ADP-ispinesib (PDB entry 4AP0) structure was complicated by twinning (supplemental Fig. S5). Detailed descriptions of the twinning problem and subsequent solution of the problem have been documented (30). This further implies that the conformation of ispinesib cannot be a factor that distinguishes the “intermediate” and “final” states of the complex.

DISCUSSION

Small Molecule Inhibitors Provide Insight into the Physiology of Kinesin Motors—Molecular motors convert the free energy of nucleotide binding and hydrolysis into movement. In the case of kinesins, they accomplish this by coupling the ATPase enzymatic cycle to corresponding cycles of forward movement by the NL. The kinesin ATPase site is separated from the NL by several nm (2, 11), so there must be an allosteric mechanism that enables these two structures to communicate their state with each other. Our prior work led us to propose that loop L5 in the mitotic kinesin Eg5 functions as a conformational relay, conveying structural information between the catalytic site and the NL (3, 12). This conclusion is supported by the finding that a variety of small molecule inhibitors of Eg5 that bind to L5, including ispinesib, not only affect its conformation but also induce movement in the switch II cluster into an orientation that permits NL docking (11). Furthermore, previous cryo-EM and crystallographic models of Eg5 have shown that the conformation of L5 in the presence of small molecule inhibitors is very similar to that in the presence of AMPPNP (12). Thus, we believe that studying the structural transitions induced by ispinesib binding can provide valuable insight into how the more physiologically relevant process of nucleotide binding induces forward movement in kinesin motors.

Crystallographic structures of Eg5 in the presence of ADP and AMPPNP have been solved, and cryo-EM reconstructions of a nucleotide-free Eg5-MT complex at significantly improved resolution have been reported recently (12). Whereas this work has provided important mechanistic insights, both crystallography and cryo-EM provide “static” pictures of discrete conformational states that do not reveal the speed or the sequence by which these state changes occur. Likewise, whereas transient kinetic studies of kinesin motors, including Eg5, have provided important information on the sequence of state changes in the ATPase cycle (3, 17, 18, 21), they do not necessarily reveal the nature of the structural transitions whose timing they monitor. We have therefore combined both approaches to generate a series of structural “snapshots” of the mitotic kinesin Eg5.

Kinetic Studies of Ispinesib Binding Identify an “Intermediate” Eg5-Ispinesib State—In order to examine the kinetics of ispinesib binding to Eg5, we utilized a series of complementary fluorescent probe systems that give robust signals even in the presence of a large excess of MTs. To do this, we generated a series of single and double cysteine-containing Eg5 mutants that our previous work had shown behave enzymatically in a manner very similar to wild type Eg5 monomeric constructs (3, 12).

Our experiments with an mBBr probe at the apex of L5 confirmed several previous findings (16), including that 1) binding of ispinesib to L5 is quite rapid in the presence of AMPPNP or ADP and does not reach a maximum rate, and 2) binding of ispinesib to a nucleotide-free preparation of Eg5 is considerably slower. These authors also noted slower binding of ispinesib to a MT-MD complex in the presence of AMPPNP. However, they used a concentration of AMPPNP in their experiments 8-fold lower than in ours. We note that AMPPNP has a relatively weak affinity for kinesin (31). This raises the possibility that in the
Ispinesib-induced Conformational Changes in Eg5

presence of MTs, a substantial proportion of MD might have been in a rigor state. Although we could not observe a maximum rate of ispinesib binding in the presence of AMPPNP or ADP, we did find that binding of drug to rigor Eg5 did approach a maximum rate (Fig. 1B). This suggests two possibilities. First, ispinesib binding to rigor Eg5 might be rate-limited by a conformational change in the loop, from one that cannot accommodate the drug to one that can. However, this would predict that the fluorescence rise associated with ispinesib binding to rigor Eg5 would be preceded by a lag, which we in fact did not observe (Fig. 1A). The second possibility is that ispinesib can bind to Eg5 in rigor but that the conformation of L5 in this state makes this process intrinsically slower. This is consistent with our earlier study, which found that L5 in a rigor MD-MT complex assumes a conformation that is relatively buried and presumably less accessible to drug (12).

Although the mBBr probe detected only one transition with ispinesib binding to MT-free Eg5, it detected two transitions with an mBBr-labeled, rigor Eg5-MT complex (Fig. 2). The first, rapid phase is kinetically similar to that seen for rigor Eg5, which we attribute to drug binding to L5. The second phase is much slower, with a rate constant of $\sim 2 \text{s}^{-1}$. This is nearly identical to the rate of ispinesib-induced NL docking, as measured both for MT-free Eg5 (Fig. 3), using a mant-ADP $\rightarrow$ Oregon Green 488 FRET strategy, and for an Eg5-MT complex (Fig. 4), monitored by means of two motor core $\rightarrow$ NL FRET pairs.

Our kinetic results are consistent with a three-step reaction pathway summarized in Scheme 1,

$$
\begin{align*}
M + l & \leftrightarrow (M \cdot l) \quad M \cdot I_1 \leftrightarrow M \cdot I_2 \\
& \\
\text{SCHEME 1}
\end{align*}
$$

where $M$ refers to the Eg5 motor domain and $l$ refers to ispinesib. Mixing motor with ispinesib leads to formation of a collisional complex, depicted as $(M \cdot l)$, which then undergoes two first order transitions, designated by the subscripts "1" and "2". We propose that the first transition corresponds to local conformational changes in L5, as measured by our mBBr probe at position 127, and the second corresponds to subsequent conformational changes in switch II and the neck linker. The general solution of this kinetic scheme follows a double exponential process, has been presented previously (33), and is included in the supplemental material.

In the absence of MTs, the fluorescent reporters that we have used monitor either the first transition (mBBr attached to L5) or the second (2’dmD $\rightarrow$ Oregon Green 488 FRET), allowing simplification of the kinetic analysis. In ADP and AMPPNP, a maximum rate for ispinesib binding to MT-free Eg5 could not be observed, implying that $k_1$ is $\gg 1000 \text{s}^{-1}$. The apparent dissociation rate constant, $k_{d}$, which is defined as the $y$ intercept of the plot in Fig. 1B, is close to zero, given the errors of the measurement (Table 1). This implies that $k_{-1}, k_{-2}$, or both are very small. Furthermore, the finding that $k_1$ is very large implies that the rate constant for ispinesib-induced neck linker reorientation, as measured by FRET (Fig. 3) is approximately equal to $k_2 + k_{-2}$. Further delineation of individual rate constants, however, requires knowing the values of the equilibrium constants $K_1$ and $K_2$, which could not be done with the fluorescence approach employed in this study. This will be the subject of a future report, using a previously described transient, time-resolved FRET approach (29).

The kinetics of ispinesib-induced NL docking for rigor MD and a rigor MD-MT complex are very similar to those measured for an MD-ADP complex (Figs. 3 and 4), implying that the value of $k_2 + k_{-2}$ is relatively insensitive to the conformation of the catalytic site. However, binding of ispinesib to L5, as monitored by the mBBr probe, is considerably slower in rigor than it is in ADP or AMPPNP, with a value of $k_1$ that is at least 3–4 times lower. These findings imply that the orientation of L5, which we have recently shown is appreciably different in rigor than in AMPPNP (12), can affect the kinetics of ispinesib binding to this loop.

Taken together, our results lead us to several conclusions. First, we propose that ispinesib-induced NL docking occurs significantly more slowly than binding of drug to L5, implying that there must be an “intermediate” Eg5-ispinesib state, one with L5 in a drug-bound conformation but with the NL undocked. This conformation would then convert to the “final”, NL-docked state at a rate of $\sim 2 \text{s}^{-1}$, in a process that is detected by distance-sensitive FRET probes in the NL and motor core. Second, our data also suggest that MT binding affects the communication pathway connecting L5 to the NL. Thus, we found that in the absence of MTs, the mBBr probe on L5 only detects local conformational changes produced by ispinesib binding. However, in an Eg5-MT complex, a second transition is detected by the mBBr probe that correlates temporally with NL docking (Fig. 2). Furthermore, although we observe a rapid rise in mBBr fluorescence when ispinesib is mixed with AMPPNP-saturated Eg5 (Fig. 1), the corresponding experiment with an mBBr-labeled Eg5-MT complex only demonstrates the slower, NL-associated transition (Fig. 2). This suggests that in an Eg5-MT complex, the binding of AMPPNP to the catalytic site induces in L5 a conformation that is very similar to what is produced by the binding of ispinesib. This result is consistent with our recent cryo-EM study of Eg5-MT complexes, which has shown that the conformation of L5 in an Eg5-MT complex in the presence of AMPPNP resembles that for monastrol-bound Eg5, determined crystallographically in the absence of MTs (12).

Finally, we note that our previous kinetic studies of monastrol binding to Eg5 are also consistent with the existence of an “intermediate” Eg5-drug state (13). In these earlier experiments, all performed with MT-free Eg5-ADP, we likewise found that drug binding to L5 was several orders of magnitude faster than subsequent NL docking. However, that study was hampered by the lack of any crystallographic structure that could correspond to such an “intermediate” state, with drug bound and the NL undocked. In order to verify the existence of such a Eg5 conformation for ispinesib, we undertook to solve a new Eg5-ADP-ispinesib structure that fulfills the features expected for this intermediate state.

Crystallographic Studies of a Novel Eg5-ADP-Ispinesib Complex Confirm the Existence of an Intermediate State—Our study defines a new conformation of Eg5 in the presence of ispinesib
that is illustrated in Fig. 6. The figure compares our new structure with previously reported structures of Eg5-ADP binary complex (PDB entry 1II6) (2) and Eg5-ADP-ispinesib ternary complex (PDB entry 4AP0) (30). Differing from the Eg5-ADP binary structure, our new structure shows loop L5 moving toward helix \( H_5 \), resulting in the closure of the inhibitor binding pocket and the trapping of ispinesib. However, our structure also shares a similarity with the Eg5-ADP structure; the switch II cluster, consisting of helix \( H_4 \), loop L12, and helix \( H_5 \), is in the obstructive or “down” position, and the NL is undocked and perpendicular to helix \( H_6 \) (Fig. 6 and supplemental Fig. S4C).

On the other hand, our new conformation differs from the previous Eg5-ADP-ispinesib structure (PDB entry 4AP0), because in that structure, the switch II cluster occupies the permissive or “up” position and thereby allows the NL to dock onto the motor domain (supplemental Fig. S4D). Therefore, we propose that our results define a new, “intermediate” Eg5-ADP-ispinesib state in which drug-induced changes in L5 have not yet led to corresponding conformational changes in the switch II cluster and the NL. We also propose that the previously published Eg5-ADP-ispinesib structure, with switch II in the permissive position and the NL docked, represents the “final” conformation that Eg5 assumes after ispinesib binding. This assignment is consistent with our kinetic studies, which demonstrate that there is a considerable delay between conformational changes in L5 and subsequent NL docking (Figs. 3 and 4). Finally, we note that we have already described an Eg5-ADP-S-trityl-L-cysteine structure that is very similar to our new Eg5-ADP-ispinesib “intermediate” (20) (PDB entry 2WOG). However, unlike the present study, this earlier report did not correlate the crystallographic model with kinetics in order to unequivocally place this S-trityl-L-cysteine-induced structure in a temporal sequence of conformational changes. These results, in conjunction with our kinetic studies on monastrol (13), however, do support our contention that the conformation of L5 per se, and not the nature of the nucleotide in the catalytic site, is what drives subsequent movement of switch II and NL docking.
L5 Is a Kinetic Regulator of the Catalytic Site and NL.—Our previous kinetic studies demonstrated that for kinesin 1, ATP binds to the catalytic site first, and NL docking follows (21), implying that a communication pathway links the catalytic site to the switch II cluster. Nevertheless, we could not identify in that study what other structural elements were also involved in this pathway. Unlike kinesin 1, ATP binding and NL docking in Eg5 occur at the same rate (17). Although this could imply that nucleotide binding produces a concerted conformational change that includes NL docking, another possibility is that both ATP binding and NL docking are gated by some other structural element that regulates the conformation of both. A logical candidate for such a structural element is L5. Point or deletion mutations of this loop uncouple nucleotide binding from NL docking (3, 5), and cryo-EM reconstructions of Eg5–decorated MTs demonstrate that in rigor, the N-terminal portion of L5 is in a position to sterically block nucleotide binding (12).

Taken together with this previous work, our current study therefore suggests that L5 acts as a kinetic regulator of both nucleotide binding and NL docking. We propose that L5 is structurally dynamic, alternating between conformations that have distinct effects on both the catalytic site and motor mechanical elements, including switch II and the NL. In one orientation (“rigor”), it blocks ATP binding, possibly through steric effects from its N-terminal segment (12), and it favors an orientation of switch II that blocks NL docking. In another (“ATP-like”), it allows ATP binding and favors NL docking. In this model, the rate of both ATP binding and NL docking would be identical and determined by how quickly these L5 orientations interconvert. ATP could thus be viewed as an allosteric modifier, driving the equilibrium of L5 orientations to one favoring NL docking. Our work thus implies that by enforcing an “ATP-like” orientation onto L5, ispinesib and other small molecule inhibitors generate a series of sequential conformational changes that ultimately lead to NL docking, even when the catalytic site is empty or contains ADP. Further studies of the energetics and kinetics of these L5 dynamic changes will be needed to obtain a more complete picture of how this interesting domain regulates the behavior of kinesin motors and will be the subject of future reports from our laboratories.

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