Measuring Kinesin’s First Step*

Received for publication, May 29, 2002, and in revised form, July 3, 2002
Published, JBC Papers in Press, July 16, 2002, DOI 10.1074/jbc.M205261200

Steven S. Rosenfeld§§, Jun Xing¶¶, Geraldine M. Jefferson‡, Herbert C. Cheung¶¶, and Peter H. King‡‡

From the Departments of Neurology and Biochemistry and Molecular Genetics, University of Alabama at Birmingham and Neurology Service, Department of Veterans Affairs Medical Center, Birmingham, Alabama 35294

A variety of models have recently emerged to explain how the molecular motor kinesin is able to maintain processive movement for over 100 steps. Although these models differ in significant features, they all predict that kinesin’s catalytic domains intermittently separate from each other as the motor takes 8 nm steps along the microtubule. Furthermore, at some point in this process, one molecule of ATP is hydrolyzed per step. However, exactly when hydrolysis and product release occur in relation to this forward step have not been established. Furthermore, the rate at which this separation occurs as well as the speed of motor stepping onto and release from the microtubule have not been measured. In the absence of this information, it is difficult to critically evaluate competing models of kinesin function. We have addressed this issue by developing spectroscopic probes whose fluorescence is sensitive to motor-motor separation or microtubule binding. The kinetics of these fluorescence changes allow us to directly measure how fast kinesin steps onto and releases from the microtubule and provide insight into how processive movement is maintained by this motor.

Several features of kinesin’s mechanochemistry contribute to its ability to move long distances on the microtubule without dissociating. These include its high duty ratio and ability to hydrolyze multiple ATP molecules per force productive encounter-features that enhance the probability that a motor will remain attached even under load (1–6). However, processive movement also requires two heads, and coordination between these two motor domains is essential if kinesin is to move in an orderly fashion along its track (7, 8). Two models have recently appeared to explain how the motor domains work together to bring about processive movement. In the hand-over-hand model (1, 9), each head alternates between a forward and rearward orientation on the microtubule, whereas in the inchworm model (10), the forward and rearward heads retain their relative positions throughout the mechanochemical cycle. The hand-over-hand model proposes that ATP binding docks the neck linker of the attached head, and this swings the tethered head forward toward the next microtubule β subunit 8 nm away (11, 12). ATP hydrolysis on the rear head and ADP dissociation from the newly attached forward head follow, and they lead to dissociation of the rearward head from the microtubule. The net effect is hydrolysis of one ATP molecule per 8-nm step in the plus end direction (11, 13). In the inchworm model, ATP binding, hydrolysis, and dissociation of ADP are associated with alternating separation and association of the two heads, only one of which is enzymatically active. However, precisely where nucleotide binding and hydrolysis fit into the overall scheme has not been determined (10).

Despite their differences, both models agree on several key features. First, ATP induces the two heads to separate by 8 nm, as one of them steps forward in the “+” direction. Second, at some point in the cycle, ATP is hydrolyzed, and phosphate is released. Third, ATP binding and hydrolysis changes the affinity of one of the kinesin motor domains for the microtubule, from “strong binding” to “weak binding,” and this strong to weak transition is associated with forward movement. Finally, the cycle is completed when the trailing head dissociates from the microtubule and rejoins its partner. However, several key questions remain unanswered by these models. What is the nature of the conformational change that produces the forward step? What enzymatic process actually produces this 8 nm separation, ATP binding or ATP hydrolysis? When and how quickly does the trailing head dissociate from the microtubule? How is the mechanical strain that is placed on the neck linkers by attachment of both heads relieved? Determining the speed and timing of kinesin’s forward step and trailing head release are therefore crucial in evaluating each of the competing models and may help in their refinement as well as in the testing of their validity.

In our previous study (14), we demonstrated that a fluorescent probe located on the neck linker of a kinesin monomeric construct could be used to measure in real time the reorientation and docking of the neck linker and to identify the step in the enzymatic cycle when this occurs. In this study, we have extended this approach to a dimeric kinesin construct to measure the kinetics of neck linker-neck linker and neck linker-microtubule separation. These measurements provide constraints that should allow us to critically examine current models of kinesin processivity.

EXPERIMENTAL PROCEDURES

Materials—Fluorescent probes were obtained from Molecular Probes (Eugene, OR). Synthesis of mant nucleotides was carried out as described (15).

Generation of K413W340F Mutant—Our goal was to generate a kinesin construct of 413 residues that contained one reactive cysteine at position 333 and in which tryptophan 340 was mutated to phenylalanine. Using a plasmid encoding wild type human K413 (16), the following PCR primers were used to amplify the native sequence: upstream primer, 5′-AAAGTTGGCATGTGCTAGTATATATGCATATGCGGGCCTGTCGCGGAGAAGCTTGGTCGAGTCC-3′; downstream primer, 5′-AAGTTGCATGTGCTAGTATATATGCATATGCGGGCCTGTCGCGGAGAAGCTTGGTCGAGTCC-3′. The PCR product was digested with PstI and XhoI to generate a fragment, which was ligated into PBSK, and the resulting product was linearized with PstI and XhoI. A PCR fragment encoding the N-terminal 349 residues of a cysteine-light mutant of kinesin (kindly provided by Dr. Ron Vale, UCSF) was amplified with the upstream primer described above and with the following down-
The kinesin dimer consists of two heavy chains that each contain a motor domain. These motor domains are depicted as the two globular structures on the left and right halves of the figure. They each contain one molecule of ADP (magenta space-filling structures on the figure). The motor domains are connected to the α helical coiled-coil at the bottom of the figure by the two neck linkers (depicted as yellow space-filling residues). Leucine 335, which is depicted in red space-filling, corresponds to valine 333 in the human sequence. It is contained within the neck linker sequence and is the site of attachment of the rhodamine probes. This model is based on the published crystal structure (20) and was drawn with RIBBONS.

within the neck linker sequence. In order to do this we engineered a kinesin dimer devoid of reactive cysteines and with a cysteine mutation at position 333. Furthermore, since we intended to perform FRET studies between microtubule tryptophans and an AEDANS probe at position 333 of kinesin, we mutated tryptophan 340 to phenylalanine. The kinesin mutant thus generated, referred to as K413W340F, demonstrated no energy transfer between the remaining tryptophan residues at positions 360 and 368 and an AEDANS probe at position 333. This is to be expected, given the 35–40 Å distance separating these residues (20) and the value of R 0 for this donor-acceptor pair (~20 Å, Ref. 14). Fig. 1 depicts the wild type kinesin dimeric structure derived from Rattus norvegicus (20). In this figure, structures relevant to our study are depicted in the space-filling format, including the bound nucleotide (magenta), the neck linker (yellow), and leucine 335 (in red and equivalent to valine 333 in the human sequence).

Measurement of ATPase Activity and Processivity—K413W340F has a microtubule-activated ATPase activity, which at 10 mM KCl has values of 19.0 ± 1.9 s^-1 and 0.25 ± 0.10 μM for kcat and K0.5,MT, respectively. Labeling cysteine 333 with either 1,5-IADANS or TMR did not appreciably alter these values (data not shown). Although the value of kcat is quite close to values reported for similar Drosophila constructs, the ratio of kcat/K0.5,MT (referred to as kcat/ATPase) is ~4-fold smaller (4, 21). We measured the kinetics of the weak-to-strong transition in this construct by mixing in the stopped flow a complex of 2'-deoxy mant-ADP-K413W340F with microtubules and 1 mM ATP and monitoring nucleotide release (2, 13, 22). The resulting fluorescence decay demonstrated a linear dependence on microtubule concentration, defining an apparent second order rate constant, kcat/(ADP) of 1.04 ± 0.07 μM^-1 s^-1 (data not shown). The ratio kcat/(ATPase)/(ADP) is a measure of the number of ATP molecules hydrolyzed per processive run. Its value is 76, which compares to the previously reported value of ~100 for native kinesin constructs of comparable size (4). Thus, although our mutagenesis of K413W340F did alter the affinity of a kinesin-ADP state for microtubules (23), it did not appear to have appreciably affected its processivity.

**Spectroscopic Studies with TMR-labeled K413W340F**—We first sought to label the K413W340F dimer at position 333 with an optical probe whose fluorescence would be sensitive to the distance between the two neck linkers of the motor. Previous reports had demonstrated that the fluorescent probe TMR dimerizes under favorable conditions (24–26). We therefore...
reasoned that labeling dimeric kinesin at position 333 with TMR might produce a motor whose rhodamine probes would alternately dimerize and separate during each mechanochemical cycle, as the motor steps along on the microtubule.

Monomeric rhodamine demonstrates a peak in its absorption spectrum at 555 nm, a shoulder at 518 nm, and a 518/555 nm ratio of 0.5 (24–26). Dimerization strongly quenches the rhodamine fluorescence emission. It also produces a blue shift in the absorption spectrum, with two discrete peaks at 555 and 518 nm and with an increase in the 518/555 nm ratio to 1.4 (24–28). Fig. 2A shows that the absorption spectrum of TMR-labeled K413W340F (dotted spectrum) has the features characteristic of rhodamine dimer. Addition of guanidine hydrochloride to 6 mM enhances the 555 nm absorbance and reduces the 518 peak (solid spectrum), as expected since unfolding of K413W340F would separate the rhodamine probes. The fluorescence emission spectrum of TMR-labeled K413W340F (Fig. 2B, dotted spectrum) is highly quenched, and addition of 6 mM guanidine hydrochloride enhances fluorescence over 10-fold (Fig. 2B, solid spectrum).

In the absence of added nucleotide, K413W340F binds to microtubules via one of its two motor domains (29, 33). The absorption spectrum of a complex of rhodamine K413W340F + microtubules under rigor conditions demonstrated a reduction in the 518/555 nm ratio (1.3 versus 1.5 for TMR-labeled K413W340F). This was reduced to 1.2 in the presence of ATP under conditions that support processive movement (10 mM KCl) and is illustrated in Fig. 2C as the dashed spectrum. In the presence of 1 mM AMPPNP, both heads of kinesin attach strongly to the microtubule (29). This is reflected in Fig. 2C by a further reduction of the 518/555 nm ratio to 1.1 (solid spectrum). This data suggests that under conditions that favor processive movement, an appreciable fraction of kinesin molecules are attached to the microtubule via both heads.

Kinetic Studies with TMR-labeled K413W340F—Our spectroscopic results allowed us to make testable predictions about the time-dependent fluorescence emission of rhodamine-labeled K413W340F as it moves along the microtubule. The first of these is illustrated in Fig. 3A. Kinesin enters its mechanochemical cycle with one head strongly attached to the microtubule. The fluorescence of this species should be relatively quenched. ATP binding to the attached head should swing the tethered head forward to attach to the next microtubule-binding site, pulling the two heads apart by 8 nm. We would therefore predict that mixing a complex of rhodamine-labeled kinesin-microtubules with ATP should produce an initial fluorescence rise, followed by a fall as dissociation of the trailing head allows the neck linkers to spring back together. The red and green transients marked 10 mM KCl and 100 mM KCl in Fig. 3B confirm this, and the inset in the figure demonstrates the rising phase over a shorter time scale. At 100 mM KCl, the fluorescence at the completion of this transient is lower than that at the start. This would be expected, since high ionic strength reduces kinesin’s affinity for the microtubule, and dissociation of the trailing head should be followed by dissociation of the motor (2, 3, 30). By contrast, at 10 mM KCl, the final fluorescence is ~50% greater than the starting fluorescence, which implies that during a processive run, the motors partition between singly and doubly attached species. Fig. 3B also shows that repeating this experiment in the absence of microtubules (blue transient marked no microtubules) produces no change in rhodamine emission.

Our second prediction is that the rate of the rising phase should vary hyperbolically with ATP concentration, and should be no faster than the rate of ATP binding. Fig. 4 (open boxes) confirms this and defines a maximum rate of 763 ± 84 s⁻¹ and apparent dissociation constant of 98 ± 24 µM in 100 mM KCl. Similar results were seen at low ionic strength (data not shown). We measured the rate of 2'-deoxy mant-ATP binding to
the attached head by monitoring FRET from kinesin tyrosine residues to the mant fluorophor (17). Fig. 4 shows the rate of mant nucleotide binding (closed triangles) was consistently as fast or faster than the rate of the rising phase of the rhodamine transient. It varied linearly with nucleotide concentration, defining an apparent second order rate constant of 4.9 \pm 1.1 \mu M^{-1} s^{-1} with an extrapolated dissociation rate constant of 73 \pm 24 s^{-1}. This dissociation rate is similar to prior measurements on native kinesin constructs (2, 21, 22).

Once kinesin has stepped forward, its trailing head dissociates from the microtubule. This should allow the neck linkers to spring back together, reform rhodamine dimers, and quench the rhodamine fluorescence. At 100 mM KCl, the rate of this phase should match the rate at which kinesin dissociates from the microtubule, since the microtubule affinity of K413W340F-ADP is reduced, and dissociation is rapid. Our third prediction is that the rate of the decrease in rhodamine fluorescence at high ionic strength should match the rate at which ATP dissociates kinesin from the microtubule. Fig. 5 confirms this. The rate of ATP-induced dissociation at 100 mM KCl, as measured by turbidity (closed circles) is close to that of the falling phase of the rhodamine transient at this ionic strength (closed triangles), with an extrapolated maximum rate of 50–60 s^{-1}.

At 10 mM KCl, the falling phase of the rhodamine transient fit a double exponential decay, and the faster phase constituted 75–80% of the total signal amplitude. This phase showed an ATP concentration dependence essentially identical to that at 100 mM KCl (Fig. 5, open boxes). The slower phase showed little ATP concentration dependence, and its rate ranged between 1.1–2.4 s^{-1}. At this ionic strength, little dissociation could be detected with turbidity over the time course of the rhodamine transient (data not shown). This strongly suggests that the rate of ATP-induced decrease in rhodamine fluorescence is controlled by (and therefore is a measure of) how fast the trailing head can dissociate from the microtubule.

Our fourth prediction, illustrated in Fig. 6A, is that mixing rhodamine-labeled K413W340F + microtubules with ADP should produce a small decrease in fluorescence, as the partially separated neck linkers reapproximate with microtubule dissociation. The orange fluorescence transient depicted in Fig. 6A is consistent with the prediction.
6C (ADP) confirms this. A small amplitude, single exponential decay at 1 mM ADP and 100 mM KCl (final concentrations) was observed, corresponding to a 4% decrease in fluorescence intensity, with a rate constant of $128 \pm 21 \text{s}^{-1}$ (n = 10). This rate is ~7–8 fold faster than the corresponding process with native kinesin, and is consistent with the lower affinity of the K413W340F-ADP for the microtubule (31, 32).

AMPPNP binding causes both heads of kinesin to attach to the microtubule (29, 33). Our fifth prediction, illustrated in Fig. 6B, is that mixing with AMPPNP should produce a fluorescence transient consisting only of a rising phase. Furthermore, the initial fluorescence intensity should be the same as that for mixing with ADP or ATP, because in each case the starting conditions are the same. The red transient marked AMPPNP in Fig. 6C confirms these predictions. Mixing with 1 mM AMPPNP and 100 mM KCl (final concentrations) produced a biphasic transient with rates of $48 \pm 19 \text{s}^{-1}$ and $1.6 \pm 1.1 \text{s}^{-1}$ (n = 10), and the amplitude of the faster phase constituted 40% of the total amplitude. The rate of the faster phase is similar to the previously reported rate of AMPPNP-accelerated ADP release (13). However, the presence of a second, slower phase suggests that attachment of the second head to the microtubule may occur in two steps. The green transient in Fig. 6C was produced by mixing with 1 mM ATP and 100 mM KCl (final concentrations). Fitting to two exponential terms, as per Fig. 3, reveals that the extrapolated amplitude of the rising phase is 87% of the total signal amplitude for AMPPNP.

Several further predictions are also supported by our results. Mixing unlabeled K413W340F + microtubules with ATP produces no signal beyond that of buffer alone (data not shown), which establishes that the signal change seen in Fig. 3B is not due to light scattering. Furthermore, repeating these experiments with a rhodamine labeled monomeric kinesin (K349) + microtubules produced no fluorescence change (data not shown).

Our conclusions from the rhodamine probe would be supported if a different probe, sensitive to neck linker association with the microtubule, could provide a direct measure of the rate of trailing head dissociation that agreed with the rhodamine data. For this purpose, we utilized an AEDANS probe on the kinesin neck linker.

FRET from Microtubule Tryptophans to AEDANS on the Neck Linker of Kinesin.—In our previous study, we demonstrated that an AEDANS probe attached in the neck linker of a monomeric kinesin construct could be excited by energy transfer from the microtubule tryptophan residues (14), and we established that this probe could monitor kinesin-microtubule association. We labeled K413W340F at position 333 on the neck linker with AEDANS, monitored microtubule binding by FRET, and found that binding produced a 21% fluorescence enhancement in the absence of nucleotide (data not shown).

In our previous study, we also had shown that binding of ATP to an AEDANS-kinesin-microtubule complex reduced the FRET efficiency of the AEDANS probe (14). This, plus the rapid
rate at which a doubly attached intermediate would be formed (>750 s\(^{-1}\), Fig. 4), means that mixing with ATP would likely produce a low amplitude rising phase that may not be observable in the stopped flow. This would then be followed by a fluorescence decay as the trailing head releases from the microtubule (Fig. 7A). Furthermore, at 10 mM KCl, the amplitude of this decay would be expected to be smaller than at 100 mM KCl, since under the former conditions, the kinesin motors would partition between singly and doubly attached species.

Fig. 7B shows that the resulting fluorescence transients at both 10 and 100 mM KCl are mono-exponential, and that the amplitude at low ionic strength is ~40% of that at high ionic strength. However, the rates at both ionic strengths show a similar hyperbolic dependence on ATP concentration, which at 10 mM KCl extrapolated to a maximum of 63.6 ± 2.5 s\(^{-1}\) with dissociation constant of 76 ± 10 \(\mu\)M. For the rhodamine data (solid curve), the corresponding parameters are 58 ± 3.9 s\(^{-1}\) and 94 ± 21 \(\mu\)M.

Anisotropy Decay of AEDANS-labeled K413W340F—The shape of the TMR-K413W340F-microtubule absorption spectrum (Fig. 2C), as well as the presence of intermediate levels of rhodamine fluorescence seen after mixing kinesin-microtubules with ATP at low ionic strength (Fig. 3B) implies that the neck linkers are in a dynamic equilibrium between dimer and monomer. This in turn suggests that the neck linkers are mobile during all phases of the mechanochemical cycle except when both heads are attached to the microtubule. In order to evaluate this, we measured the anisotropy decay of AEDANS-labeled K413W340F, both in isolation as well as complexed to microtubules in via one head (in the presence of added nucleotide) or both heads (in the presence of 1 mM AMPPNP, Refs. 29 and 33). Table I summarizes the anisotropy decay data.

For each preparation, a long and a short correlation time were observed. The short correlation time was between 1.5 and 2.5 ns in each preparation, and as discussed in our earlier study (16), is due to local probe motion. The one long correlation time represents the harmonic mean of the two correlation times that would be predicted for a prolate ellipsoid (35). Its value for AEDANS K413W340F, at 120.4 ns, is similar to our previous measurements using a mant ADP probe (16). Binding to microtubules via one head increased this correlation time to 266.8 ns. Although over 2-fold larger, this is still several orders of magnitude smaller than the rotational correlation time of a similarly sized myosin motor strongly bound to actin (34). This implies that the neck linkers in this state retain significant segmental flexibility. By contrast, binding via both heads increased the correlation time beyond what could be reliably measured with the AEDANS probe (>650 ns). For the sake of comparison, Table I also lists the long correlation time of AEDANS-labeled K349, which is nearly identical to our prior measurement using a mant ADP probe (16).
The remarkable ability of kinesin to travel along the microtubule for distances of over 1 μm without detaching has encouraged the development of several models to explain its high degree of processivity (1, 13, 22). These models agree on several key points. 1) ATP binds to the attached head and causes the tethered head to swing forward to the next tubulin-docking site, 8 nm away, generating a transient, doubly attached intermediate. 2) One molecule of ATP is hydrolyzed at some point during this process. 3) ADP is released from the newly attached forward head, and 4) at some point, the trailing head detaches and swings forward to rejoin its partner, completing the cycle.

A critical test of these models, however, requires direct measurements of the rates of several key steps in the mechanochemical cycle, including the forward step of the tethered head and the release of the rear head. This has heretofore not been done because probes that could monitor these events in real time have not been available. This has prevented determining when forward stepping by the tethered head and release of the trailing head occur in relation to ATP binding, ATP hydrolysis, and ADP release.

We have addressed this issue by developing a dimeric kinesin construct that could be specifically labeled in the neck linker with fluorescent probes. Although mutagenesis did accelerate the rate of ADP-induced release from the microtubule, it had little effect on force, step size, or processivity (29, 33). We enter the cycle with one head attached to the microtubule, and the other head tethered with ADP in its active site (13, 21, 22, 37). We had previously shown that ATP binds to the catalytic site on the attached head and causes the driving force of the neck linker to swing forward to the next microtubule-binding site in a concerted reaction. This occurs at a rate of ~750 s⁻¹ at room temperature, much faster than either ATP hydrolysis on the attached head or ADP release from the tethered head (13, 21, 22).

Several mechanical and kinetic studies have suggested that kinesin’s 8-nm step forward consists of two substeps of ~4 nm each (5, 38). It has also been suggested that the conformational change which reorients the neck linker, and which we argue allows the motor to forward, is followed by a Brownian-driven diffusion to the next tubulin-docking site. This argues that prior to attachment of the tethered head, there is considerable segmental flexibility in the neck linkers; a point confirmed by our anisotropy decay studies (Table I). Furthermore, mixing rhodamine-labeled K413 + microtubules with AMPPNP produces a biphasic fluorescence increase (Fig. 6C). This implies that AMPPNP binding redistributes the equilibrium between neck linker monomers and dimers to favor monomers and does so in two sequential steps. This is illustrated in Fig. 8 by a two-step advance of the yellow head onto the next β tubulin subunit.

The rising phase of the ATP-induced transient (Fig. 3B, inset) fit a single exponential process whose amplitude was similar to that for AMPPNP. This might at first glance suggest that, unlike AMPPNP, 8 nm of forward motion is accomplished in a single step with ATP. However, our data is also consistent with ATP-induced forward motion occurring in two substeps if the second substep were very rapid, as would be expected with a Brownian-driven diffusional process.

Release of ADP from the new, leading head has been reported by several investigators to occur at a rate of 120–150 s⁻¹ (although one report suggests a significantly faster rate, Ref. 32), and it produces a transient intermediate in which both heads are strongly bound to the microtubule. This should place the neck linkers under tension (illustrated in Fig. 8 as straightening of the magenta neck linkers) and should immobilize them. This point is supported by our anisotropy decay data in the presence of 1 mM AMPPNP (Table I). How would this strain be released? One possibility is that the proximal coiled-coil unwinds. Our anisotropy decay data suggest that the neck linker has appreciable flexibility, and this implies that neck

### TABLE I

| Sample     | Nucleotide | $r_1$ (ns) | $r_2$ (ns) | $A_1$ | $\phi_1$ | $\phi_2$ |
|------------|------------|------------|------------|-------|--------|--------|
| K413 + MT  | 1 mM ADP   | 15.2 ± 0.10| 7.1 ± 0.10 | 0.17  | 120.4 ± 8.9 | |
| K413 + MT  | No added nucleotide | 14.9 ± 0.10| 6.4 ± 0.08 | 0.20  | 266.8 ± 28.4 | |
| K413 + MT  | 1 mM AMPPNP| 15.0 ± 0.05| 6.7 ± 0.12 | 0.20  | >650   | |
| K349       | 1 mM ADP   | 14.2 ± 0.10| 5.4 ± 0.23 | 0.17  | 24.9 ± 1.6 | |

**Conditions:** 10 mM KCl, 25 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, 1 mM dithiobisreitol, pH 7.50, 20 °C. The kinesin preparations were 5 μM, and microtubules, when present, were at a concentration of 40 μM. AEDANS labeling ratio was 0.85–0.95 mol/mol active site. MT: microtubules, $r_1$, $r_2$ lifetimes (in nanoseconds); $A_1$: limiting anisotropy. $\phi_1$, long rotational correlation time (in nanoseconds). In the absence of added nucleotide, a complex of K413W340F + microtubules will have ADP attached to the tethered head, and the catalytic site on the attached head will be free of nucleotide. In the presence of 1 mM AMPPNP, both heads will be attached to the microtubule, with AMPPNP in the trailing head and the leading head in rigor (29, 33).
The first step is ATP binding to the nucleotide-free, attached head ($k_1$). Under physiologic conditions, this occurs with a pseudo first order rate constant of $>1000$ s$^{-1}$ at room temperature (14). This docks the neck linker of the attached head, separates the two neck linkers, and swings the trailing head forward toward the next tubulin-docking site at a rate of $\sim 800$ s$^{-1}$ ($k_2$). ADP release from the new, leading head ($k_3$) and ATP hydrolysis on the new, trailing head ($k_4$) follow. With release of ADP from the new leading head, a rigor bond is formed with the microtubule, which places both neck linkers under mechanical strain. This is symbolized by a straightening of the magenta neck linkers in the figure. Strain is relieved by dissociation of the trailing head at a rate of $\sim 60$ s$^{-1}$ ($k_5$). Given intracellular ATP concentrations and the rate of $k_1$, ATP would rapidly bind to the leading head, be hydrolyzed at $100$ s$^{-1}$, and dissociate the kinesin dimer before the trailing head could dissociate and swing forward. That this does not appear to happen (red X) suggests that there is a gating mechanism preventing nucleotide binding to the leading head while it is under strain.
linker-neck linker reassociation is intrinsically rapid. If this uncoiling were to precede trailing head dissociation, so too would neck linker-neck linker reassociation, and the decreasing phase of the rhodamine transient (Fig. 3B) would be predicted to be faster than the rate measured with the AEDANS probe (Fig. 7B). By contrast, if strain were relieved by dissociation of the trailing head, rhodamine quenching and trailing head dissociation, measured by the AEDANS probe, would be predicted to occur at the same rate. Our results with the AEDANS probe, which monitors motor-microtubule association, establishes this by direct measurement and confirms our prediction (Fig. 7).

We have interpreted our results within the context of a hand-over-hand model, and we find it to be consistent with the sequence of conformational changes in the neck linker that we can detect with our fluorescent probes. However, our data are also consistent, in general terms, with an inchworm mechanism that has recently been proposed (10). This in part is because the inchworm model has not identified where in the proposed mechanical sequence ATP binding or hydrolysis occur, making it difficult to critically test its validity (10). However, one aspect of this mechanism, as originally presented, does require revision. Contrary to Fig. 1 of that study (10), our data clearly demonstrate that ATP binding produces an initial separation of the two motor domains, and their reassociation follows with detachment of the trailing head from the microtubule.

Although processivity was first thought to be a unique and defining feature of the kinesin superfamily, it is now appreciated that this feature can be found in several members of the myosin superfamily as well (3). In particular, several models have recently appeared to explain the processive movement of myosins V and VI. While these models differ from those for kinesin motors in several areas, largely those reflecting the different effects that nucleotide has on polymer affinity, they share with the kinesins several important features (40–42). In particular, they propose that, like kinesin, strain is produced by two-headed binding to the actin polymer track, and it alters the kinetics of nucleotide interaction with the catalytic site. What has not been measured in these myosins, however, is the speed of motor-motor separation during a processive run, or the relation of this step to nucleotide binding and release. The approach taken in this study should be directly applicable to such myosins, and should provide further insights into how these motors accomplish processive movement.

Finally, our study raises questions relevant to the current models of kinesin processivity. Fig. 3B shows that mixing TMR-labeled K143W340F-microtubules with ATP at low ionic strength produces a final level of fluorescence that is higher than the initial level. This establishes that under conditions favoring processive movement, an appreciable fraction of kinesin molecules are attached via both heads to the microtubule. As Fig. 8 illustrates, this would lead to the accumulation of a significant population of motors in which ATP or hydrolysis products occupy the catalytic site of the trailing head, and the catalytic site of the leading head is empty. Given millimolar intracellular ATP concentrations, ATP would be expected to rapidly bind to and be hydrolyzed by the leading head, leading to dissociation of a significant fraction of kinesin molecules within a few steps. This is indicated by the dissociation reaction on the right side of the figure, separated from the main pathway by the red X. That this does not occur suggests that there is a mechanism, which regulates when nucleotide binds to the leading head. Furthermore, the slowest rate that we have measured in these studies (the rate of trailing head dissociation) is still over 3-fold faster than kcat. These observations suggest that there is another step in the kinesin ATPase pathway that gates nucleotide binding to the leading head, rate limits the ATPase cycle, and may have an important role in controlling processivity.

Acknowledgments—We thank Dr. Steven Block (Stanford University), Dr. Jonathan Howard (Max Planck Institute), Dr. Susan P. Gilbert, Lisa M. Kumpf, and Andrew T. Mackey (University of Pittsburgh) for thoughtful comments during the preparation of this article. We also thank Scott Stagg (Department of Biochemistry and Molecular Genetics, UAB) for the production of Fig. 1.

REFERENCES
1. Hancock, W. O., and Howard, J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13147–13152
2. Ma, Y.-Z., and Taylor, E. W. (1997) J. Biol. Chem. 272, 717–723
3. Visscher, K., Schnitzer, M. J., and Block, S. M. (2000) Nature 400, 184–189
4. Crevel, L., Carter, N., Schliwa, M., and Cross, R. (1999) EMBO J. 18, 5763–5772
5. Young, E. C., Mahtani, H. K., and Gelles, J. (1998) J. Biol. Chem. 374, 3477–3479
6. Vugmeyster, Y., Berliner, E., and Gelles, J. (1998) Biochemistry 37, 747–751
7. Hijmans, J. C., and Gelles, J. (2002) Science 295, 844–848
8. Coy, D. L., Wagenbach, M., and Howard, J. (1999) J. Biol. Chem. 274, 3667–3671
9. Schie, W. H., and Howard, J. (2001) Curr. Opin. Cell Biol. 13, 19–28
10. Gilbert, S. P., Moyer, M. L., and Johnson, K. A. (1998) Biochemistry 37, 792–799
11. Rosenfeld, S. S., Jefferson, G. M., and King, P. H. (2001) J. Biol. Chem. 276, 40167–40174
12. Measuring Kinesin’s First Step
Measuring Kinesin's First Step
Steven S. Rosenfeld, Jun Xing, Geraldine M. Jefferson, Herbert C. Cheung and Peter H. King

J. Biol. Chem. 2002, 277:36731-36739.
doi: 10.1074/jbc.M205261200 originally published online July 16, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205261200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 19 of which can be accessed free at http://www.jbc.org/content/277/39/36731.full.html#ref-list-1