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Direct observation of the binding state of the kinesin head to the microtubule

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Abstract

The dimeric motor kinesin-1 couples chemical energy from ATP hydrolysis into mechanical work used to transport cargo along microtubules^{1,2}. Cargo attached to the kinesin stalk moves processively in 8-nm increments³ as its twin motor domains (heads) carry out an asymmetric, hand-over-hand walk^{4–7}. The extent of individual head interactions with the microtubule during stepping, however, remains controversial^{4,8–14}. A major experimental limitation has been the lack of a means to monitor the attachment of individual heads to the microtubule during movement, necessitating indirect approaches. We developed a single-molecule assay that can directly report head binding in a walking kinesin molecule, and show that only a single head is bound to the microtubule between steps at low ATP concentrations. A bead was linked to one of the two kinesin heads via a short DNA tether and used to apply rapidly alternating hindering and assisting loads with an optical trap. The time-dependent difference between forward and rearward displacements of the bead alternated between two discrete values during stepping, corresponding to those intervals when the linked head adopted bound or unbound states. The linked head could only rebind the microtubule once ATP had become bound to its partner head.

Optical-trapping assays for kinesin typically involve tracking and loading a bead attached to the C-terminus of the common stalk of the protein³ in place of its natural cargo. Because beads attached to the stalk tend to report the position of the molecule as a whole, the displacements generated by the two separate heads remain unresolved. Single-molecule fluorescence techniques have successfully resolved motions of the heads by tagging these individually with fluorophores, but such approaches have suffered from limited spatiotemporal resolution and lack the ability to apply controlled loads, which can be used to probe binding between the heads and microtubule (MT)^{4,8,9,14}. We overcame these limitations by linking an optically-trapped bead directly to one of the two heads, rather than to the stalk (Fig. 1a), without disabling the motor function of the molecule. One end of a dsDNA oligomer (70-bp) was attached to an engineered cysteine residue (N62C) on the motor domain of an otherwise ‘cys-light’ dimer, and its opposite end was linked to a streptavidin-coated bead. Because this short oligomer subtends less than half a persistence length, the tether is stiff and transmits motions of the head directly to the bead. Control experiments where the bead was attached instead to the stalk of an oligomer-linked construct

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Author Contributions N.R.G. conceived the project, expressed and labelled the protein, and collected and analysed data. N.R.G. and S.M.B. discussed the data and co-wrote the manuscript.

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showed that the motor still stepped in 8-nm increments (Supplementary Fig. 1). With the bead linked to one of the two heads, but unloaded, motors travelled an average distance of 980 ± 80 nm (mean $\pm$ s.e.m.) before detaching (Supplementary Fig. 2), displaying a processivity equivalent to that of unlabelled kinesin¹⁵. Remarkably, the unloaded speeds of bead-linked motors under these conditions averaged 581 ± 9 nm/sec (mean $\pm$ s.e.m.), comparable to unloaded speeds recorded for wild-type motors (Supplementary Fig. 3). When head-linked beads were subjected to hindering loads under force-clamped conditions, we observed steps of 16.3 ± 0.1 nm toward the plus end of the MT (Fig. 1b and Supplementary Fig. 4a), exactly as expected for individual heads executing a hand-overhand walk along protofilaments with an 8.2-nm tubulin-dimer spacing. The stall force was halved when loads were applied to a single head instead of the stalk (falling from ~ 6 pN to ~ 3 pN)¹⁵, which is consistent with the energetic efficiency of the motor being unaffected by the head linkage, because half the load is moved through twice the distance per step (Supplementary Fig. 3).

When head-linked beads were subjected to assisting loads, we observed that the abrupt, 16-nm stepwise advances were generally composed of an ‘overshoot’ motion (~ 23 nm forward) followed by a ‘recovery’ motion (~ 7 nm backward) (Fig. 1c and Supplementary Fig. 4b). The durations of dwell intervals following the overshoot and recovery motions were sensitive to the ATP concentration, and both increased when the ATP level was reduced (Fig. 1d and Supplementary Fig. 5). This property indicates that the motor must bind ATP during both overshoot and recovery intervals. We also found that the positional variance of a head-linked bead was higher during overshoots than recoveries, suggesting that additional compliance is introduced into the linkage between the bead and the MT during overshoots (Supplemental Fig. 6). Because of their small amplitudes, a fraction of recovery motions were missed by our step-finding procedure, causing some pairs of overshoot and recovery motions to appear as a single, 16-nm transition.

The motions of head-linked beads under assisting loads may be explained by the following model (Fig. 1e), which is consistent with the established biochemical reaction cycle for kinesin. The binding of ATP to the MT-bound, linked head (state 1) triggers its unbound partner head to advance and rebind the MT (state 2). (ATP binding and head motions represent separate steps, but are shown concomitantly for simplicity.) The linked head then releases the MT and moves ahead of its bound partner (state 3). In this case, the application of assisting load not only helps to pull this head forward, but also supplies a small torque, generated between the neck linker domain (joining the head to the stalk) and the point of attachment of the DNA. To relieve torque, the linked head rotates to adopt a rearward-facing orientation, free of the MT, during overshoot dwells. The neck linker region consists of a single polypeptide chain (~ 13 residues) and can act as a free swivel. Subsequent binding of ATP to the MT-bound head allows the free head to rebind the MT in its proper orientation and release ADP (state 4). Finally, the rear head hydrolyzes ATP, releases P_i , and unbinds the MT to regenerate the starting configuration (state 1), but with the molecule advanced by 16.4 nm (2 steps). The existence of an overshoot implies that kinesin adopts a one-head-bound (1-HB) ‘waiting state’ between successive 8-nm advancements of its stalk. Furthermore, the dependence of the overshoot dwell time on ATP suggests that the unbound head cannot be induced to rebind the MT simply by coaxing it in front of its partner with the optical trap: some additional requirement must be met.

If kinesin adopts a 1-HB state between processive advances of its stalk, as implied by the foregoing, then it should be possible to swing the free, linked head backward and forward with respect to its bound partner by reversing the direction of load. To test this prediction, we modified our force-clamp apparatus to switch the sign of the constant load between hindering and assisting every 14 ms as kinesin moved in the presence of 2 μ M ATP (this

rate was chosen to be ~10-fold magnitude faster than the kinesin stepping rate at this ATP concentration, but ~10-fold slower than the update time of the force clamp). Separate records were collected for displacements under either sign of load, and from these we computed the running difference between records. The value of the running difference is expected to increase whenever the linked head releases the MT and is alternately pulled in front of and behind its bound partner. Rebinding of the linked head to the MT will eliminate any extra slack introduced in the linkage between the bead and MT as a result of head unbinding, and restore the running difference to its lower value. Accordingly, abrupt jumps in the running difference record signify that the linked head adopts both bound and unbound states during the kinetic cycle, consistent with a 1-HB waiting state. By contrast, the absence of such jumps would indicate that the linked head spends the majority of its reaction cycle bound to the MT, corresponding to a two-heads-bound (2-HB) waiting state.

When alternating loads (± 1.7 pN) were applied to a moving kinesin molecule, we observed clear transitions in the difference record, averaging 23 nm in amplitude, marking the binding transitions of the linked head (Fig. 2). (Similar transitions were observed when the magnitude of load was reduced to its lowest practical value, 0.4 pN; Supplementary Fig. 7). The presence of transitions constitutes the signature of a 1-HB state. Based on an analysis of dwell times in the running difference record, we estimate that the linked head spends ~93% of its stepping cycle in an unbound state (Supplementary Discussion). Stepwise increases in the difference record coincided with overshoot motions under assisting loads, marking events where the head released the MT and was pulled forward, then rotated about its neck linker. These unbinding events also corresponded to tiny rearward motions under hindering load, which likely results from a small increase in system compliance, as the linked head dissociates from the MT. Stepwise decreases in the difference record coincided with recovery motions under assisting load, marking events where the head bound the MT in front of its partner. These transitions corresponded to large forward motions of the bead under hindering loads.

To assess better whether the linked head remained free of the MT while its partner waited to bind ATP, we plotted the size of the transition under assisting load against the corresponding transition under hindering load (Fig. 3; central panel). Bead displacements that would normally be too small to resolve independently could be included in this analysis because the larger, readily detectable transitions under the opposite loading condition served as event markers. As expected, displacements fell into one of two populations, corresponding to unbinding (quadrant II) or rebinding (quadrant IV) events. We did not observe a population where the corresponding displacements under both assisting and hindering loads were comparable, which would have signified a 2-HB waiting state: just 6% of all transitions (mostly events in quadrant I) were in this category, and likely resulted from rare occasions when the unbound lifetime of the linked head was less than the time for load reversal of the force clamp. The small rearward motion under hindering load averaged -1.7 ± 0.2 nm. When added to the forward motion, 18.2 ± 0.2 nm, this yields a net displacement of 16.5 ± 0.3 nm, statistically indistinguishable from the expected advance of 16.4 nm (twice the tubulin-dimer spacing). Similarly, the sum of the overshoot (21.1 ± 0.3 nm) and recovery (-4.8 ± 0.3 nm) displacements yielded a net advance of 16.3 ± 0.4 nm. These results provide a strong consistency check on our interpretations and calibrations.

To exclude the possibility that the load applied by the optical trap might somehow pull the head free of the MT, we computed an estimate of the increase in head detachment rate as a consequence of load. Taking 2 nm as a (generous) estimate of the distance between the bound and transition states along the reaction coordinate for head unbinding¹⁶, the mechanical work available to detach a bound head would be less than $(1.7 \text{ pN} \times 2 \text{ nm}) = 3.4$ pN nm. Because this value is less than thermal energy ($k_B T \approx 4$ pN nm), any increase in

detachment rate would be limited to a factor of 2-3, and too small to account for our observations. This conclusion is reinforced by the persistence of transitions in the difference record when the load was reduced to 0.4 pN, which can only supply 3.2 pN nm of energy over the distance of an 8-nm step (Supplementary Fig. 7).

Our data support a model for the reaction cycle (Fig. 4) where kinesin binds ATP and takes its step from a 1-HB state. Because low ATP levels or high loads cause the transition from this state to become rate-limiting, it is the longest-lived state of the cycle (therefore the 'waiting state') under the conditions explored here. The unbound head is mobile, and can be pulled about its bound partner or even rotated with an optical trap. Our data do not support models where both kinesin heads bind to the MT until ATP binding triggers release of the rear head^{4,8}, nor where one head is parked against the forward face of its MT-bound partner in the waiting state¹⁰. However, our data can be reconciled with a recent study⁹ which concluded that one head remains poised behind its MT-bound partner head during the waiting state, and either unbound, or transiently bound, to the MT. In that study, estimates of distance between the kinesin heads were obtained from FRET measurements recorded at 100 Hz and filtered by a 100-ms window, so the proposed structure likely corresponds to an average over positions. Our data indicate that the unbound head that can move readily around its equilibrium location.

The existence of a 1-HB state implies some form of mechanochemical gating to prevent the unbound head from rebinding the MT and releasing ADP (Fig. 4; S1, S3). We propose that unfavourable strains developed in (competing) neck-linker conformations that correspond to off-pathway, 2-HB states constitute the mechanical coupling required for gating. According to this proposal, both heads will bind the MT only when the neck linker of the rear head is docked *and* the neck linker of the front head is undocked. The neck linkers are postulated to be too short, or too constrained, to adopt alternative conformations that might produce any alternative 2-HB state (Fig. 4, S1-3). Cryo-EM structures of MT-bound kinesin^{17,18} (see also C.V. Sindelar & K.H. Downing, manuscript in preparation) show that the conformation of the kinesin neck linker is tightly coupled to the state of the nucleotide bound to the head. When the head is either free of nucleotide or binds ADP, an occluding conformation of the switch-II helix prevents docking of the neck linker against the catalytic core¹⁹. By contrast, the motor core is tilted when bound to ATP or to analogues of ADP-P_i, allowing the neck linker to dock. Therefore, if both heads are to bind the MT simultaneously, the rear head must bind ATP (or ADP-P_i) while the front head holds ADP or no nucleotide. In support of this, recent work suggests that kinesin does not uniformly adopt a 2-HB configuration in the presence of apyrase⁹.

The inability of one head to bind the MT offers a natural explanation for the observation^{7,20-22} that the MT-stimulated release of ADP is inhibited until the MT-attached head binds ATP and docks its neck linker (Fig. 4, state 2). Strain produced by an unfavourable neck-linker conformation also explains the observation²³⁻²⁵ that ATP does not bind prematurely to the front, nucleotide-free head of 2-HB kinesin molecule (Fig. 4, state 3). Any tight binding of ATP is disfavoured because it is coupled to neck linker docking²⁶, and therefore to the generation of a strained configuration²⁷ where both neck linkers are docked (Fig. 4, S3). We anticipate that the single-molecule techniques presented here will be applicable to the study of dynamic properties of other motors and macromolecules that undergo analogous conformational rearrangements.

Methods Summary

Labelling kinesin with DNA

Mutation N62C was introduced into a 'Cys-light' construct consisting of the first 401 residues of the *D. melanogaster* heavy chain followed by a histidine tag. A 70-bp dsDNA oligomer was synthesized with a primary amine at the 5' end of one strand and biotin at the 5' end of the other (IDT). These labels were affixed to the DNA through hydrocarbon linkers (6-12 C atoms long) intended to serve as swivels. DNA was activated with sulfo-SMCC (Pierce) before incubation with kinesin. Labelled kinesin molecules were purified by ion-exchange chromatography (MonoQ; GE Healthcare).

Force clamp

Trap calibration and measurements under constant load were previously described²⁸. Oscillatory loading was implemented in custom force-clamp software (Labview 7.1) by reversing the sign of separation between the bead and trap every 14 ms; laser position was updated every 2 ms. To reduce settling time after a load reversal, the trapping beam was initially deflected to its approximate new position under reversed load before initiating feedback. Only data recorded after the final force-clamp update and before load reversal were included in analysis to allow time for the feedback to equilibrate.

Step sizes

Average bead displacements were estimated from the arithmetic means of records over the appropriate intervals. Uncertainties (s.e.m.) were computed by bootstrapping.

Methods

Instrumentation and analysis

Data were acquired at 20 kHz, filtered at 1 kHz, then decimated and recorded at 2 kHz. Velocity was computed by dividing the distance travelled by kinesin (the run length) by the time of a run, from unsmoothed records. Average velocities were computed from the arithmetic mean of individual velocities weighted by run lengths. The locations of step transitions in records of bead motion were detected automatically³⁰ until (apparently) misidentified steps accounted for no more than one (alternating load) or two (sustained load) position(s) in the trial step staircase. Those positions were ignored and the remainder recorded. The step detection algorithm was restricted to identifying steps separated by at least 2.5 ms. Rapid bead motions >32 nm were interpreted as dissociation events.

Under sustained hindering load, the force clamp was updated at 200 Hz and data were median filtered prior to step detection, using a window size of 3 ms. A second round of step detection was performed by marking steps separated by >4.5 ms whenever the data, median-filtered with a 9-ms window, deviated by more than 8 nm from the original data, median-filtered with a 100-ms window. The initial and final dwells of all records were not included in the analysis. Times of overshoot and recovery dwell intervals (Fig. 1d) were computed only when the intervening recovery step was smaller than -6 nm. We therefore expect our experimental estimates of the overshoot and recovery dwell times to be slightly overestimated, because the separate detection of overshoot and recovery steps is difficult when the dwell times become very short. This 'missing event problem' tends to suppress the trend of decreasing dwell times as the ATP concentration is increased (Fig. 1d) because a larger proportion of brief dwell times will be missed at high ATP levels than at low ATP levels. The actual dependence of dwell times on the ATP concentration may therefore be even stronger than that reported here (but does not change any of our conclusions).

Under alternating load, step positions detected under hindering and assisting load were merged. Any steps separated by a single load reversal (14 ms) were taken as a single step transition at the midpoint between these. The final dwell interval and step transition in any given stepping record were excluded from the analysis.

In the absence of load, video frame capture (at 30 Hz) followed by image processing was used to track the centroid of a moving bead. Bead release from the MT was determined from the increase in its positional variance.

Single-molecule assay

Plasma-cleaned, poly-lysine-coated coverslips were assembled into flow cells. MTs and 20 mg/ml BSA blocking protein were added sequentially. Kinesin covalently linked to DNA was mixed with 0.44- μ m streptavidin beads (Spherotech) and incubated overnight with ~6 mg/ml BSA. Before measurements, the ATP level was adjusted to the final concentration and an oxygen-scavenging system was introduced (235 μ g/ml glucose oxidase (Calbiochem), 42 μ g/ml catalase (Roche), and 4.6 mg/ml glucose). The experimental buffer consisted of 80 mM Pipes (pH 6.9), 50 mM potassium acetate, 4 mM $MgCl_2$, 2 mM DTT, 1 mM EGTA, and 10 μ M taxol. Data were recorded from samples where just 10-35 % of beads moved. Assisting loads greater than 1.7 pN often caused the DNA-linked kinesin irreversibly and abruptly to lose activity and then stick to the coverglass surface. Such loads may trigger unfolding of the motor and were therefore avoided. In contrast, sustained hindering loads as high as 3 pN rarely incapacitated the motor. Control beads were attached directly to the kinesin stalk by incubating kinesin with beads coated with anti-histidine antibody.

Kinesin purification and labelling

The gene for Cys-light *Drosophila* kinesin-1 (Fehr, A.N., Gutiérrez-Medina, B., Asbury, C.L., and Block, S.M., manuscript in preparation) was mutated at position 62 from Asn to Cys. This residue was selected as an attachment point because it is situated in a solvent-exposed loop on the back side of the motor domain, distal from regions known to be critical for motility, such as the neck linker and nucleotide binding pocket. Two additional constructs were generated that successfully generated 16-nm steps when bead were linked to a single head, A128C and S181C (data not shown). The N62C construct was expressed in *E. coli*. by inducing cultures grown at 37 °C to OD 0.4 with 100 μ M IPTG. Cultures were then incubated at 20°C overnight. Cell lysates were first purified with Ni-NTA affinity resin (Qiagen) and then via ion-exchange (MonoQ; GE Healthcare) using a buffer gradient composed of 25 mM Bis-tris propane (pH 7.4 at 4°C), 2 mM $MgCl_2$, 1 mM EGTA, 5 mM DTT, 2 μ M ATP, and 100-1000 mM NaCl. This procedure yielded ~1.5 mg dimeric kinesin per litre of bacterial culture. Kinesin fractions were confirmed by mass spectrometry and SDS-PAGE.

A solution of dsDNA oligomers (100 μ M) and sulfo-SMCC (2 mM) was incubated for 1 hr at 37°C in 25 mM potassium phosphate buffer (pH 7.2) containing 100 mM NaCl. The sample was passed over a desalting column (Micro Bio-Spin 6; BioRad) three times to remove unreacted sulfo-SMCC. In preparation for the reaction with DNA, kinesin was twice passed over desalting columns (Zeba; Pierce) to remove DTT and exchange it into the same buffer as the activated DNA. A mixture of ~22 μ M dimeric kinesin and ~67 μ M DNA was incubated for 12 hr at 4°C. ATP was added to bring the concentration to ~50 μ M. The reaction was quenched with 1 mM reduced cysteine and purified using ion exchange chromatography, as described above. Labelled and unlabelled kinesin was readily distinguished by a gel shift in native PAGE. The labelling efficiency was <25 %, ensuring >93 % of labelled kinesins were attached to a single DNA oligomer. We found that <10 %

of beads nominally attached to DNA-linked kinesin took 8-nm steps (rather than ~16 nm steps) under sustained hindering loads. We attributed these to beads carrying double motor linkages or to conjugation between the DNA and stalk residues, and excluded them from the analysis. The sequence of the amine-labeled strand of the 70-bp DNA oligomer was 5'-CGTTGCGCTC ACTGCCGCT TTCCAGTCGG GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG-3'.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Single-molecule observations of kinesin head motion

a, Illustration of the experimental geometry (to scale)²⁹. **b**, Four representative displacement records of head-linked beads under force-clamped conditions (1.7 pN hindering load; 2 mM ATP). Lighter traces unfiltered; darker traces median-filtered. Records offset vertically for clarity; stepwise transitions (~ 16 nm) indicated by hash marks. **c**, Same as (b), but under assisting load (1.7 pN). Overshoot (orange) and recovery (purple) transitions are indicated by hash marks. Corresponding dwell intervals are colored in the uppermost record. When separate overshoot and recovery were unresolved, the combined dwell interval and associated transition are colored green. **d**, Dependence of overshoot (orange) and recovery (purple) dwell times on ATP. Uncertainties (mean $\pm$ s.e.m.) were computed by bootstrapping. Load = 1.7 pN. **e**, Model for kinesin motions under assisting load, cycling through two steps. Heads are labelled according to the nucleotide bound (ADP, ATP, or $\emptyset$).

Figure 2. Motion of a bead linked to one of the kinesin heads under alternating loads

Four records of bead position, recorded during intervals of alternating assisting (green; top traces) and hindering (orange; middle traces) load. The running difference is shown (black; bottom traces). Cartoons (*inset*) show conformations assigned to the molecule during each plateau. Regions with shading highlight intervals where the linked head is unbound. Conditions: Load = ± 1.7 pN. [ATP] = 2 μ M.

Figure 3. 2D histograms of bead displacements under alternating load

The size of a displacement under assisting load was plotted against the size of the corresponding a displacement under hindering load (central panel), and a histogram was created for the data set. Two populations are evident, arising from unbinding (quadrant II) and rebinding (quadrant IV) of the linked head, as expected for a 1-HB waiting state (see text). A 2-HB state would populate points on the diagonal. White crosses mark centroids of the two populations. Data from these quadrants are projected onto the 1-D histograms (top and side panels). $N = 292$ (8 points landed outside the range plotted; not displayed) from 13 motors. Experimental conditions same as Fig. 2.

Figure 4. A model for strain-based mechanochemical gating

A simplified kinesin cycle with three states (green shading). In the waiting state (state 1), the rear kinesin head is unbound and mobile. Configurations postulated to produce inhibitory strain between the heads are indicated by showing neck linkers as dashed lines. This strain suppresses entry into off-pathway, 2-HB configurations (yellow shading; S1-S3). Strain is created when then neck linkers of either none (S1-S2) or both kinesin heads (S3) dock to their catalytic cores. The 2-HB configuration is only allowed (states 2-3) when the neck linker of the rear head is docked (bound to ATP or ADP-P_i) and the neck linker of the front head is undocked (bound to ADP or $\emptyset$). Structural elements (dark ovals) inhibit neck-linker docking to a head bound to ADP or $\emptyset$.