Getting in Sync with Dimeric Eg5

INITIATION AND REGULATION OF THE PROCESSIVE RUN

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Eg5/KSP is the kinesin-related motor protein that generates the major plus-end directed force for mitotic spindle assembly and dynamics. Recent work using a dimeric form of Eg5 has found it to be a processive motor; however, its mechanochemical cycle is different from that of conventional Kinesin-1. Dimeric Eg5 appears to undergo a conformational change shortly after collision with the microtubule that primes the motor for its characteristically short processive runs. To better understand this conformational change as well as head-head communication during processive stepping, equilibrium and transient kinetic approaches have been used. By contrast to the mechanism of Kinesin-1, microtubule association triggers ADP release from both motor domains of Eg5. One motor domain releases ADP rapidly, whereas ADP release from the other occurs after a slow conformational change at ~1 s⁻¹. Therefore, dimeric Eg5 begins its processive run with both motor domains associated with the microtubule and in the nucleotide-free state. During processive stepping however, ATP binding and potentially ATP hydrolysis signals rearward head advancement 16 nm forward to the next microtubule-binding site. This alternating cycle of processive stepping is proposed to terminate after a few steps because the head-head communication does not sufficiently control the timing to prevent both motor domains from entering the ADP-bound state simultaneously.

Eg5 is a homotetrameric Bim C/kinesin-5 family member that plays a vital role in the mitotic spindle and has attracted substantial interest as a potential target for chemotherapeutic agents in cancer treatment (1–9). Eg5, as with other members of this subfamily, provides a plus-end directed force necessary to both assemble and organize the mitotic spindle and contributes to microtubule (MT)² flux (10–30). If Eg5 function is disrupted prior to anaphase B, the bipolar spindle will collapse into a monoaaster from which the cell can no longer divide.

To some extent, the mechanochemistry of dimeric Eg5 appears like conventional Kinesin-1 (referred to as kinesin herein) in that it alternates catalysis on its motor domains to step processively along a MT (31–33) However, dimeric Eg5 only takes 8–10 steps on average (36–38), whereas conventional kinesin can take hundreds (39, 40). The mechanochemical cycle of kinesin is tuned such that it can step processively as soon as the first motor domain touches the MT track. In contrast, Eg5 requires a slow conformational change after MT collision to establish the intermediate poised to begin the processive run (35, 41). The velocity of Eg5 stepping is controlled by the rate of ATP hydrolysis, yet the rate of phosphate release determines the velocity of kinesin (35, 42). These initial studies indicate that the head-head communication used by Eg5 to establish and regulate a processive run is novel, suggesting that Kinesin-5 motors have additional mechanistic requirements that are not yet fully understood.

In this study, equilibrium and transient state kinetic approaches have been used to specifically address the mechanistic events that must occur to establish and coordinate the processive stepping of Eg5. This study has revealed that dimeric Eg5 begins a processive run from a nucleotide-free state with both motor domains associated with the MT. Once in a processive run, ATP binding with ATP hydrolysis signals rearward head advancement for the next step. Dimeric Eg5 processivity is proposed to terminate after a few steps because the head-head communication does not sufficiently control the timing to prevent both motor domains from entering the ADP-bound state simultaneously.

EXPERIMENTAL PROCEDURES

Standard Conditions—The experiments were performed at 25 °C in ATPase buffer: 20 mM Hepes, pH 7.2, with KOH, 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM potassium acetate, 1 mM dithiothreitol. The MTs were stabilized with paclitaxel (Sigma) in Me₂SO. For all mantAXP experiments, a racemate (Invitrogen) was used. Unless otherwise noted, mantAXP was excited at λex = 360 nm, and emission at 450 nm was monitored using a 400 nm long pass filter. Monomeric and dimeric Eg5 protein concentrations are reported as single motor domain or active site concentrations.

Cloning of R234K—A pRSETa plasmid with sequence encoding the first 513 amino acids of the human EG5 gene and a

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C-terminal His$_5$ tag was used as the template (36, 43). PCR site-directed mutagenesis using primers 5’-GCATACTCTAG- TAACTCCATCTAGTTTCC-3’ and 5’-GAAAAGTAGT- GGCTCTTACTAGAGTGTC-3’ was performed to mutate arginine 234 to lysine. Following PCR, the template was digested with DpnI, and the PCR mixture was used to transform the Nova Blue cell line (EMD Chemicals, Inc.). Sequence-confirmed plasmids were transformed into the BL21-CodonPlus (DE3)-RII cell line (Stratagene) for protein expression.

**Protein Purification**—Eg5-513 was purified by MT affinity as described (43). The R234K mutant was purified by a two-step column chromatography method utilizing S-Sepharose ion exchange followed by Ni$^{2+}$-nitrotriacetic acid affinity as described (44). Eg5-367$_{NF}$ was purified by column chromatography as described by Cochran and Gilbert (45). Protein concentration was determined using the Bio-Rad protein assay with IgG as the protein standard.

**Nucleotide-free Eg5-513**—Two methods were used to generate nucleotide-free Eg5-513$_{NF}$. One method is the treatment of the motor with apyrase (0.02 units/ml; grade VII, Sigma) as described (35). This apyrase isoform catalyzes the conversion of ADP to AMP predominantly. The affinity of Eg5-513 for AMP is so weak that apyrase treatment effectively generates a nucleotide-free state for Eg5. Steady-state experiments were performed using $[\alpha^{32}P]$ATP or $[\alpha^{32}P]$ADP to monitor the apyrase reaction and kinetics. At the conditions of the Eg5 kinetic experiments, the rate of apyrase conversion of $[\alpha^{32}P]$ADP to $[\alpha^{32}P]$AMP was 0.01 s$^{-1}$, and the conversion of $[\alpha^{32}P]$ATP to $[\alpha^{32}P]$ADP occurred extremely slowly at 0.0002 s$^{-1}$. Thus, the apyrase does not compete with the Eg5 ATPase in the ATPase buffer to remove the EDTA and to adjust the inner filter effect (47):
mantATP. After rapid mixing in the stopped flow, the fluorescence enhancement of mantATP binding to the active site was monitored (Fig. 3).

**MANTATP Binding to the MT-Eg5-513_{NF} Complex under Single Turnover Conditions**—A MT-Eg5-513 complex was treated with apyrase and then rapidly mixed in the stopped flow with mantATP. Final concentrations are as follows: 15 μM Eg5-513, 25 μM MTs, 1 μM mantATP (Fig. 4).

**MANTATP Release during Motor Stepping**—A MT-Eg5-513_{NF} complex was supplemented with mantATP immediately prior to loading the complex in the stopped flow. The time required to load the stopped-flow instrument was sufficient for Eg5-513NF to bind mantATP, hydrolyze the nucleotide to mantADP, and then rapidly mix with MgATP, MgADP, MgATPyS, or MgAMPNP. Final concentrations are as follows: 15 μM Eg5-513, 25 μM MTs, 1 μM mantATP, 500 μM MgAXP (Fig. 5). The ATP-promoted observed rates of mantATP release were fit to a hyperbola.

**MantATP Binding to the MT-R234K Complex**—A MT-Eg5-513 complex was treated with apyrase and then rapidly mixed in the stopped flow with mantATP. Final concentrations are as follows: 0.5 μM R234K/8 μM MTs for 0.5–4 μM MgMantATP and 2 μM R234K/8 μM MTs for 4–50 μM MgMantATP (Table 2 and supplemental Fig. S2). The data were fit to the following equation (Equation 3),

\[
    k_{\text{obs}}(K_a[k_{\text{max}}(\text{mantATP})/(K_a[\text{mantATP}] + 1)] + k_{\text{off}}) \quad \text{(Eq. 3)}
\]

where \( K_a \) represents the equilibrium association constant, \( k_{\text{max}} \) is the maximum rate constant of the ATP-promoted isomerization that forms the tightly bound mantATP intermediate that proceeds to ATP hydrolysis, and \( k_{\text{off}} \) is the dissociation of mantATP. The \( K_a(k_{\text{max}} \text{MantATP}) = 1/K_a \) and \( K_a k_{\text{max}} \) represents the second-order rate constant for substrate binding.

**Modeling the Kinetics of the Processive Run**—The linear kinetic model in the inset of Fig. 6 was constructed to attempt to reproduce the time course of mantATP release during the processive run shown in Fig. 5A. We followed the initial concentrations in time until the point where mantATP was released and the signal quenched. Experimentally, the system is prepared such that the predominant species is state E5 (Fig. 6). However, the fluorescence decay in the absence of ATP suggests that some dimers have ADP bound at both heads and are in state E0. After several seconds all curves in Fig. 5A asymptote to a value that is nearly constant on the time scale of the experiments; therefore, we assume that a fraction of the initial fluorescence signal is constant over time and that the amplitude of this fraction depends on the experimental ATP concentration. There are five free rate constants in the model: \( k_2 \), \( k_3 \), \( k_4 \), and \( k_5 \). The rate constant \( k_5 \) is a composite rate for species in state E0 to bind to MT, release mantATP, and enter state E2. The concentration-dependent rate of ATP binding to the nucleotide-free head was determined previously by the ATP binding kinetics presented in Ref. 35, where Equation 4 was based on Equation 3,

\[
    k_1 = 54 \text{s}^{-1} \times 0.11 \mu\text{M}^{-1} \cdot \text{ATP}^{-1} \times ([\text{ATP}]([0.11 \mu\text{M}^{-1} \cdot \text{ATP}] + 1))
\]

\[
    \text{(Eq. 4)}
\]

**TABLE 1**

<table>
<thead>
<tr>
<th>Protein</th>
<th>ADP (μM)/active site (μM)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eg5-513</td>
<td>0.64 ± 0.01</td>
<td>0.62–0.66</td>
</tr>
<tr>
<td>Dimeric kinesin K401</td>
<td>0.92 ± 0.02</td>
<td>0.83–1</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>0.01 ± 0.002</td>
<td>0.001–0.013</td>
</tr>
<tr>
<td>Eg5-513 + 1 mM MgADP</td>
<td>0.59 ± 0.04</td>
<td>0.44–0.74</td>
</tr>
</tbody>
</table>

**FIGURE 1.** MantADP titration of Eg5 motors free in solution. Titrations of nucleotide-free monomeric and dimeric Eg5 motors were performed at constant volume and motor concentration. A, 300 nM monomeric Eg5-367_{NF} was titrated with mantATP. The resultant increase in fluorescence was best fit by a single hyperbola yielding an apparent \( K_{\text{dMantADP}} = 1.2 ± 0.1 \mu\text{M} \). B, 300 nM dimeric Eg5-513_{NF} was titrated with mantADP. The resultant increase in fluorescence was best fit by a sum of two hyperbolas yielding an apparent \( K_{\text{dMantADP}} = 0.62 ± 0.14 \mu\text{M} \) for the initial phase. C, 4 μM dimeric Eg5-513_{NF} was titrated with mantADP. The resultant increase in fluorescence was best fit by a single hyperbola yielding an apparent \( K_{\text{dMantADP}} = 3.3 ± 0.1 \mu\text{M} \).

The initial concentration of molecules in states E5 and E0 and the value of the constant background fluorescence provided two more free parameters per curve in Fig. 5A. We systematically searched for a single set of rate constants that described all of the curves in Fig. 5A. For each set of initial conditions and a given ATP concentration, the kinetic model was solved using
the ordinary differential equations function ODE15 in Matlab. This was repeated for all six transients, and the root mean square differences of the model solution versus the experimental data were calculated for each curve. The root mean square difference was then used as a fitness function in a Nelder-Mead search algorithm to determine the parameter set that maximized the goodness of the fit (48). Initial rates and values were chosen from a reasonable domain with a random number generator, and well over 100,000 sets of parameters were searched. The best fit is shown in Fig. 5C, and the corresponding values are presented in the legend of Fig. 5.

**RESULTS**

Our initial kinetic analysis of Eg5-513 suggested that steady-state ATP turnover by dimeric Eg5 is governed by a slow structural transition (35, 43). This slow conformational change occurs after collision with the MT but prior to processive stepping (35, 36). To dissect the sequence of events required to establish the processive run, an analysis of Eg5-513 in the absence of MTs was pursued to assess whether the motor occurs after collision with the MT but prior to processive stepwise (35, 43). This slow conformational change (35, 43).

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These results indicate that dimeric Eg5 in solution shows an asymmetry in nucleotide affinity in that one head holds ADP tightly, whereas the partner head binds ADP more weakly. This is an intriguing observation because it implies that there is head-head communication in the Eg5 dimer prior to its interaction with the MT. Furthermore, we find that half-site behavior of dimeric Eg5-513 consistently shows ~60% high affinity sites rather than the 50% expected for a homogeneous popula-
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FIGURE 2. ADP release from head 2. The Eg5-513-mantADP or R234K-mantADP complex was preformed with mantADP on both motor domains (1:1) or predominantly on one motor domain (1:0.6). The complex was then rapidly mixed in the stopped-flow instrument with MTs plus MgATP. A, representative transients of Eg5-513 mantADP release. (Final mixture: 2 μM Eg5-513, 10 μM MTs, 500 μM MgATP.) B, representative transients of R234K mantADP release. (Final mixture: 2 μM R234K, 4 μM MTs, 500 μM MgATP.) C, observed rates for R234K-mantADP of the initial fast phase plotted as a function of MT concentration: k_{max} = 20.4 ± 0.9 s^{-1}, K_{i,MTS} = 3.9 ± 0.5 μM. Inset, representative transients obtained from mixing a R234K-mantADP complex with MTs + MgATP (final mixture: 5 μM MTs). D, an Eg5-513-mantADP (●) or an R234K-mantADP complex (○) with mantADP at one active site was mixed with MTs and varying MgATP. Final mixture: 2.5 μM motor, 25 μM MTs. The observed rate of mantADP release from head 2 did not increase as a function of increasing MgATP concentration. E, experiment was repeated but as a function of increasing MT concentration, and each data set was fit to a hyperbola as follows: Eg5-513 (●), k_{max} = 0.62 ± 0.04 s^{-1}, K_{i,MTS} = 14.6 ± 2.3 μM, and R234K (○) k_{max} = 0.79 ± 0.04 s^{-1}, K_{i,MTS} = 9.1 ± 1 μM. (Final mixture: 2 μM Eg5-513 or R234K, varying MTs, 500 μM MgATP.)

The rate of mantADP release from the second head appeared to be dependent upon the concentration of MTs in the experiment (see Fig. 8 in Ref 35). This observation suggests that the head-head communication for Eg5 may be different from the signaling mechanism for kinesin in which MT concentration only affects the rate of mantADP release from head 1, and it is ATP binding at kinesin head 1 that triggers forward stepping and mantADP release from head 2 (33–35, 51, 52).

To explore what defines the signal for the second motor domain to bind the MT and release its ADP in Eg5, we devised a series of experiments that looked carefully at mantADP release from each head of the Eg5 dimer. We engineered an Eg5-513 mutant, R234K, that was defective for ATP hydrolysis, yet with the other steps in the ATPase cycle relatively similar to wild type Eg5-513 (Supplemental Material and Table 2). Eg5-513 or R234K was incubated with mantADP either to label both sites with mantADP or to label one site with mantADP. The Eg5-mantADP complex was then rapidly mixed in the stopped-flow instrument with MTs plus MgATP, and the kinetics of mantADP release were monitored (Fig. 2, A and B). When both heads of the dimer were labeled with mantADP, the kinetics were biphasic for both Eg5-513 and R234K. To measure the mantADP kinetics from the high affinity site of the dimer, gel filtration was used to remove weakly bound mantADP. Upon collision with MTs, the mantADP release kinetics were also biphasic, but the slower second phase predominated with the initial rapid phase representing only a small fraction of the total amplitude (Fig. 2, A and B). Fig. 2C shows for R234K that the rate of mantADP release from head 1 upon MT collision increased as a function of increasing MT concentration, k_{max} = 20 s^{-1}, which is similar to the rate of mantADP release obtained for wild type Eg5-513 at 28 s^{-1} (Table 2) (35). To evaluate whether ATP and/or ATP hydrolysis signaled head 2 to bind the MT as observed for dimeric kinesin (33, 34), Eg5-513 and R234K were labeled with mantADP followed by gel filtration to generate the Eg5 dimer with mantADP only on the high affinity site of head 2 (Fig. 2D). Eg5 was rapidly mixed in the stopped-flow with 25 μM MT plus MgATP, and the kinetics of mantADP release from head 2 were monitored. Surprisingly, the rate did not change for either Eg5-513 or R234K as a function of ATP concentration, suggesting that ATP binding or ATP hydrolysis did not modulate mantADP release with MTs.
binding to Eg5 in solution to be slow (Fig. 3, A and B), mantATP binding for the MT-Eg5 complex to be rapid (54 s⁻¹; Table 2), and MT-Eg5 association to be rapid (2.8 μM⁻¹ s⁻¹; Table 2). Fig. 3A shows representative transients of mantATP binding to Eg5-513 and Eg5-513NF in the absence of MTs. The amplitude associated with the kinetics of Eg5-513NF is higher than Eg5-513 as expected because Eg5-513 retains ADP at ~60% of its sites. Fig. 3B shows that mantATP binding in the absence of MTs is very slow at 0.23 s⁻¹. For The Race (Fig. 3C), Eg5 is loaded in one syringe, and the second syringe contains MTs plus mantATP. (Final concentrations after mixing were 3 μM Eg5, 8 μM MTs, 30 μM mantATP.) At these concentrations, mantATP binding to Eg5 would occur at ~0.15 s⁻¹ in solution but at ~40 s⁻¹ if the Eg5 head were already bound to the MT (35, 44). For monomeric Eg5-367NF, mantATP binding was immediate and rapid, indicating that the motor head bound the MT first followed by rapid mantATP binding. In contrast, for both dimeric Eg5-513NF and Eg5-513, there was a lag prior to mantATP binding to the active site.

When The Race was repeated with increasing MT concentrations, the rate of mantATP binding increased to a maximum of 27 s⁻¹ for monomeric Eg5-367NF (Fig. 3D), yet was 1.6 s⁻¹ for Eg5-513NF or 1.2 s⁻¹ for Eg5-513 (Fig. 3, E and F). These rates were significantly slower than mantATP binding to the preformed MT-Eg5-513 complex at 54 s⁻¹ (Table 2), but they were similar to the rate of the slow conformational change at ~1 s⁻¹. These results indicate that the slow conformational change occurs after MT collision and is a required structural transition for mantATP binding to Eg5. Furthermore, the mantATP binding kinetics for Eg5-513 and Eg5-513NF are similar, providing confidence that we are detecting the normal sequence of events that occurs to establish the Eg5 intermediate poised for processive stepping (Fig. 6, species E2).

**Gating Mechanism during Processive Stepping**—Our previous studies indicated that ATP hydrolysis at 5–10 s⁻¹ is the rate-limiting step during a processive run (35). Thus, the conformational change that is rate-limiting for the initialization of the processive run cannot occur during subsequent stepping. The new results presented in Figs. 2 and 3 indicate that the slow ~1 s⁻¹ structural transition occurs after MT association and

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release from head 2 in this experimental design. However, when the experiment was repeated but at varying MT concentrations plus 1 mM MgATP (Fig. 2E), the rate of mantADP release increased as a function of increasing MT concentrations for both Eg5-513 (kₘₓ = 0.62 s⁻¹) and R234K (kₘₓ = 0.79 s⁻¹). These results indicate that Eg5 begins its ATPase cycle by head 1 colliding with the MT and releasing ADP rapidly, followed by a slow conformational change at ~1 s⁻¹ that limits ADP release from head 2 (Fig. 6, steps 1 and 2).

**The Race**—The results presented in Fig. 2 indicated that collision with the MT was sufficient to release ADP from both motor domains, suggesting that both heads are bound to the MT and nucleotide-free prior to ATP binding (Fig. 6, species E2). However, these experiments did not provide information to order the steps of ATP binding and the slow ~1 s⁻¹ conformational change required to establish the processive run. A novel stopped-flow experiment called The Race was designed to define the sequence of events from MT collision to ATP binding and to ask whether ATP binding or MT binding occurred first. This experimental design required mantATP increased to a maximum of 27 s⁻¹ for monomeric Eg5-367NF (Fig. 3D), yet was 1.6 s⁻¹ for Eg5-513NF or 1.2 s⁻¹ for Eg5-513 (Fig. 3, E and F). These rates were significantly slower than mantATP binding to the preformed MT-Eg5-513 complex at 54 s⁻¹ (Table 2), but they were similar to the rate of the slow conformational change at ~1 s⁻¹. These results indicate that the slow conformational change occurs after MT collision and is a required structural transition for mantATP binding to Eg5. Furthermore, the mantATP binding kinetics for Eg5-513 and Eg5-513NF are similar, providing confidence that we are detecting the normal sequence of events that occurs to establish the Eg5 intermediate poised for processive stepping (Fig. 6, species E2).

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FIGURE 4. MantATP binding under single turnover conditions. A preformed MT-Eg5-513nf complex was rapidly mixed in the stopped-flow instrument with mantATP. Final mixture: 15 μM Eg5-513, 25 μM MTs, 1 mM MgMantATP (A) or 0.25 mM MgMantATP (B, C), representative transient reflecting initial rapid mantATP binding by Eg5-513nf phase 1 $k_{\text{obs}} = 33 \pm 0.3$ s$^{-1}$; phase 2 $k_{\text{obs}} = 2.1 \pm 0.06$ s$^{-1}$. B, rapid mantATP binding to the MT-Eg5-513nf complex followed by fluorescence decay with $k_{\text{obs}} = 0.0008 \pm 0.00002$ s$^{-1}$. C, experimental design to establish the processive stepping Eg5 intermediate.

prior to ATP binding, which is consistent with this model. However, the question of how the heads of the Eg5 dimer are coordinated during a processive run remains.

To address this question experimentally, we preformed an MT-Eg5-513 complex and treated with apyrase to remove ADP. The MT-Eg5-513nf complex was rapidly mixed with mantATP in the stopped-flow instrument at single turnover conditions (Fig. 4; final concentrations: 15 μM Eg5-513, 25 μM MTs, 1 mM mantATP), MantATP binding resulted in an initial rapid phase of fluorescence enhancement, $k_{\text{obs}} = 33 \pm 0.3$ s$^{-1}$. However, the fluorescence did not begin to decrease until 50 s elapsed, and the rate was extremely slow at $k_{\text{obs}} = 0.001$ s$^{-1}$ (Fig. 4B). This observed rate is comparable with the rate of fluorescence decay for mantATP mixed with buffer in the absence of Eg5 or MTs ($k_{\text{obs}} = 0.0014$ s$^{-1}$; data not shown). For kinesins, there is no change in fluorescence associated with mantATP hydrolysis. Therefore, the kinetics in Fig. 4B suggest that a long lived Eg-513-mantADP intermediate was formed (Fig. 4C), and a nucleotide-gated mechanism prevented rapid mantADP release. Because the ADP state promotes weak Eg5 binding to the MT, we propose that the head holding mantADP is detached from the MT but tethered by the nucleotide-free head that is bound tightly to the MT (Fig. 4C).

In the next series of experiments, we used this kinetically stable MT-Eg5-513-mantADP intermediate (Fig. 4C) to determine the nucleotide signal that triggers mantADP release during processive stepping (Fig. 6, steps 6 and 7). The kinetically stable MT-Eg5-513-mantADP intermediate (Fig. 6, ES) was formed by adding mantATP to a MT-Eg5-513nf complex just prior to loading in the stopped-flow instrument. This complex was then rapidly mixed with MgATP in the stopped-flow instrument, resulting in a rapid exponential quenching correlated with mantADP release from the active site (Fig. 5A). The initial exponential rate of mantADP release increased as a function of increasing MgATP concentration with $k_{\text{max}} = 8.4$ s$^{-1}$ (Fig. 5, A and B). These results are consistent with rate-limiting ATP hydrolysis on the rearward head at ~10 s$^{-1}$, followed by rapid MT association and ADP release from the advancing head (35).

Note that the transients in Fig. 5A appear biphasic, where the exponential rate for the second slow phase is ATP-independent and occurs at ~1 s$^{-1}$ (Fig. 5B). The design of the experiment should result in single exponential kinetics because only one head of the Eg5 dimer should be occupied by mantADP. We do not know the origin of this second phase. It may reflect an off pathway isomerization of mantADP. Alternatively, there may be a population of dimers with mantADP at both sites and free in solution detached from the MT. Upon mixing in the stopped-flow instrument, this population would form the nucleotide-free MT-Eg5-513 species poised to begin a processive run (Fig. 6, E2) with the second phase in the Fig. 5A transients reflecting the slow ~1 s$^{-1}$ conformational change.

To assess whether ATP binding was sufficient to stimulate mantADP release or whether ATP hydrolysis was required, the stable MT-Eg5-513-mantADP immediate was mixed with the slowly hydrolyzable analogue, ATPγS, and the nonhydrolyzable analogue, AMPPNP (Fig. 5D). For both ATP analogues, there was an initial rapid fluorescence decrease ($k_{\text{obs}} = 20–30$ s$^{-1}$) of very small amplitude followed by a slow phase. This slow phase was of comparable amplitude to the slow phase of the ATP transient. The same experiment was also performed using the ATP hydrolysis defective mutant R234K. With AMPPNP, there was almost no initial fast phase, yet the transient for R234K with ATP mimicked the results of mixing MT-Eg5-513-mantADP with AMPPNP or ATPγS.

These results are difficult to interpret for a clear understanding of the gating mechanism. If ATP binding were sufficient in the absence of ATP hydrolysis for forward head advancement, MT association, and mantADP release, we would expect the amplitudes of the AMPPNP, ATPγS, and R234K transients to be equal to the ATP transient in Fig. 5C as observed previously for kinesin (34). However, if ATP binding followed by ATP hydrolysis were required for mantADP release, our expectation would be almost no amplitude associated with the AMPPNP, ATPγS, and R234K transients as observed for Drosophila Ncd (46). Our interpretation is that the results in Fig. 5D indicate that ATP binding is sufficient to induce some of the structural transitions required for forward advancement of the rearward
We see from the solution of the model equations (Fig. 5) behavior and the correct dependence on ATP concentration. Importantly, the rate of ATP binding (rate constant $k_1$) that the scheme presented in Fig. 6 fits the experimental data quite well. The second, slower phase of ATP concentration dependence. $C$, kinetic model in Fig. 6 (inset) was solved numerically for various ATP concentrations and matched to the experimental data in $A$. The rate constants corresponding to the fit shown are as follows: $k_1 = 5.5 \times 10^{-1} s^{-1}$, $k_2 = 1.8 \times 10^{-3} s^{-1}$, $k_3 = 1.7 \times 10^{-3} s^{-1}$, and $k_4 = 1.2 \times 10^{-3} s^{-1}$. $D$, comparison of the kinetics of mantADP release when either a MT-Eg5-mantADP or a MT-R234K-mantADP complex was mixed with ATP, ATP-yS, or AMPPNP: Eg5-513 + ATP (red), Eg5-513 + ATP-yS (dark green), Eg5-513 + AMPPNP (purple), R234K + ATP (orange), R234K + ATP-yS (light green), and R234K + AMPPNP (pink).
The Processive Run—By analogy to kinesin, we propose that the neck linker of species E2 are strained, which would favor ATP binding at the rearward head because of its docked neck linker configuration (43, 52, 56–58). ATP hydrolysis and phosphate release result in the weakly bound ADP state, leading to rearward head detachment (species E5). However, for the rearward head of species E5 to advance forward toward the MT plus-end, ATP must bind and possibly hydrolyze at the forward head (Fig. 5). Once the rearward head steps forward and collides with the MT, ADP is released rapidly. The observed rate constant for ADP release in this experimental design was 8.4 s⁻¹, indicative that ATP hydrolysis was controlling the rate of Eg5 stepping during its processive run.

In the next ATP cycle (steps 6 and 7), we propose that ATP binding at the forward head during the processive run results in rapid movement of the neck linker to the docked configuration followed by a MT plus-end directed advancement of the rearward head to the next MT-binding site 8 nm ahead of the ATP-bound head. The results from Figs. 4 and 5 indicate that during processive stepping, ATP binding and ATP hydrolysis at the forward head are required for rearward head advancement to generate the tightly bound E7 intermediate (35, 36, 38). This model accounts for the exceedingly slow steady-state ATP turnover at 0.5 s⁻¹, yet at the same time it is consistent with the velocity measurements from the single molecule results of Valentine et al. (36–38) at 12 s⁻¹. Further support comes from the simulations of the kinetics shown in Fig. 5C. This model predicts ATP hydrolysis at 10.7 s⁻¹, followed by rapid ADP release at >185 s⁻¹.

Short Processive Runs of Eg5—The results presented here do not define the point in the cycle where Eg5 is most likely to terminate its processive run. However, we speculate that the short run lengths of dimeric Eg5 in comparison with kinesin are because of the inability of Eg5 to keep the motor domains out-of-phase such that both heads reach the ADP state simultaneously. This could occur at steps 3–5 (Fig. 6) if ATP were to bind and hydrolyze at the forward head prematurely, or at steps 6 and 7 if ATP were hydrolyzed on the rearward head with ADP remaining at the forward head. The geometry or structural state of the neck linker may also play a role in loss of coordination and motor detachment (43). These are but two of a number of possibilities. We propose that the head-head communication of the Eg5 dimer leads to the short run lengths observed experimentally, and this behavior may be required for the functional roles of the Eg5 tetramer in the spindle. This type of mechanism may prevent the homotetramer from stalling at a roadblock by terminating the processive run on one microtubule with Eg5 remaining attached to the other MT. This behavior may also permit MT dynamics promoted by other proteins and/or kinesin motors and may be essential to achieve the balance of plus-end and minus-end directed forces for the metaphase spindle.

Although this study provides a mechanistic understanding of Eg5 mechanochemistry and head-head communication, one intriguing question ahead is to understand the mechanistic advantage of one rate-limiting transition to establish the processive run and another, rate-limiting ATP hydrolysis, to control the velocity of the processive run.

motor to a docked configuration (54, 55), approximately parallel with the MT (species E2). What is unique for Eg5 is that it will begin its processive run from an intermediate with both heads bound to the MT and both free of nucleotide.
Dimeric Eg5 Cooperativity

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