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Differential effect of multiple kinesin motors on run length, force and microtubule binding rate

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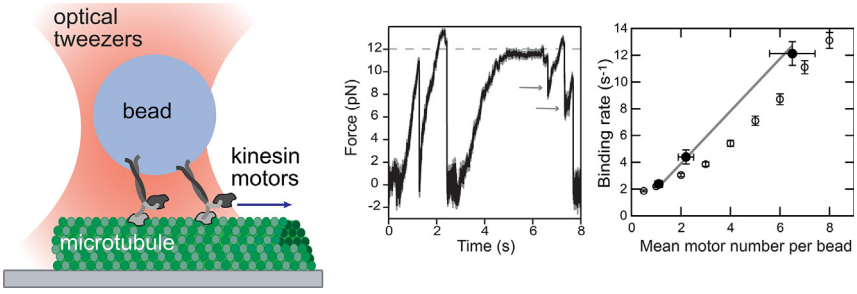
HIGHLIGHTS

- Multiple kinesin motors produce events of increased run length and force.
- Mean run length and force values change little as motor number per bead increases.
- Binding rates of beads to microtubules increase linearly with motor number.

GRAPHICAL ABSTRACT



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ABSTRACT

The *in vitro* transport of cargo by motor proteins constitutes a model system to understand mechanisms of vesicle trafficking inside cells. Here we apply the classic bead assay with a short, stiff kinesin protein to test the effect of multiple motors on essential transport parameters: distance, force and microtubule binding rate. Measurements of unloaded run length show that the transition from single- to multiple-motor behavior can be characterized by the appearance of extended runs, in accordance with a recently proposed model that quantifies the probability of multiple-motor engagement. In this transition, application of mechanical load using optical tweezers allows us to register maximum force values above single kinesin levels (8 pN). Yet, averages of run length and maximum force undergo little change as the probability of multiple-motor participation increases. In contrast, the measured rate of bead binding to microtubules scales linearly with the average number of motors per bead. These observations suggest that multiple motors bound randomly to the same cargo mainly increase the probability of attachment of these cargoes to the cytoskeletal filament network.

1. Introduction

The intracellular transport of vesicles and organelles by cytoskeletal motor proteins often occurs in geometries such that multiple motors act on the same cargo [1–3]. To understand how cooperative effects may arise in such scenario, experimental and theoretical approaches have been pursued. On the experimental side, *in vitro* studies of micron-sized beads carried by teams of motors have provided a wealth of

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information. In particular, it has been observed that multiple motor copies can carry cargo for longer distances and develop higher forces compared to individual motors [4,5]. One of the main challenges in the field has been to control the number and disposition of motors attached to cargoes. In the classic bead-assay motor proteins bind to micron-sized particles in a random fashion, making it difficult to assign transport to specific numbers of motors. This problem has been addressed by specifically linking motors to designed DNA scaffolds [6–9], among other strategies.

Despite potential drawbacks in studying motor cooperativity, the bead-assay remains of relevance as a model of cytoskeletal trafficking not only due to its simplicity, but because it possibly best mimics how motors bound to vesicles and organelles inside cells effect transport. By observing beads carried by motor proteins it is possible to measure how the distribution of quantities such as run length, velocity, and force developed change with increased motor concentrations used during preparations. To rationalize these measurements it becomes important to know the probability of having two or more motors involved, $P(\geq 2)$. Recently, a quantitative model for this probability was developed and shown to agree with experimental data [10]. This model is based on the Poisson probabilities that there are k motors on a bead and that at least two of those k motors can participate in bead displacement. In that study the length and the flexibility of the motor molecule were found to be important parameters that influence the resulting probability $P(\geq 2)$.

Here, we use the bead assay to quantify run length, maximum force and bead-microtubule attachment rate involving multiple kinesin molecules. Kinesin-1 or conventional kinesin is a dimeric motor protein that ferries cargo along microtubules (MTs), advancing in 8-nm steps in a hand-over-hand fashion [11]. Each kinesin polypeptide consists of a catalytic and microtubule-binding head domain followed by an extended dimerization stalk region. We probe a short, stiff kinesin construct whose use removes uncertainty in the extension length of the molecule and facilitates assessment of the probability for multi-motor participation. We find that although many motors produce events with significantly longer run lengths and higher forces compared to single motors, average quantities undergo small change within the range of P ($\geq$ 2) values explored. In contrast, linear behavior for the attachment rate of beads to MTs as a function of motor number is found.

2. Materials and methods

2.1. Kinesin expression and purification

All reagents purchased from Sigma-Aldrich unless otherwise specified. Protein was obtained following standard procedures [12]. Briefly, BL21(DE3) *Escherichia coli* cells transformed with the *Drosophila melanogaster* kinesin-1 heavy chain gene DmK401 (containing the first N–terminal 401 amino acids followed by a 6 × His tag) were grown at 37 °C in 5 mL Luria Bertani medium (LB) supplemented with ampicillin, under constant shaking for 16 h. 2.5 mL of the pre-inoculum was transferred to 250 mL of LB, and the culture was kept under agitation at 37 °C (~5.5 h) until reaching an $O.D._{600} = 0.6$. Kinesin-1 expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 12 h at 18 °C and 180 rpm shaking. Harvested cells were lysed using pulsed sonication. Kinesin was purified to homogeneity using Ni-NTA agarose beads (30,210, Qiagen) and stored at −20 °C in elution buffer (50 mM NaH_2PO_4, 250 mM NaCl, 500 mM imidazole, 1 mM $MgCl_2$, 1 mM DTT and 20 μM ATP, pH 6.7) supplemented with glycerol (50% v/v).

2.2. Bead assays

We followed procedures previously reported [12]. 440-nm (run length, binding experiments) or 550-nm (force development experiments) diameter streptavidin-coated polystyrene beads (Spherotech) were functionalized with PentaHis Biotin Conjugate (34440, Qiagen). Functionalized beads were incubated with kinesin overnight in assay buffer (2 mg/mL BSA, 0.05 M potassium acetate, 1 mM ATP, 1 mM DTT, 80 mM PIPES, 1 mM EDTA, 4 mM $MgCl_2$, 0.02 mM Taxol, pH 6.9) at 4 °C. Flow chambers were prepared using a coverslip and a glass slide separated using double-sided tape, and taxol-stabilized microtubules were immobilized on the coverslip using poly-L-lysine (run length experiments) or glutaraldehyde on APTES-treated surfaces (force development experiments). Kinesin-bead preparations were introduced and the flow chamber was sealed using valap, ready for experiments. For bead-MT binding 1 mM ATP was substituted by 1 mM adenylyl-imidodiphosphate (AMP-PNP) in the assay buffer. Observations of samples at room temperature were limited to 1 h.

2.3. Measurements and analysis

Assays were executed using a homemade optical tweezers apparatus [12]; position and force calibrations were performed every day of instrument use. To observe bead motion, kinesin-coated beads were kept close to microtubules using optical tweezers. Fraction moving was determined by waiting ~3 min and scoring bead motion reported by change in the position photodetector voltage within this time window. To record run length, the laser trap was automatically turned off when a threshold in the photodetector voltage change was reached (typically 0.5 V, corresponding to a bead displacement from the equilibrium position of 32 nm) and video microscopy frames were acquired at 30 frames per second. To determine bead displacement, video frames were averaged 5-fold and two-dimensional position tracking was performed by first establishing an intensity threshold and then computing the center of mass [13] of the bead image. The run length was obtained by the distance travelled from the time the trap was turned off to the last frame before observing bead detachment off the microtubule; an extra 32 nm was added to each run to account for the distance travelled within the optical trap. Due to uncertainty in determining small run distances, only displacements longer than 200 nm were included in the analysis. To record force development, the laser trap held a bead against the microtubule and photodetector voltages were recorded at 20 kHz. Raw photodiode voltage data was converted to displacement and force (typical distance-voltage calibration parameter: 65 nm/V; trap stiffness: 0.065 pN/nm), and an 18-point median smoothing was applied to data. Maximum force events were determined manually. To measure bead-MT binding rate, beads were positioned 30 nm above the coverslip surface by observing signature changes in the z–voltage signal of the trap photodiode [14]. Trap stiffness used in binding experiments: 0.01 pN/nm. Video and force data were analyzed using LabView and IgorPro, respectively.

2.4. Simulation of rotational Brownian motion

We consider a position-stationary sphere undergoing rotational diffusion and compute the mean time a rotational random walker takes to be within reach of kinesin molecules randomly attached to the surface of the sphere. The initial angular position of the walker is also random. In the lab frame of reference, the position of the random walker corresponds to the MT location site. Provided the mean number of motors per bead (n), a bead was assigned a random number of motors consistent with the Poisson probability of having exactly k motors per bead $P(k) = (n^k/k!)\exp(-n)$. Motors were distributed on the surface of a sphere 220 nm in radius by imposing random rotations. Simulation of rotational random motion for the angular coordinate θ was performed using a finite differences approach [15]: $\theta_i = \theta_{i-1} + \sqrt{2D_r\Delta t}\, w_i$, where D_r is the rotational diffusion coefficient, Δt is the time step, and w_i is a series of Gaussian random numbers with zero mean and unit variance. For a sphere: $D_r = k_B T/8\pi\eta R^3$, where k_B is Boltzmann's constant, T temperature, η viscosity of the immersing medium, and R radius of the sphere. Parameters used: $T = 295$ K, $R = 220$ nm, $\Delta t = 10^{-4}$ s, and $\eta = 1.2 \times 10^{-3}$ Pa s. The viscosity value was estimated to include a small