Mechanical Properties of a Single-Headed Processive Motor, Inner-Arm Dynein Subspecies-c of *Chlamydomonas* Studied at the Single Molecule Level

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Abstract. Dynein from inner arms of *Chlamydomonas* flagella contains seven distinct subspecies, a through g. Several lines of evidence suggest these subspecies play important roles in generating flagellar beating and that the different subspecies are functionally diverse. To evaluate their roles and diversity, the mechanical properties of subspecies-c, which is a single-headed motor, were examined using optical trap nanometry. A polystyrene bead coated with a small number of subspecies-c molecules was captured with the optical trap and brought into contact with a microtubule fixed to a coverslip. Movements of the bead were measured by a quadrant photodiode sensor with sub-nanometer- and millisecond-resolution. Beads carrying a single active subspecies-c molecule moved processively along the microtubules in 8-nm steps but slipped backwards under high loads. Force-velocity relationships of single subspecies-c molecules were almost linear and the shapes of the normalized curves at 5 μM and 100 μM ATP were similar. These results indicate that dynein subspecies-c functions in a very different way from conventional motor proteins, such as myosin and kinesin, and has properties that could produce self-oscillation *in vivo*.

Key words: axonemal dynein, force-velocity relationship, optical trap, processivity, single molecule measurement

1. Introduction

Axonemal dyneins are the force-generating ATPases responsible for movement of eukaryotic cilia and flagella [1]. They consist of many isoforms: three heavy chains (α , β and γ) in the outer-arm dyneins and eight in the inner-arm dyneins of a *Chlamydomonas* flagellar axoneme [2, 3]. Analyses of dynein mutants of *Chlamydomonas* suggest that these dynein heavy chains have distinct functions, each of which is crucial for proper functionality of the axoneme [3]. Several studies also suggest that inner-arm dyneins mainly produce an active bending moment in flagellar movement [3, 4] and that these axonemal dyneins themselves have an inherent property of oscillation in their generation of force and movement [5–8].

Therefore characterization of the mechanical parameters of inner-arm dyneins is necessary for elucidation of the mechanism of flagellar movement.

The eight distinct inner-arm dynein heavy chains of *Chlamydomonas* axoneme are organized with various intermediate chains and light chains into seven different molecular complexes, one two-headed isoform and six single-headed isoforms, termed a to g [2]. These single-headed inner-arm dyneins have a large globular head with both a slender stalk and a long stem emanating from it [9, 10]. This head is similar to the structures of dynein heads that have been observed in phylogenetically and functionally diverse forms of dynein [9, 11–13]. The head domain of the inner-arm dynein subspecies-c consists of several globular subdomains encircling a hollow central core [10], closely similar to cytoplasmic dynein [13]. Subspecies-c incubated with microtubules in the absence of ATP were observed by negative-stain EM as head domains with stems aligned obliquely to the microtubule but without direct contact of either the head domain or the stem with the microtubule [10]. These observations suggest the microtubule-binding site of subspecies-c is segregated away from the head domain and located at the tip of a stalk [10], as indicated for cytoplasmic dynein [14, 15].

This single-headed subspecies-c showed high processivity in gliding microtubules on glass surfaces and in moving beads on a microtubules [10]. Outer-arm dynein of *Tetrahymena* cilia 22S dynein also shows processive movement in an ATP-dependent manner [16]. At low concentrations of ATP, 22S dynein showed processive movement while at higher concentration, it showed pulse-like force generation such as is observed in skeletal muscle myosin [17, 18]. These reports suggest that dynein is radically different in organization and mechanochemical properties from other motors such as myosin and kinesin. Hence, we focus here on mechanical properties of inner-arm dynein subspecies-c from *Chlamydomonas* at the single molecule level; this is a single-headed motor and is the fastest known among inner-arm dyneins [2].

2. Materials and Methods

2.1. PROTEIN PREPARATION

Isolation and purification of inner-arm dynein subspecies-c were carried out as described previously [10]. Subspecies-c was isolated from wild-type or the outer-armless mutant of *Chlamydomonas reinhardtii* (strain 137c or *oda1*, respectively) essentially according to Kagami and Kamiya's method [2]. Flagellar axonemes and the high salt extract of axonemes were obtained by the methods of King *et al.* [19]. Crude dynein extract was fractionated using a high-pressure high-performance liquid chromatography system (HPLC, MonoQ HR5/5 analytical anion exchange column, Amersham Pharmacia) eluted with a linear gradient of 200 to 500 mM KCl in a solution containing 30 mM HEPES/KOH (pH 7.4), 5 mM MgSO₄, 1 mM dithiothreitol (DTT), and 1 mM EGTA. Pooled fractions of subspecies-c were rechromatographed by the anion exchange column (MiniQ PC 3.2/3, Amersham

Pharmacia) in an HPLC system. Purity and aggregate formation of subspecies-c were examined by using 3% or 5–15% SDS-PAGE with silver staining and sucrose density gradient centrifugation, respectively. No significant contamination of other subspecies and no aggregation were detected [10]. Bovine brain tubulin, purified according to Vallee [20], was polymerized into microtubules in assembly buffer (80 mM PIPES/KOH, 1 mM MgCl_2 , 1 mM EGTA, 1 mM GTP, 10% (v/v) dimethyl sulfoxide, pH 6.9) at 37 °C, and was subsequently stabilized by adding 10 μM taxol. Polarity-marked microtubules, the minus-ends of which are much more fluorescent, were prepared using NEM tubulin, according to the method of Howard and Hyman [21]. Subspecies-c and tubulin concentrations were determined using the Bradford dye-binding assay.

2.2. OPTICAL TRAP NANOMETRY

Polystyrene carboxylated beads were preincubated in buffer, 30 mM HEPES/KOH, 5 mM MgSO_4 , 1 mM EGTA, pH 7.4, supplemented with 1 mg ml^{-1} casein. Beads were then incubated for 10 min with varying concentrations of subspecies-c. A flow cell, volume 10 μl , was made from a two coverslips (24 mm $\times$ 40 mm), after cleaning with 0.1 M HCl and 70% ethanol. The subspecies-c coated beads were then added to taxol-stabilized rhodamine-labeled microtubules (10 $\mu\text{g ml}^{-1}$ taxol) adsorbed onto a coverslip of the flow cell. The experimental setup for the optical trap based on a Nd:YAG laser (Spectra Physics, 500 mW at 1064 nm), was almost the same as that of Kojima *et al.* [22]. Displacement of the bead was measured at a sampling rate of 1 kHz using a quadrant photodiode system with a spatial resolution of 0.1 nm (noise-equivalent value) and response time of ca. 0.02 ms. Trap stiffness (0.017 pN nm^{-1}) and bead-to-dynein linkage stiffness (0.1–0.3 pN nm^{-1}) were determined from the variances of the thermal fluctuations of beads. Displacement of beads within the trap was converted to displacement of subspecies-c by multiplying by the factor of attenuation, which is the ratio of the sum of bead-to-dynein linkage stiffness and trap stiffness to bead-to-dynein linkage stiffness [22]. To determine the frequency of slippage, the displacement of the bead from the trap center was divided into 20-nm bins and in each bin we counted backward movements of the bead, which were more than 4 nm and were followed by more than 40 ms dwell state. These counts of backward movement were divided by the total time during which the bead stayed in each bin. The force-velocity relationship was calculated from the displacement of the bead as follows: 7–62 runs of bead displacements were averaged, and the displacement of dynein molecules was calculated according to Kojima *et al.* [22]. The velocities were calculated from the slopes of the displacement.

Figure 1. The probability ($P(n)$) of beads binding and moving in the presence of $5 \mu\text{M}$ ATP as a function of the molar mixing ratio, n , of subspecies-c molecules to beads at incubation. The fitted curve (solid line) is $P(n) = 1 - e^{-n/\lambda}$, with the efficiency of binding, $\lambda = 480 \pm 73$ ($\chi^2 = 10.3$, $p > 0.1$, 6 df); a fit to a two-molecule model (dotted line) yields $\chi^2 = 81.7$, $p < 0.001$. The probability $P(n)$ is expressed as $P(n) \pm \sqrt{P(n)(1 - P(n))/N}$, where N is the number of beads brought into contact with microtubules. Figure reproduced from Nature [10] with permission.

3. Results and Discussion

3.1. BINDING OF SUBSPECIES-C-COATED BEADS TO A MICROTUBULE

Since precisely measuring movement in detail should give an insight into the mechanisms of the movement of subspecies-c, bead motion was assayed using the optical trap [22]. First, polystyrene beads coated with various densities of subspecies-c molecules were prepared by mixing subspecies-c solutions with the bead solutions at various molar ratios. A diffusing bead was captured with the optical trap and brought into contact with a single microtubule that was bound to the glass surface. The bead would often attach to the microtubule showing markedly reduced Brownian motion. After a few seconds, the light from the trap laser was blocked by a shutter, whereupon beads that had failed to bind to a microtubule generally diffused away. These beads could be recaptured and re-tried several times. A reduction of Brownian motion by 50% was the criterion for observation of binding of a bead to a microtubule.

The relative frequency of these outcomes depends on the number of subspecies-c molecules carried by a bead. Assuming that one subspecies-c molecule attached to a bead with a geometry favorable for interaction and in an active form suffices to bind the bead to a microtubule, the fraction of beads bound to microtubules should follow Poisson statistics [23]. Our data are fitted well by the probability

form $P(n) = 1 - \exp(-n/\lambda)$, where n is the number ratio of subspecies-c molecules to beads (see Figure 1, with $\lambda = 480 \pm 73$, $\chi^2 = 10.3$, for 6 degrees of freedom). The fitting parameter λ corresponds to the efficiency of binding. A fit to two-molecule model ($P(n)1 - \exp(-n^2/\lambda)$) yielded a much worse $\chi^2 = 81.7$, 6 degrees of freedom. From this and geometric considerations of dynein molecules on bead surface, the two-molecule model was excluded (Figure 1 dotted curve). The measurements of force and movement were performed at such a dilution where the probability of binding and movement was less than 0.5, so that the Poisson probability that a bead carried two or more active subspecies-c molecules was less than 0.15. Assuming a random distribution of subspecies-c molecules over the bead surface and allowing a 50-nm reach for the subspecies-c molecule tether [9, 24], the probability that two or more subspecies-c molecules simultaneously interacted with a microtubule is estimated to be less than 0.01.

3.2. BEAD MOVEMENT BY DYNEIN SUBSPECIES-C MOLECULES

The stiffness of the trap was chosen to be as large as possible to decrease the amplitude of Brownian motion of the beads whilst not exceeding the force that would impede a subspecies-c molecules from producing its full displacement. In measurements of a subspecies-c-coated beads displacement, it was necessary to use a relatively compliant optical trap (0.017 pN nm^{-1}). When a bead was brought into contact with a microtubule, the trapped bead attached and showed transient movements along the direction of the microtubule (Figure 2). These movements occurred in such a manner that several runs occurred as a cluster once movement was elicited. In such clusters, beads progressed for various periods and were then drawn rapidly back to the trap center, whereupon they rebound and began to move again. Multiple cycles of attachment, movement, and release were observed with the same bead. The average distance moved was $69 \pm 25 \text{ nm}$ at $5 \mu\text{M}$ ATP, which corresponded to a force of $1.2 \pm 0.4 \text{ pN}$ (mean $\pm$ SD, $n = 77$). This force does not correspond to a stall force but a yielding force since beads often detached from a microtubule before slowing down substantially. These progressive displacements were almost without exception in one direction: minus-end directed movement, which was confirmed by using polarity-marked microtubules. Although bead displacements perpendicular to the microtubule axis were noisy owing to the low stiffness of the trap, subspecies-c moved laterally over a range of about 30 nm. These features of bead movement were observed both in the presence of $5 \mu\text{M}$ ATP (Figure 2a) and $100 \mu\text{M}$ ATP (Figure 2b).

In a run of bead displacement, thermal noise superimposed on the displacement signal decreased as the beads developed greater force, indicating a nonlinear elasticity; the total (glass-to-microtubule-to-dynein-to-bead-to-trap) linkage stiffness becomes greater with increasing extension (Figure 3a). This phenomenon was also reported in experiments using kinesin-coated beads [22, 24, 25]. At forces ranging from 1 to 2 pN, step movement of beads was clearly observed. The histogram

Figure 2. A typical record of force and movement generated by a subspecies-c-coated bead captured by the optical trap in the presence of $5\ \mu\text{M}$ ATP (**a**) and $100\ \mu\text{M}$ ATP (**b**). Several runs occurred as a cluster once movement was elicited. All traces were processed using a low-pass Butterworth filter (2-pole, $-3\ \text{dB}$ @ $100\ \text{Hz}$). Figure reproduced from Nature [10] with permission.

of all pairwise distance shows that the step size of the movement occurred was in integral multiples of $8.3\ \text{nm}$ step (Figure 3b). In the higher force range (more than $1\ \text{pN}$), the bead often slipped backward by one or two steps (arrows in Figure 3a). The frequency of such slippage increased as the load on the bead increased (Figure 4). If the transition from the bound state of subspecies-c to the detached one is associated with the displacement at the interface between subspecies-c and microtubule in the direction of the force, by modifying the Equation 2 in reference [26] we obtained the interaction distance to be $3.3\ \text{nm}$, over which the bond energy is dissipated [26, 27]. This value is an order of magnitude greater than the length of

Figure 3. **a:** Detail of movement of a subspecies-c-coated bead at 5 μ M ATP with horizontal gridlines spaced at 8 nm. Multiple steps were observed. Arrows indicate backward movement of the bead 'slippage'. Displacement of the bead from the trap centre was measured over 100 s. The number of active subspecies-c molecules on the bead was 0.06 at $n = 30$. **b:** Pair-wise distance distribution of bead movement. The continuous curve is the sum of seven Gaussian components fitted to the data: peak position of the unit step was 8.3 nm (SD 3.5 nm). Arrowheads indicate peak positions of each component. This trace was filtered using a low-pass Butterworth filter (2-pole, -3 dB @ 100 Hz). Figure reproduced from Nature [10] with permission.

Figure 4. Relationship between frequency of slippage and force on a subspecies-c-coated bead obtained from three traces. Error bars indicate SDs. The force on dynein accelerated the frequency of slippage. This would imply an interaction distance of the order of 3.3 nm. The continuous curve indicates the relation, $r(F) = A \exp(-(\Delta G_a - Fx)/k_b T) = r(0) \exp(Fx/k_b T)$, where $r(F)$ is the frequency of slippage at the force F , ΔG_a , an activation free energy, k_b the Boltzman constant and T the absolute temperature. Figure reproduced from Nature [10] with permission.

Figure 5. Force-velocity relationship of dynein subspecies-c in the presence of 5 μM ATP (a) and 100 μM ATP (b). Sixty-two runs and 7 runs of bead displacements were averaged for 5 μM ATP and 100 μM ATP, respectively and the velocities calculated from the slopes of the displacement. The solid lines represent linear fits to the data.

individual bonds at the molecular interface but is similar to the interaction distances reported in myosin in rigor [28].

3.3. THE FORCE-VELOCITY RELATION OF DYNEIN SUBSPECIES-C MOLECULES

Force-velocity relationship curves of single subspecies-c molecules were obtained at two different ATP concentrations (Figure 5). These were almost linear at both ATP concentrations: i.e. the velocity decreased linearly with increasing load. Since the beads often dissociated before slowing down substantially, the force-velocity curves do not reach the force-axis (zero velocity). A linear fit to the data gave an extrapolated maximal force of 2.3 pN at 5 μM ATP and 2.6 pN at 100 μM ATP. The extrapolated maximal velocities obtained from the linear fits, $0.030 \mu\text{m sec}^{-1}$ at 5 μM ATP and $0.47 \mu\text{m sec}^{-1}$ at 100 μM ATP, agreed with those for microtubules gliding on single dynein molecules measured in *in vitro* motility assays [10]. These features were generally consistent with those of the force-velocity curves of microtubules sliding on ensemble of dynein molecules in a demembranated flagellar axoneme of sea urchin sperm [6]. In contrast, the force-velocity curve of outer-arm dynein obtained at the single molecule level was downwardly convex [16]. The difference in the shape of the curves between inner-arm dynein and outer-arm dynein could reflect differences in their mechanochemical cycles.

According to the phenomenological theory of Svoboda and Block [24], we assumed two biochemical rates, one of which is independent of ATP concentration and the other of which is inversely proportional to the ATP concentration. If one or both of these rate constants were load-dependent, the shapes of the force-velocity curves predicted according to the theory at saturating and limiting ATP concentration would be quite distinct [24]. In the force-velocity relation obtained in this

study, however, the shapes of the curves were almost similar irrespective of ATP concentrations. In addition, dynein was observed to slip backward along a microtubule more frequently at high loads than at low loads and to have longer dwell times at high loads than at low loads. These observations imply that the load dependence of velocity could be due to a decrease in the net displacement per ATP molecule hydrolysed. Although the precise comparison of the shape of the force-velocity relations at various concentrations of ATP would provide detailed information of mechanochemical properties [24], scatter in the data precludes detailed discussion.

To move processively, it is essential that a subspecies-c molecule remains bound to the microtubule throughout the movement, because if the motor detaches it quickly diffuses away. The head domain of subspecies-c is substantially larger than both kinesin and myosin, and given that it contains multiple ATP-binding sites like other dyneins, the mechanism of movement may be complex. The negatively charged C-terminal domain of tubulins could interact with the cluster of positively charged residues in dynein by electrostatic interaction [29, 30]. This weak interaction may play an important role in the processive movement of subspecies-c. Since the ionic strength of our experimental conditions was much lower (ca. 50 mM) than physiological (150 mM), the electrostatic interaction between dynein and microtubules *in vitro* should be stronger than *in vivo*.

In flagellar beating, the conversion of microtubule sliding into rudimentary oscillatory bending waves can occur in small subsets of the axonemal structure [3]. The low detachment rate and strain-dependent slippage of subspecies-c therefore suggest that a structural component capable of limiting sliding resides in these dyneins. In addition, this strain-sensitive slippage could cause cooperative detachment of dynein molecules from a microtubule, leading to backward movement of the microtubule until enough strain is released allowing the dyneins to re-engage. This cooperativity may be a source of the oscillatory flagellar movement.

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