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Two-state displacement by the kinesin-14 Ncd stalk

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ABSTRACT

The nonprocessive kinesin-14 Ncd motor binds to microtubules and hydrolyzes ATP, undergoing a single displacement before releasing the microtubule. A lever-like rotation of the coiled-coil stalk is thought to drive Ncd displacements or steps along microtubules. Crystal structures and cryoelectron microscopy reconstructions imply that stalk rotation is correlated with ADP release and microtubule binding by the motor. Here we report FRET assays showing that the end of the stalk is more than ~9 nm from the microtubule when wild-type Ncd binds microtubules without added nucleotide, but the stalk is within ~6 nm of the microtubule surface when the microtubule-bound motor binds an ATP analogue, matching the rotated state observed in crystal structures. We propose that the stalk rotation is initiated when the motor binds to microtubules and releases ADP, and is completed when ATP binds.

1. Introduction

Cytoskeletal motor proteins use energy from ATP hydrolysis to produce force and move along microtubules or actin filaments, or disassemble microtubules, regulating their dynamics. How the motors do this is not well understood, but is thought to involve generation of strain by a spring-like or elastic element of the motor under load, followed by a strain-relieving step that produces force [1]. The motors are believed to undergo changes in conformation caused by changes in nucleotide binding and hydrolysis that, in turn, cause changes in motor-filament binding affinity — thus, specific states of the ATP hydrolysis cycle are postulated to generate strain and drive changes in the motor that result in motor binding to its filament, displacements or steps along the filament, and release from the filament. The structural changes of the motor are expected to be small, but, in myosin, are thought to be amplified by the relatively rigid α-helical light-chain binding region or regulatory domain, which acts like a lever arm to produce the working strokes of the motor [2–4].

Although considerable evidence now supports the hypothesis that rotation of the myosin lever arm is the primary mechanical component of the power stroke [2,5–10], less evidence is available for the kinesin motors. Two crystal structures have been reported that show the stalk of a kinesin motor in a different conformation than previous structures; both are structures of the kinesin-14 motor Ncd and exhibit the same or similar conformation [11,12] — the Ncd stalk is rotated by ~70–75°, together with one head, and the other head is unstably bound to ADP. One of the crystal structures is of a mutant that releases ADP faster than wild type [12] and the other, a mutant that binds more tightly to microtubules than wild type [11]. The altered nucleotide or microtubule binding by the mutants implies that ADP release and microtubule binding are correlated with stalk rotation. Wild-type Ncd can also be crystallized in a stalk-rotated conformation [11] although at lower resolution, possibly due to lower stability of the conformation in the absence of microtubules.

Unlike previous Ncd crystal structures with an unrotated stalk and head, the stalk-rotated structure fits well when docked into cryoelectron microscopy (cryoEM) density maps of dimeric Ncd bound to microtubules without nucleotide [13], although density corresponding to the stalk was not visible in the cryoEM reconstructions used in the dockings [11]. The stalk-rotated Ncd structures have been interpreted to resemble the motor as it releases ADP and binds to a microtubule [11,12]. Rotation of the stalk could amplify force produced by the motor, representing a power stroke. However, conflicting evidence from docking Ncd crystal structures with a rotated or unrotated stalk into other cryoEM maps has led to the proposal that the stalk rotates when the microtubule-bound motor binds ATP [14,15].

Identifying the motor conformational changes and the ATP hydrolysis state at which they occur is critical to understand how the motors work. Here we use fluorescence resonance energy transfer (FRET) assays of wild-type Ncd and a mutant that promotes stalk rotation to determine the position of the stalk relative to the microtubule in different nucleotide states of the motor.

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2. Materials and methods

2.1. Plasmids and proteins

Plasmids to express EGFP-Ncd proteins in bacteria were constructed by standard methods and confirmed by DNA sequencing. EGFP-Ncd proteins for TIRF assays were partially purified by SP-Sepharose chromatography and dimer motor concentration was determined from EGFP absorbance at 489 nm ($\varepsilon = 53{,}000\ M^{-1}\ cm^{-1}$) [16].

2.2. TIRF assays

Slides and coverslips for total internal reflection fluorescence (TIRF) assays were cleaned by immersion in Piranha solution (30% H_2O_2/H_2SO_4, 1:3) [17] and sonication in deionized water or by sonication in 1 M KOH, 3 M HCl and 95% ethanol followed by sonication in deionized water. Slides were silanized in 0.15% (v/v) (3-aminopropyl)trimethoxysilane (Alfa Aesar, Ward Hill, MA) in hexane [18], rinsed in hexane, sonicated in 95% ethanol and deionized water, and stored in deionized water up to a week.

Microscope chambers were made by sealing clean coverslips onto silanized slides with double-sided tape. To immobilize microtubules on the slide, chambers were incubated with anti-α-tubulin monoclonal antibody (Chemicon International, Inc., Temecula, CA, >0.3 mg/ml) diluted 1:50 (v/v) in 0.5× PM buffer (PM buffer = 100 mM PIPES pH 6.9, 1 mM $MgSO_4$, 2 mM EGTA) + 400 nM taxol. Microtubules, either unlabeled or labeled with TMR as described below, were diluted to 100 nM (tubulin concentration) with 0.5× PM buffer + 20 μM taxol and added to the chamber.

The chamber was flushed with blocking buffer (0.5× PM buffer, 2.5 mg/ml α-casein, 2.5 mg/ml BSA, 400 nM taxol) or 1% Pluronic F-127 + 400 nM taxol in 0.5× PM buffer to prevent motor adsorption to the glass. EGFP-motor was diluted for microtubule binding (20 nM) or FRET (10 nM) assays in reaction buffer (HEM50 = 10 mM HEPES pH 7.2, 1 mM $MgCl_2$, 1 mM EGTA, 50 mM NaCl, 1 mM DTT, 1 mg/ml BSA, 400 nM taxol) without nucleotide or with 0.5 mM Mg·ADP or Mg·AMP·PNP (adenosine 5′-[β, γ-imido]triphosphate), a non-hydrolysable ATP analogue, and added to the chamber. After 1–2 min incubation, the chamber was rinsed once with reaction buffer before observation. Motor–microtubule binding in the TIRF assays was assumed to be at equilibrium so that motor binding to microtubules occurs at the same rate as dissociation from microtubules.

2.3. Rhodamine-labeled microtubules

Bovine brain tubulin was obtained from Molecular Probes, Inc. (Eugene, OR) covalently labeled with TMR to a stoichiometry of 2.6 moles TMR/moles α,β-tubulin dimer. The labeling was performed by incubation of microtubules with *N*-hydroxy succinimidyl 5-carboxytetramethylrhodamine ester, followed by two cycles of polymerization–depolymerization [19,20]. Bovine brain α,β-tubulin dimers contain 34 lysines distributed essentially randomly on the surface. The activated succinimidyl TMR esters are thought to label random surface lysines [20]. Because the labeling was done on microtubules rather than tubulin, the TMR fluorophores are expected to be predominantly on the outer microtubule surface rather than lumen. Purification of the labeled tubulin through two assembly–disassembly cycles ensures that the TMR-tubulin is competent to assemble into microtubules. TMR-microtubules for TIRF binding assays and FRET assays were assembled from 10 μM TMR-tubulin + 30 μM unlabeled porcine brain tubulin (1:3).

2.4. TIRF microscopy

Time-lapse images were acquired at 22 °C using a prism-based TIRF microscope custom-built on a Zeiss Axioskop 2FS microscope (Thornwood, NY) equipped with a Zeiss 100×/1.45 NA Plan-Fluar oil immersion objective. Specimen excitation was by the 488-nm line of an Ar laser (Melles Griot, Carlsbad, CA, Model 532-MAP-A01) measured at ~0.4 mW before the prism. Images were recorded with an Andor iXon 897 EM-CCD camera (Belfast, UK) driven by Andor iXon v 4.4.0.0 software at a gain of 300, 250 ms exposure time and 1 s/frame. GFP and rhodamine/FRET fluorescence was collected with 510/30BP and 620/50BP emission filters (Chroma Technology Corp., Rockingham, VT), respectively. A beamsplitter with 510/30BP, 565DCLP and 650/75BP filters was used in microtubule-binding assays for simultaneous collection of GFP and rhodamine images.

2.5. TIRF data analysis

Microtubule fluorescence was measured by manually tracking the time-lapse images using ImageJ v 1.38t (NIH, Bethesda, MD). Nonspecific fluorescence in the images was subtracted from the microtubule fluorescence to correct for background. Because of the difference in emission filter and motor concentration, GFP bleed through into the rhodamine/FRET channel was determined in separate controls for the microtubule-binding and FRET assays using unlabeled microtubules (100 nM) and the corresponding motor concentration (20 nM EGFP-motor for microtubule-binding assays or 10 nM EGFP-motor for FRET assays). Bleed-through fluorescence was determined to be ~11–16% of the GFP fluorescence in microtubule-binding assays of the EGFP-motors without nucleotide; it was subtracted from the rhodamine fluorescence before determining the ratios between the GFP and rhodamine fluorescence. For the FRET assays, bleed-through fluorescence was found to be negligible — only ~2–3% or less of the GFP fluorescence appeared in the rhodamine/FRET channel in control assays with unlabeled microtubules; the FRET data were therefore not corrected for GFP bleed through.

Mean fluorescence from several FRET assays was averaged and normalized, then fit to

$$f(t) = m3 + m2\ e^{-m1*t} \tag{1}$$

where f(t) = fluorescence at t = time (s), m2 = total fluorescence lost and m3 = fluorescence at t = ∞ to determine a photobleaching rate constant, m1 = k_{PB}. Donor quenching and acceptor sensitization effects were determined by comparing photobleaching rate constants for the donor or acceptor alone, and with each other under the same nucleotide conditions. For the acceptor photobleaching experiments, m3 was assumed to be 0; the microtubules are expected to photobleach without recovery as t goes to infinity, given that the TMR fluorophores are covalently bound to tubulin and photobleaching recovery would require assembly of TMR-tubulin into microtubules.

2.6. FRET calculations and modeling

The EGFP-Ncd emission spectrum, f(λ), at $\lambda_{ex} = 400$ nm and $\lambda_{em} = 420$–600 nm and TMR-tubulin absorbance spectrum, E(λ), at $\lambda_{ex} = 400$–600 nm and $\lambda_{em} = 620$ nm were read in a Varian Cary Eclipse fluorimeter, normalized and used to calculate the overlap integral, J, the overlap between the donor emission spectrum and acceptor absorbance spectrum, and the Förster distance, R_0, the distance at which the FRET efficiency for the EGFP-Ncd (donor) and TMR-microtubule (acceptor) FRET pair is 50% [21,22]. The overlap integral, J, was calculated from the wavelength λ by normalizing the EGFP emission spectrum f(λ) and TMR absorbance spectrum E(λ) such that

$$\int_{420\ nm}^{600\ nm} f(\lambda)d\lambda = 1 \quad \text{and} \quad \max_{\lambda} E(\lambda) = 1. \tag{2}$$