We have previously shown that the mitotic motor centrosome protein E (CENP-E) is capable of walking for more than 250 steps on its microtubule track without dissociating. We have examined the kinetics of this molecular motor to see if its enzymology explains this remarkable degree of processivity. We find that like the highly processive transport motor kinesin 1, the enzymatic cycle of CENP-E is characterized by rapid ATP binding, multiple enzymatic turnovers per diffusive encounter, and gating of nucleotide binding. These features endow CENP-E with a high duty cycle, a prerequisite for processivity. However, unlike kinesin 1, neck linker docking in CENP-E is slow, occurring at a rate closer to that for Eg5, a mitotic kinesin that takes only 5–10 steps per processive run. These results suggest that like kinesin 1, features outside of the catalytic domain of CENP-E may also play a role in regulating the processive behavior of this motor.

The mitotic kinesins are a group of microtubule-based molecular motors that help orchestrate the complex dynamics of chromosomal and spindle movements during the process of mitosis (1). Among these is centrosome protein E (CENP-E).2 CENP-E is markedly up-regulated during the G2 and M phases of the cell cycle, and it appears to be necessary both for proper chromosomal congression to the metaphase plate and for binding of spindle microtubules to the kinetochore (2–5). Furthermore, it had been proposed that CENP-E functions by actively transporting chromosomes along kinetochore fibers (5–7). This putative role would therefore predict that CENP-E works as a processive transport molecular motor. As such, it would therefore be expected to share features with another, better characterized processive kinesin, kinesin 1. Consistent with this, we had previously shown that a dimeric construct of CENP-E is capable of walking processively for over 250 steps on the microtubule in a hand over hand manner, with stall forces very similar to what had been previously measured for kinesin 1 (8).

In the case of kinesin 1, processivity is in part a result of specific features of this motor’s enzymology. These include a high duty ratio, rapid ATP binding, the ability to undergo multiple enzymatic turnovers per diffusive encounter, and strain-dependent gating of the motor ATPase (9–13). These features reduce the probability that the kinesin 1 motor will detach prematurely from its microtubule track. This connection between enzymology and processivity also applies to Eg5, a mitotic kine- sin responsible for formation of a bipolar spindle. We had previously shown that neck linker docking for Eg5 gates ATP binding, and it is 20-fold slower than for kinesin 1, and these differences explain the much shorter run lengths for this processive mitotic motor (14). In this study, we examine the ATPase cycle of CENP-E to see if, like kinesin 1, its enzymology is consistent with its remarkable degree of processivity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides for PCR were synthesized by Invitrogen. Paclitaxel, *Taq* DNA polymerase and DNA-modifying agents, FlAsH, and ReAsH were purchased from Invitrogen. Restriction enzymes were purchased from New England BioLabs, Inc. (Ipswich, MA). Culture medium reagents were purchased from EMD Chemicals, Inc. and Fisher. Buffer reagents were purchased from Sigma and Fisher, except for imidazole, which was purchased from EMD Chemicals, Inc. Protein assay reagents and gels were purchased from Bio-Rad. 2’dmT and 2’dmD were synthesized and purified from the unlabeled 2’deoxyribonucleotides as described previously (15).

**Mutagenesis**—The full-length *Xenopus* xCENP-E cDNA in pBLUESCRIPT was obtained from Dr. Yinghui Mao and was used as a PCR template to create a 342-amino acid length monomer (CENP-E 342) to be inserted into the bacterial expression vector pET21a (Novagen, Madison, WI) between the NdeI and XhoI sites with the following primers: GGAGAATATACATATTGTCGAGCTCGAGTGTTAAATGTTGGATACATCCAGAAGCTTACATTTAACGACATGGGG. A COOH-terminal CCPGCC sequence (for binding FlAsH and ReAsH) was added with the following PCR primer sequence: GTGGTGTGCTGAGACGCAACCGGGGACGAGCAACCTCCATCATCAGGACCTCATTACATTTAACGACATGGGG. The PCR product was ligated into pET21a by GenScript (Piscataway, NJ). An NN₂-terminal CCPGCC sequence was subsequently added with the following PCR 5’-primer sequence: GAGAATATACATATTGAGGAGGGTGGCTGT-
CENP-E ATPase Cycle

FIGURE 1. Sedimentation coefficient distributions for three CENP-E constructs. Top, monomer CENP342 g(s) distributions show no concentration dependence from 6.4 to 84.8 μM total protein. Direct boundary fitting with Sedanal returns an Mₐ of 38,292 with a 95% confidence interval of (37,246, 39,358) consistent with the expected Mₐ of 39,082, s = 4.598 (f/f₀ = 1.30) and root mean square of the fit = 0.00533. Middle, g(s) distributions show the weak monomer-dimer behavior of CENP392 over the range of 8.1–138.7 μM. The Kᵥ estimated by direct boundary fitting is at least 100 μM. Bottom, dimeric CENP392zip g(s) distributions show no concentration dependence over the range of 1.5–25.5 μM total protein. Direct boundary fitting with Sedanal returns an Mₐ of 101,500 with a 95% confidence interval of (93,399, 110,407), consistent with the expected Mₐ of 101,543, s = 4.598 (f/f₀ = 1.66), and root mean square of the fit = 0.00445. The wide confidence interval is due to the presence of ~4% low Mₐ contamination.

CENP-E ATPase Cycle

The first 1176 bp of xCENP-E, corresponding to amino acids 1–392, was fused to a GCN4 leucine zipper (accession number AF416613) and cloned into the pET21a vector in order to express the construct in bacterial BL21 cultures and to purify the protein using the encoded COOH-terminal His₆ tag. NdeI and Kpnl restriction sites were introduced into the 5’- and 3’-ends (respectively) of the xCENP-E cDNA by using PCR with the following primer set: 5’-GCCGCGATTCGCCGAGGAGATGCAGT-TAAAGTGTTGATGAGG-3’ and 5’-GCCGCGGGTTACCTCTTTGTGAGTTTGGAT-3’. Similarly, Kpnl and XhoI restriction sites were introduced into the 5’- and 3’-ends, respectively, of GCN4 cDNA by PCR using the following primer set: 5’-GCCGTTACCACATGAAACAGCTTGAGGACAAA-CGC-3’ and 5’-TGCACAAAGCTGGCGCCGACCTCGAGTT-AATAGCC-3’. The PCR transcripts were digested and subsequently triple-ligated to NdeI/XhoI-cut pET21a vector. All DNA was confirmed by sequencing.

Bacterial Cell Culture and Protein Expression—Expression plasmids were transformed into BL21(DE3) RIL cells (Stratagene, Inc., La Jolla, CA) in an enriched medium (2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, 50 mM Na₂HPO₄, 50 mM KH₂PO₄, 50 mg/liter ampicillin, 50 mg/liter chloramphenicol). Nine liters of culture were grown to an A₉₀₀ of 1.8 and induced with 0.5 mM isopropylthio-β-galactoside at 19 °C for 20 to 24 h. Protein purification was described as previously (9, 10). Typical yields were 20 mg protein/liter of culture. After thawing, proteins were filtered through a prepacked PD-10 column containing Sephadex G-25 M (GE Healthcare) and used within 6 h of thawing.

Steady State ATPase—The ATPase rate was determined with the EnzChek phosphate assay kit (Invitrogen) using a Varian Cary 300 BIO UV-visible spectrophotometer and the CARY kinetics software. The reaction conditions were 100 nM CENP-E, 2 mM Na₃ATP, and 0.1–20 μM polymerized tubulin in ATPase buffer (50 mM KCl, 25 mM HEPES, 2 mM MgCl₂, 1 mM dithiothreitol, pH 7.5). The data were fitted by nonlinear regression to a hyperbola using GraphPad Prism software version 4.00 for Windows (GraphPad Software, San Diego, CA).

Sedimentation Studies—Sedimentation studies were performed in a Beckman Optima XLA analytical ultracentrifuge equipped with absorbance optics and an An60 Ti rotor at 19.7 °C. Temperature was calibrated as described previously (16). Buffer density was measured to be 1.00283 on an Anton Paar DMA 5000. Velocity data were collected at 42,000–50,000 rpm using 278 nm at a spacing of 0.002 cm with one flash at each point in a continuous scan mode. Below 1 OD, samples were run in 1.2-cm centerpieces; above 1 OD and up to ~5 OD, samples were run in 0.3-cm centerpieces. Velocity data were analyzed with DCDT⁻² to produce g(s) distributions (16) (Fig. 1). Direct boundary fitting to extract Mₐ was done with Sedanal (17). Sedntrap was used to estimate ν and extinction coefficients for each construct (17).

Fluorescence Methodologies—We labeled a CENP342 construct with the sequence CCPG GCC at both the carboxyl and amino termini with FlAsH by incubating with a 0.2:1 FlAsH/CENP342 molar ratio for 4 h at 4 °C in labeling buffer. ReAsH was then added at a 5:1 ReAsH/CENP342 molar ratio, the sample was incubated for another 4 h at 4 °C, and excess fluorophore was removed by gel filtration. We found that labeling was stoichiometric under these conditions.

Fluorescence lifetime measurements were performed with a Fluorolog 3 spectrofluorimeter equipped with an IBH 5000 photon-counting lifetime system (Horiba Jobin Yvon). Data from donor-acceptor decays were analyzed as a static Gaussian distribution of distances, as described previously (18). The value of Rₑ for a FlAsH-ReAsH donor acceptor pair was calculated using the quantum yield dissociation fluorescent in 0.01 M NaOH as a reference (0.91) (18). This generated a quantum yield for FlAsH of 0.285, an overlap integral of 5.9 × 10¹⁴ M⁻¹ cm⁻¹ nm⁻², and a value of Rₑ of 37.1 Å at 20 °C, assuming a value of κ² = 3%.

Kinetic Methodologies—Kinetic measurements were made using a KinTek stopped flow spectrophotometer with instrument dead time of 1.1 ms. Dissociation of CENP-E from microtubules was monitored by turbidity at 350 nm. Mant nucleotide fluorescence was excited at 295 nm (for tryptophan energy...
CENP-E ATPase Cycle

TABLE 1
Rate constants for the ATPase cycles of monomeric CENP342, kinesin I (Kin349), and Eg5 (Eg367) constructs in the presence of microtubules

Conditions were as follows: 100 mM KCl, 25 mM HEPES, 2 mM MgCl2, 1 mM dithiothreitol, pH 7.50, 20 °C. Rate constants are illustrated in Fig. 8A.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>kcat (s−1)</td>
<td>CENP342</td>
<td>21.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Kin349</td>
<td>59.7 ± 6.9</td>
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<tr>
<td></td>
<td>Eg367</td>
<td>8.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>CENP342</td>
<td>22.0 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Kin349</td>
<td>34.6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Eg367</td>
<td>6.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>CENP342</td>
<td>21.9 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>Kin349</td>
<td>19 ± 3</td>
</tr>
<tr>
<td></td>
<td>Eg367</td>
<td>1.1 ± 0.1</td>
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<tr>
<td></td>
<td>CENP342</td>
<td>1.9 ± 0.2</td>
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<tr>
<td></td>
<td>Kin349</td>
<td>3.4 ± 1.5</td>
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<tr>
<td></td>
<td>Eg367</td>
<td>6.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>CENP342</td>
<td>5.0 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Kin349</td>
<td>19 ± 3</td>
</tr>
<tr>
<td></td>
<td>Eg367</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>CENP342</td>
<td>4.9 ± 0.7</td>
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<tr>
<td></td>
<td>Kin349</td>
<td>19 ± 3</td>
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<td></td>
<td>Eg367</td>
<td>1.1 ± 0.1</td>
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<tr>
<td></td>
<td>CENP342</td>
<td>12.2 ± 0.7</td>
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<td></td>
<td>Kin349</td>
<td>97 ± 7 to 116 ± 4</td>
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<td></td>
<td>Eg367</td>
<td>6.3 ± 0.3 to 7.5 ± 0.6</td>
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<td>CENP342</td>
<td>28.7 ± 3.9</td>
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<td></td>
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<td>Eg367</td>
<td>62 ± 17 to 78 ± 6</td>
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<td>CENP342</td>
<td>11.4 ± 0.9</td>
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<td></td>
<td>Kin349</td>
<td>92 ± 1</td>
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<td>Eg367</td>
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<td>CENP342</td>
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<tr>
<td></td>
<td>Kin349</td>
<td>~4.6</td>
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<td></td>
<td>Eg367</td>
<td>17.3 ± 1.7 to 20.1 ± 3.8</td>
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<td>1.4 ± 0.4</td>
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<td>Kin349</td>
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<td></td>
<td>Eg367</td>
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<tr>
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<td>CENP342</td>
<td>193 ± 35</td>
</tr>
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<td></td>
<td>Kin349</td>
<td>~300</td>
</tr>
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<td></td>
<td>Eg367</td>
<td>76 ± 15</td>
</tr>
<tr>
<td></td>
<td>CENP342</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Kin349</td>
<td>219 ± 16</td>
</tr>
<tr>
<td></td>
<td>Eg367</td>
<td>46 ± 10 to 51 ± 18</td>
</tr>
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</table>

*a* Unless otherwise indicated, data are from Ref. 32.
*b* Unless otherwise indicated, data are from Ref. 14.
*c* Experiments performed under the above conditions except [KCl] = 50 mM.
*d* Measured with the CENP342 construct with CCGFGCC at the carboxyl and amino termini.
*e* From Ref. 33.
*f* From Ref. 19.
*g* From Ref. 9.

RESULTS

Structural and Enzymatic Studies of CENP-E Constructs—In the studies to be discussed below, we utilized two CENP-E constructs. The first is a monomer containing the first 342 residues (CENP342) and includes the motor domain and neck linker (20). The second contains the first 392 residues followed at the carboxyl terminus by a leucine zipper sequence from GCN4 (CENP392zip) and includes the motor domain, neck linker, and ~7 heptads of putative coiled coil. We confirmed through sedimentation velocity studies that CENP342 is monomeric and CENP392zip is dimeric (Fig. 1) and found in a previous study that this construct could move processively as a single molecule on immobilized microtubules (8). These studies have also revealed that the leucine zipper on CENP392zip is necessary to ensure dimerization because a 392-residue CENP-E construct without the leucine zipper is a mixture of monomer and dimer (Fig. 1) and does not demonstrate single molecule motility.3

Dimeric CENP392zip has a kcat that is approximately half of and a K0.5,Mt that is 37-fold smaller than the corresponding values for monomeric CENP342 (Tables 1 and 2). These features suggest that the ATPase activity is gated in dimeric CENP-E, consistent with its demonstrated ability to move processively on microtubules (8). Reducing the KCl concentration from 100 to 50 mM increased kcat by ~5% (data not shown).

The motor domain of CENP-E contains four cysteine residues, each of which is necessary for normal enzymatic function of this motor. Because we wished to fluorescently label the CENP-E neck linker to monitor the timing of neck linker docking, we generated an additional monomeric construct in which

3 H. Yardimci and P. Selvin, personal communication.

FIGURE 2. Kinetics of binding of 2′dMT and 2′dMD to a complex of CENP342-microtubules. A complex of 2 μM CENP342 and 5 μM polymerized tubulin was made nucleotide-free with 0.2 units/ml apyrase and then mixed in the stopped flow with varying concentrations of 2′dMT or 2′dMD. Binding of nucleotide was monitored by FRET from vicinal tryptophan residues to the mant fluorophore. The resulting fluorescence enhancement occurs in a single phase, and the rate constant is plotted as a function of [2′dMT] (red) or [2′dMD] (blue). The linear variation of rate constant with nucleotide concentration defines apparent second order rate constants, and the extrapolation of the lines to zero nucleotide concentration define apparent dissociation constants that are summarized in Table 1. Conditions were as follows: 100 mM KCl, 25 mM HEPES, 2 mM MgCl2, 1 mM dithiothreitol, pH 7.50, 20 °C.

Downloaded from www.jbc.org at Columbia University on January 2, 2010
we added the sequence CCPGCC to both the carboxyl and amino termini. This sequence forms a tight bond with FlAsH and ReAsH-arsenical derivatives of the fluorophores fluorescein and resorufin. Furthermore, because the quantum yield of ReAsH in our system is very low (≤0.03), we could use ReAsH as a fluorescence quencher. Table 1 demonstrates that the microtubule-activated ATPase activity of this construct is similar to that for the unmodified CENP342.

Kinetics of Nucleotide Binding, Phosphate Release, Microtubule Dissociation, and Microtubule-activated ADPase for Monomeric CENP342—We examined the kinetics of ATP binding by mixing a 1:5 CENP342-microtubule complex with the fluorescent ATP analogue 2’dmT and monitoring mant fluorescence emission by FRET from two vicinal tryptophan residues (9, 10, 18). The resulting fluorescence increase occurs in a single phase, and the rate constant varies linearly with [2’dmT], defining an apparent second order rate constant (k₅k₇ in Table 1) of 4.9 ± 0.7 μM⁻¹ s⁻¹ (Fig. 2, red). A maximum rate was not observed. Extrapolation of the line to zero [2’dmT] defines an apparent dissociation rate constant (k₋₁ in Table 1) of 122.5 ± 50.8 s⁻¹. A similar experiment was performed using 2’dmD, and the fluorescence increase also occurs as a single phase. Its rate constant also varies linearly with [2’dmD], yielding an apparent second order rate constant of 1.4 ± 0.4 μM⁻¹ s⁻¹ and a dissociation rate constant (k₋₁ in Table 1) of 191 ± 35 s⁻¹ (Fig. 2, blue).

The rate of the phosphate release step was measured by mixing a 1:5 CENP342-microtubule complex with varying concentrations of ATP in the presence of MDCC-labeled phosphate-binding protein. The resulting fluorescence transient consists of an initial exponential phase, representing phosphate release in the first turnover, followed by a linear phase, reflecting phosphate release in the steady state. A plot of the rate constant of the exponential phase versus [ATP] varies hyperbolically with nucleotide concentration, defining a maximum rate of 11.4 ± 0.9 s⁻¹ (Fig. 3, inset, magenta) and Table 1).

ATP- and ADP-induced dissociation from the microtubule was measured by rapidly mixing a 1:1 CENP342-microtubule complex with ATP or ADP. The resulting turbidity decrease at 350 nm consists of a single exponential process, and the rate constant varies hyperbolically with nucleotide concentration, defining maximum rates for dissociation induced by ATP (kd in Table 1) and ADP (kd in Table 1) of 12.0 ± 0.6 and 6.8 ± 0.7 s⁻¹, respectively (Fig. 3, inset, red and blue curves, respectively).

Microtubule-activated ADP release was monitored by mixing a preformed complex of CENP342–2’dmD with an excess of microtubules + 2 mM ATP and observing the mant fluorescence decrease by FRET. This produces a monophasic FRET transient (data not shown), and the rate constant for this process varies linearly with [tubulin], defining an apparent second order rate constant (k₅k₇ in Table 1) of 0.9 ± 0.2 μM⁻¹ s⁻¹. A maximum rate was not observed.

Kinetics of Neck Linker Docking Measured by FRET—In kinesin 1, ATP binding induces neck linker docking and subsequent forward stepping with a rate constant in the range of 800–1000 s⁻¹ at 20 °C (9, 10). Dissociation from the microtubule, on the other hand, is at least 40–50 times slower (9, 10, 21, 22). This disparity in rates means that forward stepping is much more likely than dissociation, and it contributes to the high degree of processivity of this motor. By contrast, neck linker docking is only 8–10 times faster than dissociation in Eg5 (14), and not surprisingly, the mean run length for this motor is 10–20 times shorter than for kinesin 1 (23).

We used a different FRET approach to measure the kinetics of neck linker docking in CENP342 from that used in our prior studies of kinesin 1 and Eg5 because we found that mutating the four CENP342 cysteines reduced kcat by over 4-fold (data not shown). In this approach, we monitored the change in separation between the amino and carboxyl termini of CENP342 by

![Figure 3. Kinetics of ATP and ADP-induced microtubule dissociation and of ATP-induced phosphate release for CENP342.](image)
labeling these positions with FlAsH and ReAsH, using a CENP342 construct with the CCPGCC sequence at the COOH and NH₂ termini. We measured a FRET separation distance between FlAsH and ReAsH of 42 Å for a rigor complex of labeled CENP342-microtubules, with a half-width of 18 Å. Adding AMP-PNP increased the FRET to 49 Å and the half-width slightly to 21 Å. The only crystallographic structure of CENP-E is with the neck linker in a plus end-directed, docked orientation, and the separation distance between residues Glu⁴ and Ser³³⁹ is 26 Å (20). The larger separation distance that we measure is probably the result of two additional factors: 1) our construct extends from residue 1 to 342, and 2) the sequence CCPGCC is added at both the amino and carboxyl termini. The increase in separation between amino and carboxyl termini of the motor domain with neck linker docking is consistent with crystallographic models of kinesin 1, which demonstrate that docking of the neck linker is associated with a 4.3-Å increase in amino-to-carboxyl terminus separation (24).

Our results therefore predict that mixing ATP with nucleotide-free FlAsH and ReAsH-double-labeled CENP342 that is bound to microtubules should produce an increase in FlAsH fluorescence emission that corresponds to the neck linker docking. This prediction is illustrated schematically in Fig. 4A and is confirmed in Fig. 4B (red dotted curve). When we repeat this experiment with a CENP342 construct labeled only with FlAsH, we see no change in fluorescence emission (Fig. 4B, inset), which establishes that the fluorescence increase that we observe with the FlAsH and ReAsH-FRET pair is due to energy transfer and not simply a change in donor quantum yield. The fluorescent transient produced by mixing with ATP is biphasic, and adequate fitting requires a double exponential function (Fig. 4B, solid yellow curve). The rate con-
stants for both phases vary with [ATP] (Fig. 4C, closed and open red circles) in a hyperbolic manner, defining maximum rates of 28.7 ± 3.9 and 7.3 ± 1.2 s⁻¹. That both rate constants vary with ATP concentration implies a branched pathway (depicted in Fig. 4A). This in turn implies that ATP-induced dissociation can occur before neck linker docking and, therefore, that there is loose coupling between neck linker docking and dissociation. Repeating the same experiment with ADP generates a monophasic fluorescence increase (Fig. 4B, dotted blue curve), and the corresponding rate constant varies hyperbolically with [ADP] (Fig. 4C, blue triangles), defining a maximum of 8.3 ± 1.0 s⁻¹.

**Kinetics of ATP Binding to and ADP Release from Dimeric CENP392zip—**Mixing CENP392zip with 2'dmT in the stopped flow produces a fluorescence increase that occurs in a single phase (Fig. 5A), and its rate constant varies hyperbolically with [2'dmT], defining a maximum rate of 807 ± 189 s⁻¹ (Fig. 5C, red). The red smooth curve extrapolates to the origin, indicating that the dissociation rate constant is negligible. By contrast, when this experiment is repeated with a 1:5 CENP392zip (active site)/tubulin molar ratio, the resulting fluorescence increase occurs in two phases of similar amplitudes (Fig. 5C, inset). The rate constant for the faster phase varies linearly with [2'dmT], defining an apparent second order rate constant (Kₚ₋substrate in Table 2) of 1.7 ± 0.3 M⁻¹ s⁻¹, and its extrapolation to zero [2'dmT] defines a dissociation rate constant (k₋substrate in Table 2) of 100.8 ± 13.1 s⁻¹ (Fig. 5C, closed blue circles). The slower phase shows little ATP concentration dependence and averages 13.7 ± 2.7 s⁻¹. We had previously observed a similar finding in kinesis 1 and in that study demonstrated that the slower phase reflects binding of 2'dmT to the lead head, which is rate-limited by two preceding steps. These are ADP release from the leading head and dissociation of the trailing head, which releases the rearward strain on the leading head and allows ATP binding (9).

Nucleotide-induced release of 2’dmT from the tethered head of CENP392zip-2’dmT was monitored by generating a complex of 2 μM CENP392zip-2’dmT with 14 μM microtubules and mixing this with ATP or ADP. Mixing with ATP produces a fluorescence decrease in two phases (Fig. 6A, red). The faster phase makes up ~65% of the total amplitude, and its rate constant varies hyperbolically with [ATP], defining a maximum rate of 56.8 ± 3.5 s⁻¹ (Fig. 6B, closed red circles). The rate constant of the slower phase shows little variation with ATP concentration and averages 0.9 ± 0.3 s⁻¹ (Fig. 6B, inset, open red circles). Mixing with ADP instead generates a fluorescence decrease that consists of a single phase (Fig. 6A, blue). The rate constant for this process shows no appreciable dependence on ADP concentration and averages 0.8 ± 0.2 s⁻¹ (Fig. 6B, inset, blue triangles). Repeating this experiment with 1 μM CENP392zip and 14 μM microtubules produced essentially identical results (data not shown).

**Kinetics of Nucleotide-induced Dissociation of CENP392zip from the Microtubule—**We monitored the kinetics of nucleotide-induced dissociation of CENP392zip from the microtubule by mixing 3 μM CENP392zip plus 2 μM microtubules plus 0.2 unit/ml apyrase with ATP or ADP in the stopped flow. We used this stoichiometry to enhance the probability that the motor will be sterically impeded from taking a forward step and will dissociate after the first turnover. With both nucleotides, the resulting turbidity decrease can be described as the sum of two exponential terms (Fig. 7, red for ATP, blue for ADP). The rate constant of the faster phase varies hyperbolically with both [ATP] and [ADP], defining maximum rates of 2.1 ± 0.2 s⁻¹ for ADP (Fig. 7, inset, closed blue boxes) and 4.7 ± 0.4 s⁻¹ for ATP (Fig. 7, inset, closed red circles). The rate constant for the slower process, on the other hand, averages 0.30 ± 0.16 s⁻¹ for ATP and 0.31 ± 0.11 s⁻¹ for ADP and shows no appreciable nucleotide concentration dependence (Fig. 7, inset, open red circles and open blue boxes). Repeating this experiment with 10 μM ADP instead of apyrase, in order to occupy the catalytic site of the tethered head with nucleotide produces similar results (data not shown).

**DISCUSSION**

The Enzymologies of Processive Kinesins Reflect Their Physiologic Roles—The two best studied processive kinesins, kinesin 1 and Eg5, serve very different functions. Kinesin 1 works in isolation and transports its cargoes long distances (25). By contrast, Eg5 slides anti-parallel spindle microtubules against each other, generates intramolecular tension, and works in ensembles (26). These functional differences are reflected in corresponding kinetic differences in the motor ATPases. The ATPase cycle of kinesin 1 is characterized by rapid ATP binding, a rate of forward stepping that is >100-fold faster than dissociation, and strain-dependent gating of ATP binding to the leading head (9, 10), features that in aggregate reduce the chance that the motor will detach prematurely. By contrast, ATP binding to the attached head of an Eg5 dimer is at least 10-fold slower, and it is gated by docking of the neck linker in a process that is only 8–10-fold

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**FIGURE 4. Measurement of the kinetics of neck linker docking in CENP342 by FlAsH → ReAsH FRET. A, schematic of the experimental design and results. The neck linker is depicted as the blue line and the amino terminus as the black line projecting from the side of the motor domain (magenta). The amino terminus of the motor domain and the carboxyl terminus of the neck linker are randomly labeled with FlAsH and ReAsH (red and cyan discs). The small green wavy arrow in the leftmost image represents the shallow degree of fluorescence emission of FlAsH in the rigor state, due to its proximity to the minimally fluorescent ReAsH acceptor. Binding of ATP reorients the neck linker, increases the separation distance, and enhances FRET emission from the FlAsH donor (represented by the enlargement of the green wavy arrow). Dissociation can occur either with or after neck linker docking to generate an ADP-bound state. B, fluorescence transient produced by mixing a complex of FlAsH and ReAsH-labeled CENP342 + microtubules with 100 μM ATP (red) or 100 μM ADP (blue). Fitting of the ATP-induced transient to a single exponential (magenta solid line) is inadequate, whereas fitting to a double exponential function (yellow solid line) yields rate constants of 9.9 and 3.7 s⁻¹. By contrast, the ADP-induced transient (blue) is adequately fit to a single exponential function (magenta solid line), defining a rate constant of 2.3 s⁻¹. The inset illustrates that labeling the CENP342 only with FlAsH in this experiment produces no fluorescence change. C, plots of rate constants for the transients produced in this experiment versus [nucleotide]. The two rate constants with ATP (closed and open red circles) both demonstrate hyperbolic dependence on ATP concentration (solid and dashed red lines), defining maximum rate constants and apparent dissociation constants summarized in Table 1. The single rate constant with ADP (closed blue triangles) also varies hyperbolically with ADP concentration, defining a maximum rate constant and apparent dissociation constant summarized in Table 1. Conditions are as in Fig. 2.
faster than microtubule dissociation (14). These features are consistent with the motor’s proposed role in generating microtubule sliding because this occurs with ensembles of Eg5 molecules that therefore do not require individual Eg5 motors to be highly processive. In the current study, we have examined the ATPase cycle of CENP-E to see if its enzymology explains its remarkable degree of processivity, with run lengths in excess of 2 μm (8).

The ATPase Cycle of CENP342—The kinetic scheme for the monomeric CENP342 is summarized in Fig. 8A and Table 1. As

![Graph](https://via.placeholder.com/150)

**FIGURE 5.** Kinetics of binding of 2’-dmt to CENP392zip and to a complex of CENP392zip + microtubules. A, 4 μM CENP392zip was made nucleotide-free by incubating with 0.2 unit/ml apyrase for 30 min and then mixed in the stopped flow with 40 μM 2’-dmt. The resulting fluorescence increase is depicted as the red dotted curve, which could be fit to a single exponential process (solid black line), defining a rate constant of 147.3 s⁻¹. B, a complex of 2 μM CENP392zip and 10 μM polymerized tubulin was generated, and 3 μM ADP was added to saturate the catalytic site of the tethered head. This was then mixed in the stopped flow with 20 μM 2’-dmt, and the resulting fluorescence enhancement is depicted as the blue dotted curve, which required fitting to the sum of two exponential processes (solid black line), defining rate constants of 109 and 11.5 s⁻¹. C, plots of rate constant versus [2’-dmt] for CENP392zip (red) and CENP392zip + microtubules (blue). Rate and dissociation constants derived from these plots are summarized in Table 1. For the complex of CENP392zip + microtubules, the faster rate constant (closed blue circles) varies linearly with [2’-dmt], defining rate constants summarized in Table 1. The slower rate constant shows little [2’-dmt] concentration dependence and averages 13.7 ± 2.7 s⁻¹. Inset, ratio of amplitude of the faster phase (A_f) divided by the total signal amplitude (A_t) as a function of [2’-dmt] for mixing CENP392zip-microtubule complex with 2’-dmt. The amplitude of the faster phase constitutes slightly more than 50% of the total signal amplitude over the range of 2’-dmt concentrations used in this study. Conditions are as in Fig. 2.
Fig. 3 demonstrates, microtubule dissociation by ATP is nearly 2-fold faster than for ADP, which suggests that dissociation largely occurs from the ADP-Pi intermediate. Several features of this ATPase cycle resemble those of kinesin 1. Like kinesin 1, ATP binding to microtubule-bound CENP342 is very rapid (Fig. 2) and is not rate-limited by neck linker docking. Furthermore, the ratio \( k_{cat}/k_{d} \) at \( t \sim 2 \), indicates that CENP342 is capable of undergoing on average approximately two enzymatic turnovers before dissociating from the microtubule. However, the kinetics of neck docking in CENP-E are much more like Eg5, with rates that are only 3–4-fold faster than for ATP-induced dissociation (Fig. 4 and Table 1). This appears to be inconsistent with the kinesin 1-like run lengths that we had previously measured for CENP392zip (8), and we therefore next examined the ATPase cycle of this dimer.

CENP392zip Is Chemically Processive—As in the case of the CENP342 monomer, several features of the CENP392zip dimer are very similar to kinesin 1 and are consistent with this motor’s processive behavior. First, the value of \( k_{cat} \) for CENP392zip is approximately half of that for the CENP342, suggesting that the ATPase of the former is gated. Second, the bimolecular association constant defined by the ratio of \( k_{cat}/K_{0.5,MT} \) for CENP392zip is \( \sim 134 \mu M^{-1} s^{-1} \), which is nearly 5-fold larger than the theoretical limit for bimolecular association of a molecule the size of CENP392zip to the microtubule \( \sim 30 \mu M^{-1} s^{-1} \) (27), implying that CENP392zip can undergo multiple cycles of ATP hydrolysis per diffusional encounter with the microtubule.

The Mechanisms Underlying the Processivity in CENP-E—Mixing CENP392zip with 2’dmT produces an increase in mant fluorescence in a single phase (Fig. 5A), whereas in the presence of microtubules, two phases of roughly equal amplitude can be distinguished (Fig. 4, B and
CENP-E ATPase Cycle

![CENP-E ATPase Cycle](image)

FIGURE 7. Kinetics of nucleotide-induced dissociation of CENP392zip from the microtubule. A complex of 3 μM CENP392zip plus 2 μM microtubules plus 0.2 units/ml apyrase was mixed in the stopped flow with nucleotide, and the turbidity at 350 nm was measured as a function of time. The resulting time-dependent concentration, defining maximum rates and apparent dissociation constants of 4.7 s⁻¹/11006 M for ATP and 2.1 s⁻¹ for ADP. The rate constant for the slower process averages 0.30 ± 0.16 s⁻¹ for ATP (open red circles) and 0.31 ± 0.11 s⁻¹ (open blue boxes) for ADP, and neither rate shows any nucleotide concentration dependence. CENP392zip under conditions where forward stepping and mechanical strain can occur before neck linker docking is somewhat loose, because as Fig. 4 illustrates, dissociation from the microtubule can occur before neck linker docking. This conclusion is also consistent with our studies of the CENP392zip dimer. Our data suggest that ATP induces forward stepping and rapid ADP release from the tethered head of the CENP392zip dimer in only about 65% of motor molecules, producing the faster phase in the transient illustrated in Fig. 6A. This implies that the remaining

C). Similar findings have been observed in kinesin 1 and have been interpreted to mean that binding of ATP to the lead head is gated by intramolecular strain and can only occur once strain is relieved by dissociation of the trailing head (9, 11). We propose a similar mechanism for CENP-E, which is illustrated in Fig. 8B. As Fig. 6 shows, ATP-induced release of ADP from CENP392zip occurs with two rate constants, and only the faster one (with a maximum rate of 57 ± 4 s⁻¹) is rapid enough to be on the main mechanochemical pathway. If the slower phase in the 2’dmT binding transient (13.7 ± 2.7 s⁻¹, Fig. 5C, open blue circles) reflects binding of ATP to the leading head, as it does in kinesin 1, then we can calculate the value of $k_d$, the dissociation rate constant of the trailing head under mechanical strain, from Equation 1,

$$k_{obs} = \frac{k_3k_d}{k_3 + k_d} \hspace{1cm} (Eq. 1)$$

because under processive conditions, both $k_3$ and $k_d$ are essentially irreversible. If we set the values of $k_{obs}$ and $k_3$ in Equation 1 to those measured in Figs. 4 and 5 ($k_3 = 57 ± 4$ s⁻¹, $k_{obs} = 13.7 ± 2.7$ s⁻¹) then we can calculate a value for $k_d$ of 18 s⁻¹. This is about 50% larger than the rate constant for dissociation of the unstrained monomeric CENP342 and, as will be discussed below, ~3-fold larger than dissociation of dimeric CENP392zip under conditions where forward stepping and generation of a strained intermediate are unlikely. This situation is also similar to that of kinesin 1, because although 35% of motors bind and hydrolyze ATP and dissociate from the microtubule before forward stepping can occur. Because we observe similar findings at motor/microtubule stoichiometries as low as 1:14, it seems unlikely that the slow phase in the transient represents an artifact of motor crowding. Rather, it appears instead that as in the monomer, there is a relatively loose coupling between ATP binding and forward stepping in the dimer.

Our results suggest that some other feature of the dimer keeps it closely associated with the microtubule for multiple mechanochemical cycles, so that if the motor fails to take a forward step, it will not diffuse away. A clue as to what this might be comes from the kinetics of the turbidity change produced by dissociating CENP392zip from the microtubule with nucleotide. Although dissociation of CENP342 from the microtubule occurs in a single step (Fig. 3), that for CENP342zip occurs in two steps with either ATP or ADP (Fig. 7). The faster phase (inset, closed circles and boxes) demonstrates a hyperbolic dependence on nucleotide concentration, and, as in monomeric CENP342, it is about 2-fold faster for ATP. We propose that this faster component of the transient reflects dissociation of the rigor-bound CENP392zip head in the absence of strain. As noted above, this rate is ~3-fold slower than the value of $k_d$ that we calculate from Equation 1. By contrast, the slower component in the dissociation transient (Fig. 7, open boxes and circles), which is not seen in monomeric CENP342, has no appreciable nucleotide concentration dependence. This suggests that...
it occurs subsequent to or independent of nucleotide binding and dissociation of the rigor head. We propose that this phase in the transient reflects the contribution of a structure not present in the CENP-E monomer that slows the complete detachment of the dimer from the microtubule and reduces the probability that the motor will diffuse away. One possibility is that the tethered head might interact with the microtubule surface, producing additional stability against dissociation. This would be consistent with recent evidence from kinesin 1 that the tethered head can synthesize ATP from ADP and Pi (29) as well as with earlier evidence that ADP release from the tethered head is accelerated compared with kinesin in the absence of microtubules (19). However, this explanation is not consistent with our finding that the kinetics of microtubule dissociation are the

FIGURE 8. Schematic of the CENP-E mechanochemical cycle. A, the CENP342 monomeric ATPase cycle. The CENP342 motor domain is depicted in magenta, the neck linker as the blue line, nucleotide and phosphate as the colored, labeled spheres, and αβ-tubulin as the gray ellipses. Docking of the neck linker is depicted as a change from a curved to a straight conformation of the blue line, toward the plus end of the microtubule. Based on analogies to other processive kinesins, such as kinesin 1 and Eg5, and on the data presented in Figs. 1–3, we propose that binding of ATP to the leftmost, rigor motor, with apparent second order rate constant $K_0$, is followed by neck linker docking ($k_2$) and ATP hydrolysis ($k_h$). Dissociation ($k_d$) is favored in the ADP-Pi state and occurs concomitantly with phosphate release. This leads to reorientation of the neck linker, rebinding to the microtubule, and release of ADP to complete the cycle. However, our finding that the ratio $k_{cat}/k_d < 1$ implies that CENP342 is capable of releasing phosphate while still bound to the microtubule. This is shown by the dashed black arrow.

$B$, the CENP392zip dimeric ATPase cycle. The two CENP392 motor domains are depicted as the dark blue and cyan ellipses and the coiled coil as the cyan double helix, and the other features of the figure have the same meaning as in Fig. 2. Binding of ATP to the leftmost, rigor motor, with apparent second order rate constant $K_0$, is followed by neck linker docking and forward stepping ($k_2$). Release of ADP from the lead head and hydrolysis of ATP on the trailing head follow, generating intermediates in which both heads are attached to the microtubule. In this configuration, the trailing head experiences forward mechanical strain, and the leading head experiences backward mechanical strain (represented by the red arrows and straightening of the neck linkers). We propose that, as in the case of kinesin 1, rearward strain on the lead head blocks ATP binding to that head (×) and can only occur once the rear head dissociates ($k_d$). This relieves internal strain, allows ATP to bind to the lead head, and completes the cycle.
same, both in the presence of apyrase, which should allow a strong contact between the tethered head and the microtubule, and in 10 µM ADP, which should dissociate this contact.

A second possibility is that a structure outside of the motor domain contributes to stabilizing the microtubule-bound state. A candidate for such a structure is the proximal coiled coil. In kinesin 1, it has been proposed that a sequence of three lysines that bridge the first two heptads of the coiled coil interact with negatively charged residues in the carboxyl terminus of tubulin. Amplifying this region in recombinant kinesin 1 or adding additional positively charged residues was found to enhance run length severalfold, whereas inserting negative charges reduced it severalfold (30). This arrangement would effectively tether the kinesin 1 motor near the microtubule surface and allow motors that have dissociated to rebind rapidly. Like kinesin 1, the first two heptads of CENP-E also contain a cluster of positive charges (KRKYR), and we postulate that this may provide a similar tethering effect.

The Mechanisms of CENP-E Processivity in Vitro Have Implications for How It Functions in Vivo—Based on our finding that CENP392zip is a highly processive, plus end-directed motor, we had proposed that it functions, at least in part, to transport chromosomes to the metaphase plate. However, CENP-E also maintains spindle microtubules at the kinetochore (2–7). These microtubules are dynamic at the plus, kinetochore-associated ends, and a plus end-directed, processive transport motor would therefore continually be at risk of running off of these microtubules. One way of preventing this would be to ensure that CENP-E remains tethered to the microtubule via interactions through a second site, its coiled coil. In this way, even if the motor domains walk off of the plus ends, CENP-E and its attached chromosome would always be in close proximity to these kinetochore microtubules.

Finally, we note that a prior study found that a longer CENP-E construct has different motility properties. These authors found that a 473-residue-long construct moved processively along microtubules in discontinuous runs that were 20–40-fold slower than for CENP392zip (31). We note that the region between residues 392 and 473 contains the sequence RVKRKRR (residues 415–421). This sequence is located in a region between residues 392 and 473 contains the sequence.

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