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Unidirectional movement of fluorescent microtubules on rows of dynein arms of disintegrated axonemes

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SUMMARY

Tetramethylrhodamine-labelled microtubules were observed to move on rows of dynein arms of sea urchin sperm axonemes exposed by elastase-induced sliding disintegration. The microtubules moved towards the flagellar tip at a velocity of 3.1±2.1 μm second^{-1} (mean ± s.d., *n*=53) in the presence of 0.1 mM ATP at 22°C, but none moved towards the sperm head. We also examined the polarity of microtubule binding to axonemal doublet microtubules in the absence of ATP by using microtubules brightly labelled at their minus-ends. In 140 of 210 microtubules studied, they bound to axonemal microtubules with a parallel polarity. These results suggest that tightly packed dynein arms on the outer doublet microtubules of sperm axoneme preferentially bind microtubules to themselves with the same polarity as that of the axoneme and that they generate a force to move only these microtubules in the direction away from the sperm head.

Key words: Dynein-microtubule interaction, Polarity, Flagellum

INTRODUCTION

It is well established that the beating of eukaryotic cilia and flagella is generated by dynein arms, which are regularly arranged on the A-tubules of outer doublet microtubules and interact with adjacent B-tubules to generate active sliding while hydrolyzing ATP (Gibbons, 1981). Indeed, when demembranated axonemes are briefly digested with trypsin and subsequently exposed to ATP, they disintegrate into separate doublet microtubules by a process of active sliding that takes place between the doublets (Summers and Gibbons, 1971).

The direction of force generated by dynein was studied by observing the protease-treated axonemes under an electron microscope (Sale and Satir, 1977; Fox and Sale, 1987). These observations indicated that force generated by dynein arms slid the adjacent outer doublet microtubule away from the base of an axoneme. Furthermore, when purified dynein from *Tetrahymena* cilia was adsorbed on a glass surface in an in vitro motility assay, microtubules slid with their minus-end markers trailing (Vale and Toyoshima, 1988). These studies suggest that the direction of the movement was determined by the polarity of microtubules and that dynein moves towards the minus-ends of microtubules.

In the actomyosin motility system, it is commonly accepted that the polarity of actin filaments determines the direction of their movement on myosin (Spudich et al., 1985). In addition, tight packing of myosin molecules in myosin filaments could influence the movement of actin filaments: fluorescently labelled actin filaments were observed moving much faster towards the centre of myosin filaments than away from it on isolated molluscan thick filaments (Sellers and Kachar, 1990; Yamada et al., 1990) and synthetic rabbit skeletal muscle myosin filaments (Yamada and Wakabayashi, 1993).

By analogy, it is hypothesized that the regular arrangement of dynein molecules on doublet microtubules would modulate the movement of microtubules. In this study, we examine the movements of fluorescence-labelled singlet microtubules on the dynein rows of axonemes exposed by sliding disintegration and show that microtubules moved only towards the tip of the flagella and not in the opposite direction. Our results strongly suggest that dynein molecules in flagellar axonemes are oriented in such a way that they can efficiently move adjacent outer doublet microtubules away from the base of flagella.

MATERIALS AND METHODS

Fluorescent microtubules

Tubulin was purified from bovine brain according to the method of Vallee (1986), and was then labelled with tetramethylrhodamine (TMR) succinimidyl ester (Hyman et al., 1991). *N*-ethylmaleimide (NEM) tubulin was prepared according to the method of Hyman et al. (1991). For the preparation of fluorescent microtubules, a mixture of non-labelled tubulin (1.8 mg ml^{-1}) and TMR-labelled tubulin (0.1 mg ml^{-1}) was incubated at 37°C for 15 minutes in a solution containing 1 mM $MgCl_2$, 1 mM GTP, 1 mM ethyleneglycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 80 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (Pipes) buffer, pH 6.9, and 10% (v/v) dimethylsulfoxide. After incubation, 0.1 mM taxol was added to stabilize the microtubules for lower temperatures and concentrations. The polarity-marked microtubules, whose minus-ends contain much more fluorescence, were prepared using the NEM tubulin, according to the method of Howard and Hyman (1993).

Rows of dynein arms on doublet microtubules of disintegrated axonemes

Sea urchins, *Hemicentrotus pulcherrimus*, were collected near the Misaki Marine Biological Station, University of Tokyo. Spermatozoa were obtained by intracoelomic injection of either acetylcholine or KCl, and placed on ice until used. They were demembranated with 0.05% (w/v) Triton X-100 (Gibbons and Gibbons, 1972) in a standard buffer containing 0.15 M K-acetate, 5 mM $MgCl_2$, 4 mM EGTA, 1 mM dithiothreitol (DTT), and 10 mM Tris buffer, pH 8.0. After gently mixing for 30 seconds at room temperature (22°C), the suspension was diluted with 20 volumes of the standard buffer. Demembranated spermatozoa were used for experiments within a day. We also used glycerinated spermatozoa (Brokaw, 1966) in this study. We will indicate when the results were obtained from the glycerinated spermatozoa; otherwise, they were from the Triton-extracted spermatozoa. The demembranated spermatozoa were treated with 2 μg ml^{-1} elastase (Sigma, type IV, from porcine pancreas, E-0258) for 3 minutes at room temperature (Brokaw, 1980). Digestion was terminated by adding phenylmethylsulfonyl fluoride (final concentration was 2 mM). The digested spermatozoa were introduced into a flow cell made of coverslips as described by Kron et al. (1991). To elicit sliding disintegration of the axonemes, the flow cell was perfused with 0.1 mM ATP, 0.1 M K-acetate, 5 mM $MgCl_2$, 4 mM EGTA, 1 mM DTT, 50 mg ml^{-1} bovine serum albumin (BSA), and 50 mM Tris buffer, pH 8.0. The TMR-labelled microtubules suspended in 10 μM taxol, 0.5 mg ml^{-1} BSA, 6 mg ml^{-1} glucose, 60 μg ml^{-1} glucose oxidase, and 10 μg ml^{-1} catalase in the standard buffer either with or without 0.1 mM ATP were added to the disintegrated axonemes.

Light microscopy

Light microscopic observation of both sperm axonemes and fluorescent microtubules was carried out at room temperature with an inverted microscope (Nikon TMD, Japan) equipped with a Nikon Fluor ×100 objective lens (NA=0.8-1.3) and a Hamamatsu Photonics C2400-08 SIT camera. The spermatozoa and the fluorescent microtubules were observed by dark-field illumination and epi-fluorescence illumination, respectively, and the images were recorded on s-VHS video tapes.

RESULTS

Binding of fluorescent microtubules to axonemes before and after sliding disintegration

We first examined the binding of TMR-labelled microtubules to the axonemes of demembranated sperm flagella in the absence of ATP (Fig. 1). Elastase-treated demembranated spermatozoa were introduced into a flow cell. They spontaneously attached to the glass surface, and were observed by dark-field light microscopy (Fig. 1A). TMR-labelled microtubules were then added to the flow cell, but none of them bound to the axonemes (Fig. 1B). We also added them to demembranated spermatozoa without elastase treatment, but no binding was observed (data not shown). The addition of 0.1 mM ATP to the elastase-treated spermatozoa elicited sliding disintegration (Fig. 1C). After ATP was washed out, TMR-labelled microtubules were added. They were observed to bind to disintegrated areas of axonemes (Fig. 1D). These results indicate that the components that bound microtubules were not on the surface of the demembranated axoneme, but were inside the axoneme, which were exposed by the sliding disintegration. The components were most likely to be the inner and outer dynein arms on axonemes.

The axonemal microtubules that extruded from the elastase-treated spermatozoa were rather straight than curled. These straight areas provided good tracks for the movement of singlet microtubules in the presence of ATP (see the following section). In addition, since sperm heads still remained after elastase digestion and sliding disintegration, the polarity of the axonemal microtubules was easily determined by dark-field observation. In contrast, the microtubules that extruded from the trypsin-treated axonemes were curled, and trypsin digested the sperm heads (data not shown). Therefore, the trypsin-digested spermatozoa were not applicable for the purpose of the present study.

Movement of TMR-labelled microtubules

Fluorescent microtubules were added to disintegrated axonemes in the presence of 0.1 mM ATP. Observation by epi-fluorescence illumination showed that they moved along the disintegrated axonemes (Fig. 2). We observed the disintegrated axonemes, by dark-field illumination, in the same field of view (Fig. 2, top panel). We identified the polarity of axonemes by examining the position of sperm heads. The movement shown in Fig. 2 was from base to tip and, therefore, the direction of

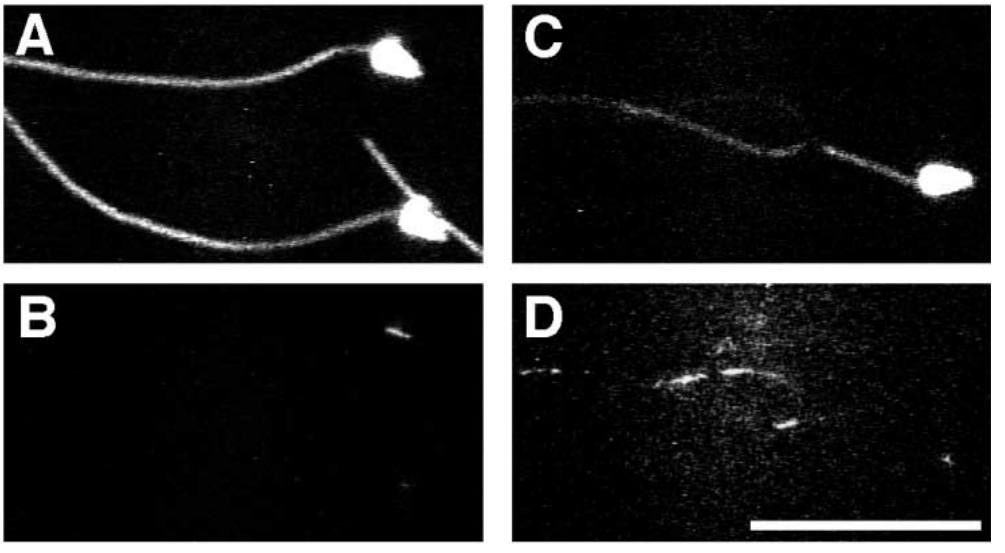

Fig. 1. Binding of fluorescent microtubules onto the disintegrated areas of the sea urchin sperm axonemes. Elastase-treated spermatozoa were bound to the glass surface. Before (A and B) and after (C and D) 0.1 mM ATP was introduced, TMR-labelled microtubules were added in the absence of ATP. (A and C) Dark-field images showing the spermatozoa. The axonemes were disintegrated by the ATP treatment (compare A and C). (B and D) Fluorescence images in the same fields of views of A and C, respectively. Note that the fluorescent microtubules bound to the axonemes only in the disintegrated areas in C. All photographs are at the same magnification. Bar, 20 μm.

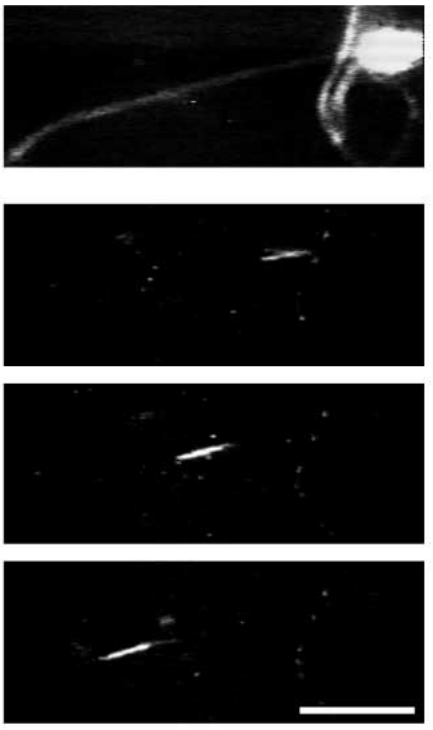

Fig. 2. Movement of a fluorescent microtubule along a disintegrated sperm axoneme. The top photograph is a dark-field image showing a disintegrated axoneme. The other three are fluorescence images taken at one-second intervals, showing a microtubule moving towards the tip. All photographs are in the same field of view. Bar, 10 μm.

force generation in this case was the same as that which occurs in living flagella and cilia.

We observed 43 microtubules moving along disintegrated axonemes whose polarity were identified by dark-field illumination. All of them moved towards the tip of the flagella, and none moved towards the base. Although some microtubules intermittently stopped, we determined their velocities when they continuously moved as being more than 1 μm. Fig. 3 shows the distribution of velocities (3.1±2.1 μm second^{-1}, n=53). We obtained similar results from glycerinated spermatozoa (3.2±2.9 μm second^{-1}, n=74; see also Table 2). In addition to the moving microtubules, in spite of the presence of ATP, many microtubules were observed to bind to the axonemes and not to move. These suggest the presence of some non-dynein components in the axonemes which bind to the TMR-labelled microtubules.

Polarity of microtubule binding to disintegrated axonemes

Occasionally, a microtubule bound to an axoneme showed no movement at first, and then flipped 180° by Brownian motion and started to move towards the tip of the spermatozoon (Fig. 4). Since a microtubule moving along a disintegrated axoneme towards the tip of the flagellum was considered to interact with the axoneme with a parallel orientation, this microtubule first bound to the axoneme in the anti-parallel orientation. These observations imply that microtubules can bind to disintegrated axonemes in both a parallel and an anti-parallel manner.

To confirm the polarity of these bindings directly in our

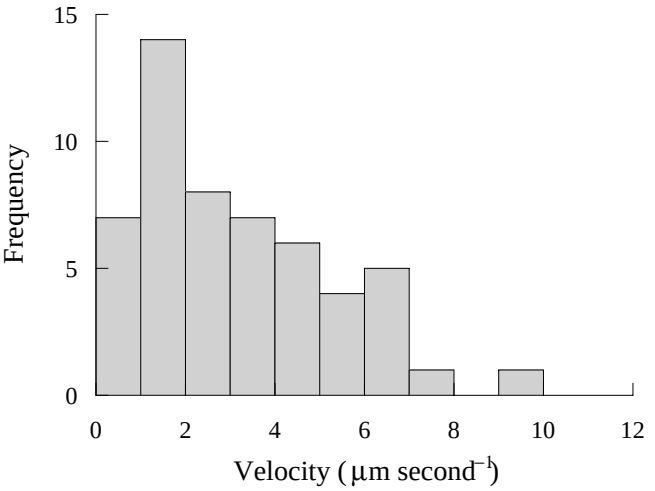

Fig. 3. Velocity distribution of the sliding movement of fluorescent microtubules along disintegrated sperm axonemes demembranated by Triton X-100 in the absence of free Ca^{2+}.

system, we prepared fluorescent microtubules which have more fluorescence in their minus-ends (Howard and Hyman, 1993). We observed the binding of these polarity-marked microtubules under three different conditions: in the absence of ATP, in the presence of 0.1 mM ATP, and in the presence of both 0.1 mM ATP and 10 μM sodium vanadate. Since the affinity between dynein and microtubules in the presence of ATP and vanadate is very weak, it is not considered that dynein would be able to rebind microtubules (Sale and Gibbons, 1979; Shimizu and Johnson, 1983).

Fig. 5 shows examples of polarity-marked microtubules binding to disintegrated sperm axonemes in the absence of ATP. The brighter region indicates the minus-ends of the fluorescent microtubules (Fig. 5B,D), and the flagellar base is the minus-ends of the axonemal microtubules. Both parallel (Fig. 5A,B) and anti-parallel (Fig. 5C,D) bindings were observed under all three conditions.

Table 1 summarizes the numbers of observed microtubules categorized in two different orientations under three conditions. Many TMR-labelled microtubules bound to disintegrated axonemes in the presence of ATP and vanadate, indicating that non-dynein components had considerable affinity to TMR-labelled microtubules. In addition, these components bound to TMR-labelled microtubules regardless of their polarity. In the absence of ATP, where both dynein and non-dynein components contribute to the binding of microtubules with disintegrated axonemes, the microtubules

Table 1. Numbers of observed microtubules bound to disintegrated axonemes in parallel and anti-parallel manners

Condition	Parallel binding	Anti-parallel binding
Without ATP*	140	70
0.1 mM ATP†	86	117
0.1 mM ATP + 10 μM vanadate	149	125

*,†The numbers of microtubules were different between parallel and anti-parallel binding at a significance level of 1%(*) and 5%(†) in the χ^2-test.

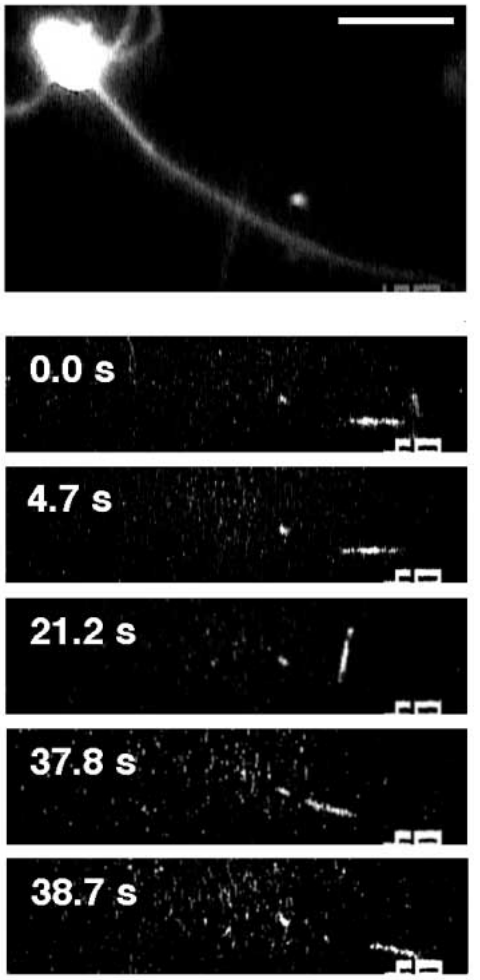

Fig. 4. Sequence showing a microtubule starting to move just when it reversed its orientation by 180° on a disintegrated axoneme of a glycerinated spermatozoon. The top photograph is a dark-field image showing the disintegrated spermatozoon. The other five photographs are fluorescence images showing the microtubule with time in seconds after the first fluorescence image was taken. First, for about 5 seconds, the microtubule bound to the axoneme but did not move ('0.0 s' and '4.7 s'). While the solution inside the flow cell was perfused from the bottom to the top in the images, one end detached from the axoneme and swung around for about 30 seconds ('21.2 s'). Then, it bound to the axoneme in the reverse orientation ('37.8 s') and started to move towards the tip of the spermatozoon ('38.7 s'). All photographs have the same magnification and the bottom lines are in the same position. Bar, 10 μm.

tended to bind to axonemes in a parallel orientation. This is consistent with the results of Warner and Mitchell (1981), which showed that the outer doublet made after the sliding disintegration of axonemes reassembled into bundles in both parallel and anti-parallel manners, though the former was preferred. Since non-dynein components bind microtubules regardless of their polarity, dynein was considered to bind them preferentially in a parallel manner. However, from the present results, it is still unclear whether dynein molecules on the outer doublet microtubules can bind the anti-parallel microtubules, since the anti-parallel binding under this condition might be caused by non-dynein components. Warner and Mitchell (1981), however, showed that anti-parallel binding microtubules were also dissociated by adding ATP, and strongly suggested that anti-parallel binding was mediated by dynein arms. In the presence of ATP but no vanadate, where dynein could actively generate force, significantly more microtubules binding in the anti-parallel orientation were observed than those in the opposite orientation (Table 1). This may have been because some parallel microtubules were moved by dynein and then dissociated from the axonemes.

Spermatozoa demembranated in the presence of Ca^{2+}

Although we revealed that fluorescence-labelled microtubules move only towards the tip of the flagella, Ishijima et al. (1996) have shown electron micrographs of disintegrated sperm axonemes, suggesting that the dynein arms moved the outer doublet towards the sperm head. They concluded that when the flagella were demembranated in the presence of millimolar free Ca^{2+} and then treated with elastase, dynein can move microtubules towards the sperm head. To see whether microtubules move towards the sperm head, we used sea urchin axonemes demembranated in the presence of millimolar Ca^{2+} in the same solution as they described (Ishijima et al., 1996), instead of the standard buffer. We observed 198 TMR-labelled microtubules moving along disintegrated axonemes whose direction was determined. All of them, however, moved towards the tip of the flagella, indicating that dynein arms on the axoneme generate force only in the direction from base to tip of the flagella.

DISCUSSION

We observed the binding and movements of TMR-labelled microtubules on dynein rows of axonemes demembranated under three different conditions: demembranated by Triton X-100 in the absence of free Ca^{2+}, by glycerol in the absence of free Ca^{2+}, and by Triton X-100 in the presence of millimolar free Ca^{2+}. There has been no attempt to have microtubules interact with dynein molecules on outer doublet microtubules in anti-parallel orientation, except in electron microscopic observations by Warner and Mitchell (1981) in which disintegrated doublet microtubules reassembled into anti-parallel bundles in the absence of ATP. Our results, which showed that the polarity-marked microtubules bound to disintegrated axonemes in both a parallel and an anti-parallel manner are consistent with their observations.

In addition, we examined the possibility that microtubules could move towards the flagellar base under the condition where the microtubules were able to interact with dynein arms on an axoneme in both parallel and anti-parallel orientations. In all cases, the movements were from the base to the tip of the sperm flagella (Table 2) and no movement towards the base was observed. This direction of movement was consistent with the electron microscopic observations of Sale and Satir (1977) and Fox and Sale (1987), but inconsistent with the observations of Ishijima et al. (1996) showing that dynein produced force from the tip to the base when axonemes were demembranated in the presence of Ca^{2+}.

This inconsistency might pose the possibility that the force of dynein moving microtubules towards the flagellar base could be so weak that internal resistance might hamper the movement

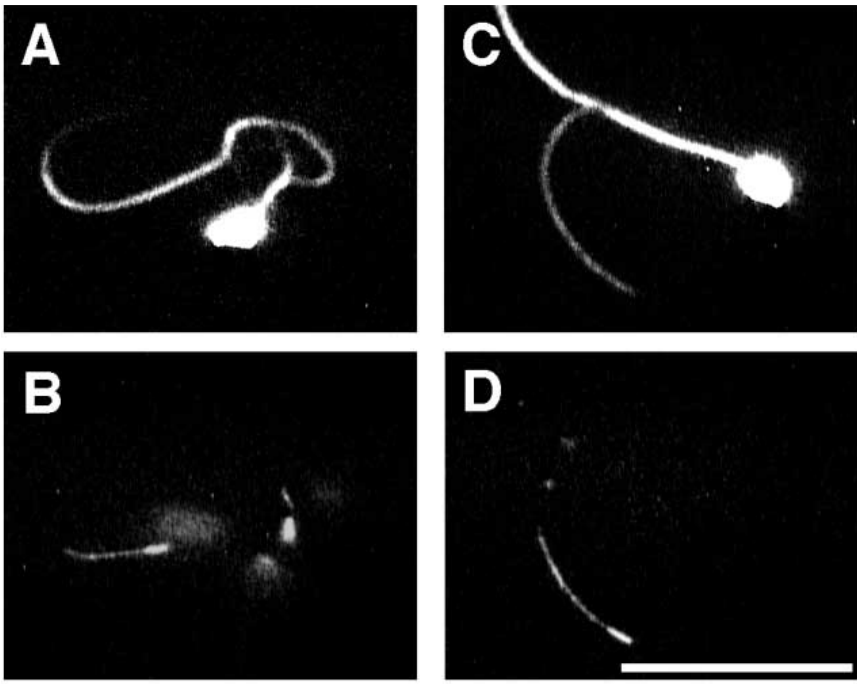

Fig. 5. The binding of fluorescent microtubules to the disintegrated axonemes in parallel and anti-parallel manners in the absence of ATP. (A and C) Disintegrated spermatozoa observed under dark-field illumination. (B and D) Fluorescence images in the same fields of views of A and C, respectively. The polarity-marked fluorescent microtubules whose minus-ends were brighter than other parts (B and D) bound to axonemes in parallel (B) and anti-parallel (D) manners. All photographs have the same magnification. Bar, 20 μm.

of microtubules in that direction. Since vanadate in addition to ATP detaches microtubules from dynein (Sale and Gibbons, 1979; Shimizu and Johnson, 1983), the binding of microtubules on an axoneme observed in the presence of vanadate and ATP in the present study is due to non-dynein components on outer doublet microtubules. These components, therefore, might behave as an internal resistance that slows down the sliding. Actually, the velocity of microtubule sliding showed a broad distribution ranging from 0.1 to 9.5 μm second^{-1}. Nevertheless, about one third of the microtubules observed moving had velocities of more than 4 μm second^{-1} and were thus comparable to those observed in sliding disintegration, about 5 μm second^{-1} at 0.1 mM ATP (Yano and Miki-Noumura, 1980). Therefore, if dynein arms generate force towards the base, we should have observed some microtubules moving towards the base in our experiments. However, we observed no movement under any of the demembranation conditions we studied (Table 2), and thus dynein rows on the axonemes are most likely unable to generate force towards the base.

As mentioned above, we found some properties of the components that impeded the movement of microtubules. They bound to microtubules even in the presence of vanadate and ATP, and thus they were considered to be different from dynein components themselves. We also found that they bound to microtubules irrespective of their polarity. These non-dynein components could play important roles in the formation of the flagellar beating since the flagellar beating could not occur unless the interdoublet sliding generated by dynein arms are resisted by structures intrinsic to the axoneme (Shingyoji et al., 1977). On the other hand, dynein complexes could have a modulatory function. Actually, in an in vitro microtubule gliding assay, the movement of microtubules on the glass surface covered with the β-heavy chain/intermediate chain 1 (β/IC 1) subunit of the sea urchin sperm outer arm dynein was faster than that on the glass surface covered with intact outer arm dynein (Sale and Fox, 1988). Furthermore, it was shown that while the β/IC 1 subunit could make neither rigor nor structural binding to microtubules, the α subunit could make both bindings (Moss et al., 1992a,b). Thus, the α subunit probably behaved as a modulator against the movement of the β/IC 1 subunit. The modulatory function has to provide both spatial and temporal control against the sliding movement occurring between the outer doublet microtubules in order to make the flagellar beating. It could be considered that microtubule movements on the axonemes in this study were affected by the modulatory function and, as a result, we found both fast and slow velocities in the microtubule movements. If the disintegrated axonemes retain the modulatory function, further study with the new assay system presented here would provide information about axonemal functions including their beating mechanisms.

The result that dynein molecules on axonemal microtubules generate force only in one direction differed from observations that showed myosin molecules arranged on thick filaments move actin filaments in both directions (Sellers and Kachar, 1990; Yamada et al., 1990; Yamada and Wakabayashi, 1993). This might be because the rotational flexibility of dynein molecules on axonemal doublet microtubules is different from that of myosin molecules in thick filaments. Reedy et al. (1989) showed electron micrographs of *Drosophila* mutant muscles

Table 2. Numbers of observed microtubules that moved from base to tip and from tip to base along disintegrated axonemes demembranated under three different conditions

Demembranation condition	Base to tip	Tip to base
Triton X-100, Ca^{2+} free	43	0
Glycerol, Ca^{2+} free	63	0
Triton X-100, millimolar Ca^{2+}	198	0

which suggested that myosin heads in thick filaments can bind to thin filaments whose polarity is opposite to that of normal ones, by rotating 180°. In contrast, dynein arms on axonemal microtubules seemed to have rigid structures even when they were dissociated from neighbouring outer doublet microtubules (Goodenough and Heuser, 1982, 1985).

In the present study, we succeeded in moving fluorescent microtubules on dynein molecules arranged on axonemes in a native manner. Our results indicate that the orientation of dynein molecules affects microtubule movement. In most in vitro motility assays, the motor protein molecules were adsorbed on a glass surface in a random manner and actin filaments or microtubules were observed moving while changing their directions (Kron and Spudich, 1986; Paschal et al., 1987; Vale and Toyoshima, 1988). Mimori and Miki-Noumura (1994) reported the movement of microtubules on dynein rows artificially formed on a glass surface. Though they reported that the movement of microtubules on dynein rows was faster than that on randomly oriented dynein molecules on a glass surface, the polarity of dynein molecules might be incomplete since they were able to move microtubules in both directions with similar velocities. Hence, dynein molecules should be arranged in a uniform orientation when mechanochemistry of dynein molecules is studied in an in vitro motility assay, as rabbit skeletal muscle myosin molecules were successfully arranged in a uniform orientation more than 20 μm in length (Yamada et al., 1997). Our system using native dynein rows of axonemes serves as a prototype of such an array system.

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