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Translocation of microtubules caused by the αβ, β and γ outer arm dynein subparticles of *Chlamydomonas*

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SUMMARY

Three kinds of subparticles of *Chlamydomonas* outer-arm dynein containing the αβ, β and γ heavy chains were isolated and assayed for their activities to translocate microtubules in vitro. All of them had activities to form bundles of microtubules in solution in an ATP-dependent manner and, when adsorbed on an appropriate glass surface, translocated microtubules. The αβ subparticle readily translocated microtubules on a silicone-coated glass surface with a velocity of 4.6 μm/second at 1 mM ATP. The β subparticle translocated microtubules after it had been preincubated with tubulin dimer and when the Brownian movement of microtubules was suppressed by addition of methylcellulose. The velocity was on average 0.7 μm/second. The γ subparticle translocated microtubules after being preincubated with tubulin dimer and adsorbed onto a silicone-coated glass surface. The velocity was about 3.8 μm/second. The tubulin dimer appeared to facilitate in vitro motility by blocking the ATP-insensitive binding of dynein subparticles to microtubule. The αβ, β and γ subparticles were thus found to have different properties as motor proteins. In addition, these subparticles showed different dependencies upon the potassium acetate concentration. Hence the outer-arm dynein of *Chlamydomonas* is a complex of motor proteins with different properties.

Key words: Outer dynein arm, Dynein heavy chain, In vitro motility assay

INTRODUCTION

Flagellar dyneins are molecular motors that produce a sliding force between adjacent outer doublet microtubules (reviewed by Gibbons, 1981; Porter and Johnson, 1989; Mitchell, 1994). Dyneins contain heavy chains (M_r >500,000) with ATPase activities, and smaller proteins of unknown functions. A single flagellum contains several dynein heavy-chain isoforms; for example, a *Chlamydomonas* flagellum contains three outer-arm heavy chains and at least eight inner-arm heavy chains.

Studies with *Chlamydomonas* mutants showed that different heavy chains contribute to flagellar motility in different manners. Lack of inner-arm dynein subspecies results in a decrease in bend angle of flagellar beating while lack of outer-arm dynein results in a decrease in beat frequency (Brokaw and Kamiya, 1987). The velocity of microtubule sliding in mutant axonemes lacking the inner-arm subspecies, during sliding disintegration upon addition of ATP and protease, is similar to the velocity in the wild-type axoneme, whereas the sliding velocity in the mutant axonemes that lack the outer-arm dynein is as low as 1/5 that of wild type (Kurimoto and Kamiya, 1991). These observations indicate that inner and outer dynein arms differ in function. Furthermore, studies with mutants that lack part of the outer-arm heavy chains suggest that functional differentiation is present among the outer-arm dynein heavy chains. The mutant *oda11* which lacks the α heavy chain swims at a speed that is intermediate between the swimming velocities of wild type and a mutant lacking the entire outer arm, whereas the mutant *oda4-s7* which lacks the functional β heavy chain swims at a speed that is close to that of the mutant lacking the entire outer arm (Sakakibara et al., 1991, 1993). These findings indicate that the α heavy chain and the β heavy chain have different roles in outer-arm function. In addition, it has been demonstrated that the outer arm dynein of sea urchin and *Tetrahymena* dissociates into two subparticles after appropriate treatments, but that only one of the two particles has the activity to translocate microtubules (Sale and Fox, 1988; Vale and Toyoshima, 1989). Moss et al. (1992a,b) reported that the β subparticle of sea urchin translocates microtubules but does not produce a rigor complex in the absence of ATP, whereas the α subparticle does not translocate microtubules but produces a rigor complex. These observations also suggest that each of the outer arm heavy chains has a distinct mechanochemical property.

Thus far, in vitro motility properties of outer arm dynein subparticles have been studied only in limited species, such as *Tetrahymena* and sea urchin. Thus, although *Chlamydomonas* mutants have offered information as to the functional difference of different outer-arm dynein heavy chains, no studies have been performed to examine in vitro motility properties of its outer arm dynein. Therefore, we have no information as to whether its individual heavy chain has a motor activity, or how different heavy chains differ in the velocity of translocation. In this study, we isolated three kinds

of subparticles that contained the outer arm α and β heavy chains, the β heavy chain, or the γ heavy chain, and assayed them for the activity to translocate microtubules in vitro. We found that all of the αβ, β and γ subparticles have motor activities but their properties greatly differ from each other.

MATERIALS AND METHODS

Strains

Chlamydomonas reinhardtii wild type (137c), an outer arm-less mutant (*oda4*) (Kamiya, 1988), and a mutant lacking the α heavy chain (*oda11*) (Sakakibara et al., 1991) were used.

Isolation of the outer arm dynein subparticles

Isolation of the outer arm dynein subparticles was essentially after the method of Kagami and Kamiya (1992) used for fractionation of inner arm dyneins. For the preparation of dynein subparticles, wild-type or mutant cells were cultured on 90 1.3% agar plates (600 cm^2) containing the TAP medium (Gorman and Levine, 1965) under continuous illumination at 23°C. Axonemes were isolated by the method of Witman (1986). Dyneins were extracted from axonemes with 5 ml of 0.6 M KCl in HMDE solution (30 mM Hepes, 5 mM $MgSO_4$, 1 mM DTT, 1 mM EGTA, pH 7.4) containing protease inhibitors (0.5 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The high salt extract was first diluted 5-fold with HMDE solution containing protease inhibitors to lower its ionic strength, spun at 40,000 ***g*** for 30 minutes to remove aggregates, applied to a MonoQ anion-exchange column (Pharmacia, Inc., Uppsala, Sweden), and eluted with a KCl gradient made in HMDE solution containing 0.5 mM PMSF. 0.25 ml fractions were collected. Fractions containing the αβ or β subparticles were diluted 3-fold with HMDE, and applied to MonoQ chromatography again to remove inner-arm dyneins. Each sample of subparticle was concentrated to about 300 μg/ml. The samples were diluted to 100 μg/ml with HMDE before use to lower their KCl concentration.

Microtubule and tubulin binding

Bovine brain tubulin was purified by 2 cycles of temperature-dependent polymerization and DEAE-Sepharose (Pharmacia, Inc., Uppsala, Sweden) ion exchange chromatography (Vallee, 1986). Microtubules were prepared from purified tubulin (5 mg/ml) at 37°C in 0.1 M Pipes, 1 mM $MgSO_4$, 1 mM EGTA, 1 mM GTP, pH 6.9, for 30 minutes. Following incubation, polymerization was stopped and microtubules were stabilized by 10-fold dilution with HMDE solution containing 20 μM taxol. Before assays, microtubules were further diluted with the HMDE solution containing taxol to adjust microtubule concentration. Microtubule binding was assayed at 30°C. For the quantification of microtubule bundling activity, dynein subparticles (final concentration: 20 μg/ml) and microtubules (100 μg/ml) were mixed and incubated for 30 minutes. Bundles of microtubules were observed under a dark-field microscope. To assay ATP-insensitive binding, dynein subparticles (20 μg/ml) and microtubules (400 μg/ml) were mixed and incubated for 1 hour. After incubation, 1 mM ATP and 10 μM sodium vanadate were added to eliminate ATP-sensitive binding. Samples were layered on a cushion of 30% glycerol in HMDE solution containing 0.2 mM ATP. Microtubules and associated proteins were pelleted by centrifugation at 100,000 ***g*** for 1 hour. Precipitates and supernatants were analyzed by SDS-PAGE. Binding of tubulin to dynein subparticles was assayed by sucrose density gradient centrifugation. A 5-20% sucrose gradient in HMDE containing 0.2 mM ATP was used. Dynein subparticles (100 μg/ml) and tubulin (100 μg/ml) were mixed and incubated in the presence of 1 mM ATP at 0°C for 1 hour. The samples were layered on sucrose gradients and spun at 180,000 ***g*** (44,000 rpm) for 5.5 hours at 4°C in an RPS55T-2 rotor (Hitachi Koki Co., Ltd, Tokyo, Japan) and collected in 0.25 ml fractions from the bottom of the tube.

In vitro motility assay

Each dynein subparticle was examined for the activity to translocate microtubules at 23°C according to the method of Kagami and Kamiya (1992) with modifications. Each subparticle (100 μg/ml) was either mixed or not mixed with tubulin (100 μg/ml) in the presence of ATP (1 mM), and left standing at 0°C for 1 hour. A 20 μl aliquot of the sample was perfused into an observation chamber made of a glass slide and a coverslip (18 mm × 18 mm), of which two opposite sides were sealed with vaseline. The chamber was flushed with 40 μl HMDE, then loaded with HMDE solution containing 1 mg/ml BSA, and left standing for 2 minutes to allow the glass surface to adsorb BSA. Then taxol-stabilized microtubules (about 50 μg/ml in HMDE solution) were introduced into the chamber and attached to the dynein-coated glass surface. Microtubule translocation was initiated by perfusing the chamber with 40 μl of HMDE solution containing 1 mM ATP. For examination of ATP concentration-dependence of motility, we used various concentrations of ATP and an ATP-regenerating system (70 units/ml of creatine phosphokinase and 5 mM phospho-creatine) in place of the 1 mM ATP. We separated the procedure for loading microtubules and addition of ATP, because we wanted to avoid microtubule bundling, which often occurred when microtubules were mixed with reactivation solutions containing methylcellulose. Glass slides for the motility assay were coated with silicone or treated with HCl. Silicone-coated glass slides were prepared by immersing slides in 50% Sigmacote solution (Sigma Chemical Co., St Louis, USA) for a few seconds. Treatment of glass slides with HCl was performed by immersing them in 0.1 N HCl for 1 day. Glass slides, the surface of which had been thus treated, were rinsed with ethanol prior to use to clear frost on the glass surface.

The movement of microtubules on the glass slide was examined by dark field microscopy using an Olympus BH microscope equipped with a 100 W mercury light source, a UV filter, a heat filter, an Olympus BH-DCW dark field condenser (1.2-1.4 NA) and an Olympus ×100 DPlan apochromatic oil-immersion lens with an adjustable iris. The image was projected onto a CCD with a ×3.3 eyepiece lens, contrast-enhanced with an Argus-10 image processor (Hamamatsu Photonics, Hamamatsu, Japan) and recorded with an S-VHS VCR. The sliding velocity was measured with a personal computer as described by Kagami and Kamiya (1992).

Other methods

SDS-PAGE was carried out by the method of Laemmli (1970) as modified by Jarvik and Rosenbaum (1980). The gel was composed of a 3% to 5% acrylamide gradient and a 3 M to 8 M urea gradient. To analyze the intermediate- and low-molecular-mass protein, the samples were also run on a 5% to 20% acrylamide gradient gel. All the gels were silver-stained (Merril et al., 1981). Protein concentrations of the samples of tubulin and outer-arm-dynein subparticles were determined by the method of Bradford (1976).

RESULTS

Isolation of the αβ, γ and β subparticles

Outer-arm dynein was separated into the αβ and γ subparticles by extracting axonemes with a high salt concentration solution (Piperno and Luck, 1979; Pfister et al., 1982). For in vitro motility assays, we purified the subparticles from the high-salt extract by HPLC on a MonoQ column. Fig. 1 shows the elution profile. As reported by Goodenough et al. (1987), the γ subparticle appeared at 200-220 mM KCl as the first peak and the αβ subparticle appeared between the peaks of the inner arm

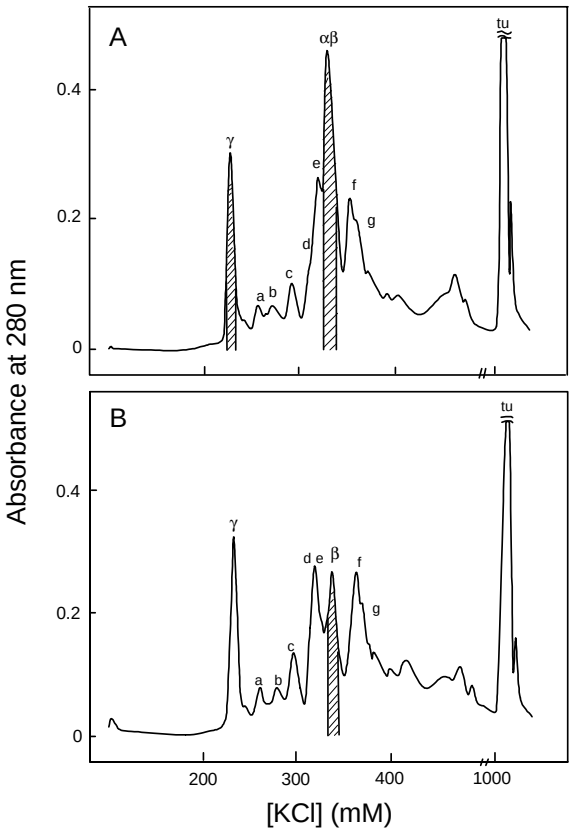

Fig. 1. Fractionation of the high-salt extract from wild-type (A) and *oda11* (B) axonemes by HPLC on a MonoQ column. (solid line) Absorbance at 280 nm. The fractions of the peaks (αβ, γ, and β) indicated by shaded area were used as the samples of subparticles. (a-g) Peaks of inner-arm subspecies named after Kagami and Kamiya (1992).

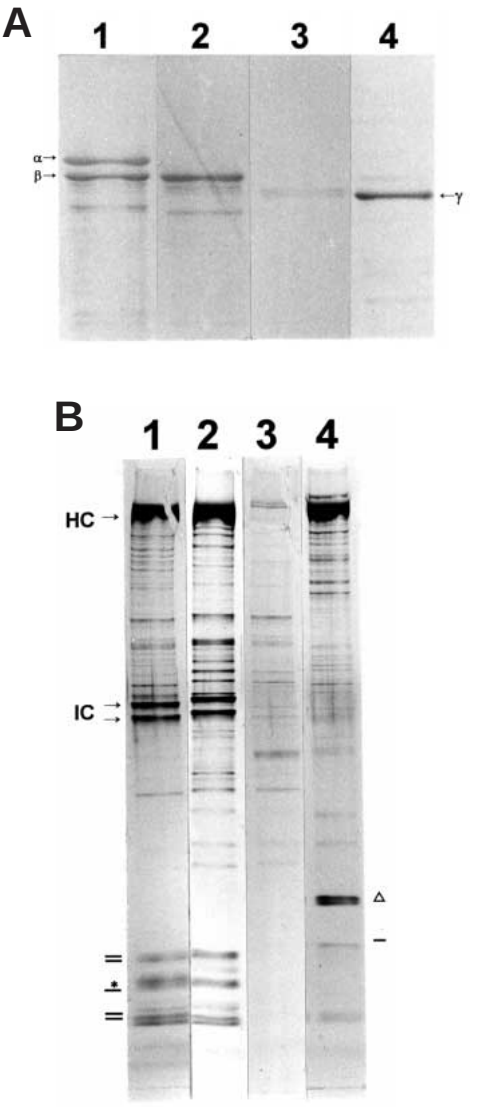

Fig. 2. SDS-PAGE patterns of the peak fractions of the αβ (lane 1), β (lane 2), and γ (lane 4) subparticles. (lane 3) a HPLC fraction from the axonemes of *oda4* lacking the outer arm dynein, eluted at the same position as that of the αβ subparticles. (A) Part of a 3-5% SDS-urea PAGE pattern showing the high molecular mass bands. (B) 5-20% SDS-PAGE separating intermediate-sized and low molecular mass peptides. Stained with silver. (A) Arrows indicate the three (α, β and γ) outer-arm dynein heavy chains. (B) HC and IC, heavy chains and intermediate-sized chains of outer arm dynein. Bars on the left indicate low molecular mass chains normally associated with the β heavy chain. Asterisk, the 16 kDa light chain normally associated with the α heavy chain. Bar on the right indicates the 22 kDa light chain normally associated with the γ heavy chain. (△) Contaminating protein which is not associated with the γ heavy chain.

subspecies e and f (Fig. 1A). We isolated the β subparticle from the axoneme of the mutant *oda11* which lacks the α heavy chain (Fig. 1B). The β subparticle was eluted at almost the same position as that of the αβ subparticle. The αβ and β subparticles obtained were subjected to the MonoQ column again to remove the e subspecies of the inner arm dynein, whose peak partially overlapped with the peaks of αβ and β. The SDS-PAGE patterns of the αβ, γ, and β subparticles are shown in Fig. 2. The αβ and β subparticles contained two intermediate-sized chains and light chains (Fig. 2B, lanes 1 and 2) as reported by Pfister et al. (1982) and Sakakibara et al. (1991). The γ subparticle contained a 22 kDa chain, but lacked the 18 kDa light chain that is contained when prepared by sucrose-density gradient centrifugation (Pfister et al., 1982) (Fig. 2B, lane 4). It may have been lost during the process of preparation. Although the sample of the αβ and β subparticles appeared to have some cross-contaminating proteins, they should be negligible in the motility assay because the same amount of those proteins obtained from the outer arm-less mutant *oda4* (Fig. 2A and B, lane 3 each), did not affect the in vitro motility when mixed with the outer arm sample.

Binding of dynein subparticles to microtubules

In the flagellar axoneme, dyneins are bound to doublet microtubules through both ATP-sensitive and ATP-insensitive binding sites. Crude dynein from *Chlamydomonas* axonemes and 22S dynein from *Tetrahymena* have been found to cross-bridge microtubules in vitro, suggesting both binding sites are present in these dyneins (Haimo et al., 1979; Porter and Johnson, 1983). We examined whether each of the αβ, β and

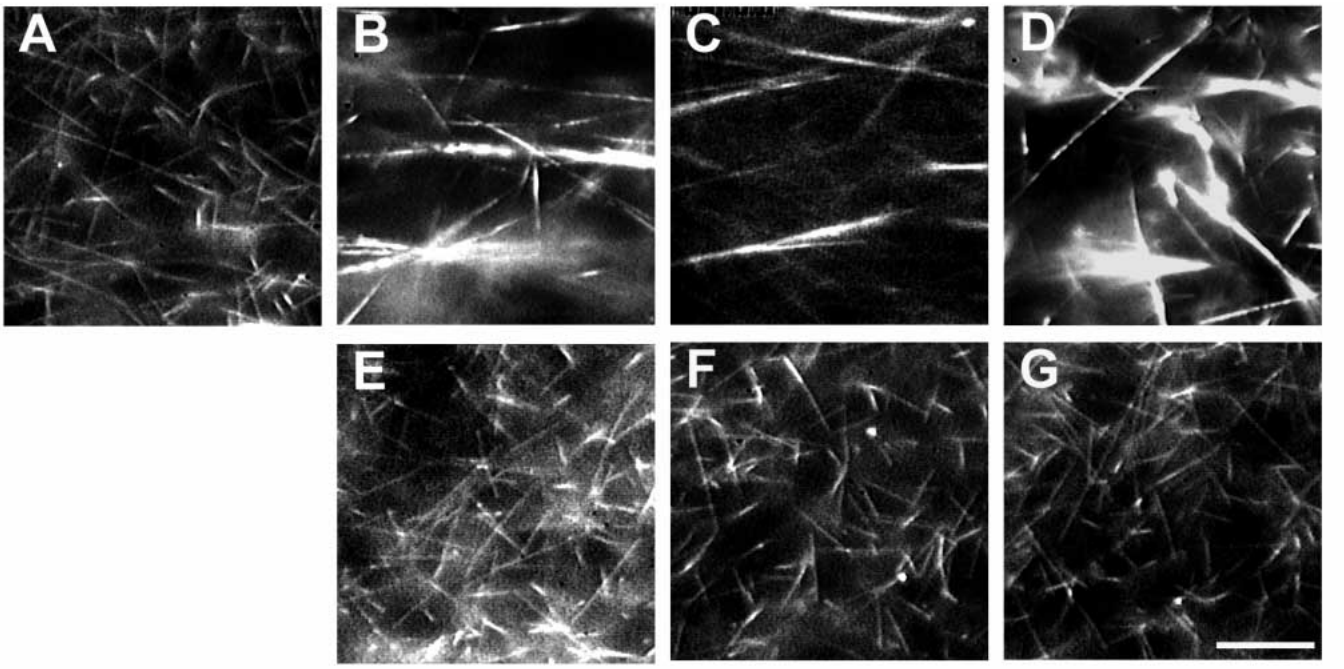

Fig. 3. Dark-field microscopy of microtubule bundles induced by the αβ, β and γ subparticles. (A) Microtubules assembled in vitro. (B,C,D) Microtubule bundles formed in the outer arm dynein subparticles. (E,F,G) Dissociation of microtubule bundles into single microtubules upon addition of 1 mM ATP. (B and E) αβ subparticle; (C and F) the β subparticle; (D and G) the γ subparticle. Bar, 10 μm.

γ subparticles has such an activity. In fact we found that, when one of the αβ, β and γ subparticles (20 μg/ml each) was mixed with microtubules (100 μg/ml), all of the three subparticles bundled microtubules (Fig. 3B,C,D). The microtubule bundles dissociated into single microtubules upon addition of 1 mM ATP (Fig. 3E,F,G). These observations suggest that each of the αβ, β and γ subparticles has two or more microtubule binding sites, of which at least one is ATP sensitive. Next, we examined whether the αβ, β and γ subparticles have ATP-insensitive binding sites also. Each of the three subparticles (20 μg/ml) was mixed with microtubules (400 μg/ml) and incubated for 1 hour. The sample was then centrifuged after ATP-sensitive binding was removed by addition of ATP and vanadate. As shown in Fig. 4, all of the three subparticles precipitated when microtubules were present, indicating that they actually have ATP-insensitive binding sites. The amount of dyneins precipitated with microtubules was somewhat greater in the absence of ATP than after addition of ATP. In the absence of ATP, dyneins were probably bound to microtubules by both ATP-sensitive and ATP-insensitive binding sites.

As described below, while examining the conditions for in vitro motility assays, we found that the β and γ subparticles translocated microtubules only when they were preincubated with tubulin dimer. Therefore, we also examined whether tubulin dimer binds to the β and γ subparticles in an ATP-independent manner, as shown previously with *Tetrahymena* 22S dynein and the crude dynein of *Chlamydomonas* (Haimo and Fenton, 1988; Mimori and Miki-Noumura, 1994). When only tubulin dimer was centrifuged on a 5-20% sucrose gradient, tubulin appeared in fractions 12-18 and did not appear in the bottom fraction (fraction 11) (Fig. 5). When tubulin dimer was incubated with each of the β and γ subparticles and subjected to sucrose density gradient centrifugation in the presence of ATP, tubulin appeared in fractions 6-9 for the β subparticle (Fig. 5), and 9-11 for the γ subparticle (Fig. 5). These results strongly suggest that both of the β and γ subparticles bind to dimeric tubulin in an ATP-insensitive manner.

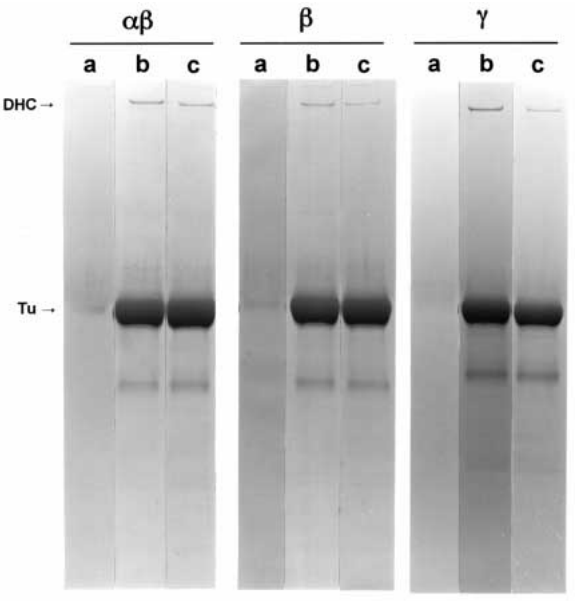

Fig. 4. ATP-insensitive binding of the αβ, β and γ subparticles to microtubules. SDS-PAGE (5-20%) patterns of the precipitate from centrifugation at 100,000 g for 1 hour. (a) Without microtubules. (b) Microtubules and dyneins without ATP and vanadate. (c) Microtubules and dyneins with 1 mM ATP and 10 μM sodium vanadate. DHC, heavy chains of outer arm dynein. Tu, tubulin.

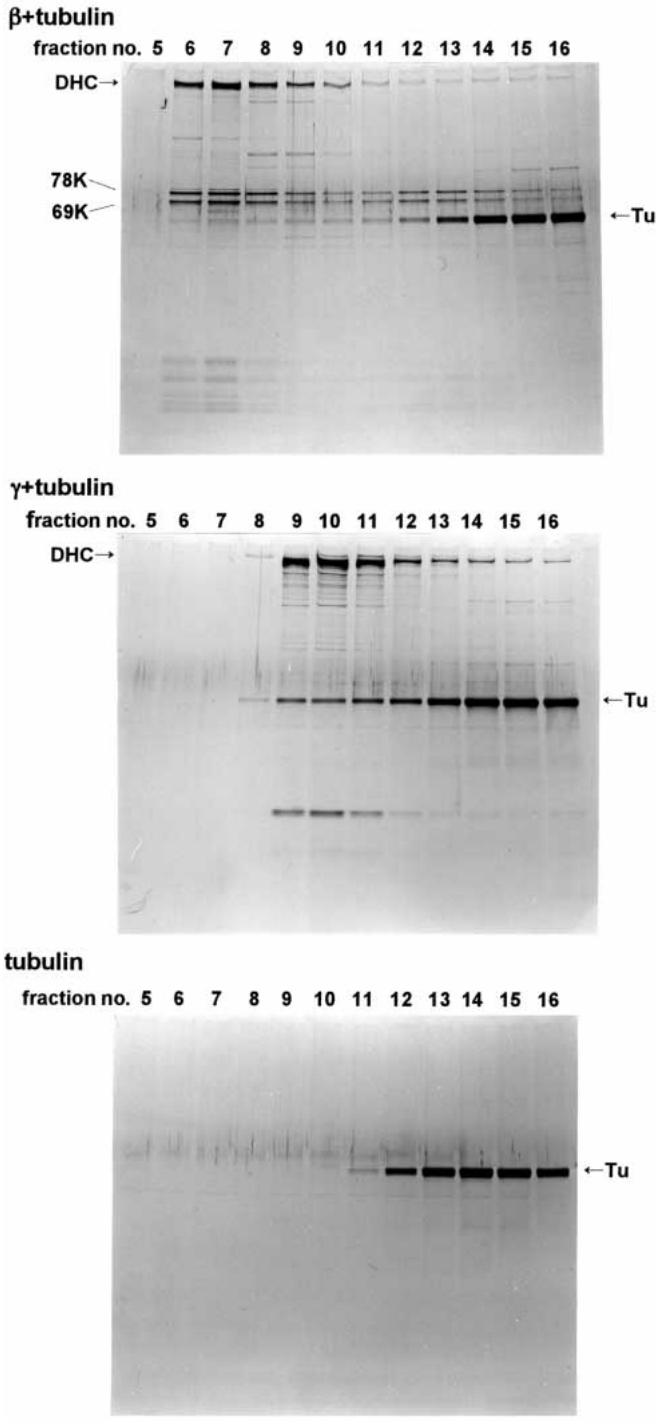

Fig. 5. ATP-insensitive binding of tubulin dimer to the β and the γ subparticle. The β and the γ subparticles were mixed with tubulin dimer, respectively, and centrifuged on a 5-20% sucrose density gradient in the presence of 0.2 mM ATP. As a control, tubulin was also subjected to the sucrose gradient centrifugation in the absence of dynein subparticles. SDS-PAGE patterns of all the fractions are shown. The bottom of the gradient is at left. DHC, dynein heavy chain. Tu, tubulin; 69K, 78K, intermediate chains. Stained with silver.

In vitro motility assay

We next examined the αβ, β and γ subparticles for microtubule-translocating activity in vitro. The three subparticles were each introduced into an observation chamber and left standing for a few minutes, to allow time for adsorption onto the glass surface. The chamber was then perfused with a buffer solution containing 1 mg/ml of BSA. The use of BSA prevented the microtubules from directly sticking to the glass surface (Kagami and Kamiya, 1992). Microtubules were then perfused into the chamber and attached to the surface of the glass slide. The chamber was then perfused with a reactivation solution containing 1 mM ATP.

We examined motility while taking into consideration various factors that might affect the motility. First, we examined the conditions of the glass surface. Since hydrophobicity and charge should be most important, we tested three kinds of glass slides that were coated with silicone, washed with 0.1 N HCl, or washed with 0.1 N NaOH. We found glass slides coated with silicone or washed with HCl gave good results. Second, to block the ATP-insensitive microtubule binding site of dyneins, we tested preincubation of subparticles with tubulin dimer (Haimo and Fenton, 1988; Mimori and Miki-Noumura, 1994). Third, we tried to suppress the Brownian movement of microtubules while not preventing their active movements. If the binding of dynein subparticles to microtubules is weak, microtubules would diffuse away from dynein subparticle coated glass surface in the in vitro motility assays. Following previous studies (Uyeda et al., 1990; Nakata et al., 1993), we used methylcellulose.

Table 1 shows the results of the experiments under different conditions. All of the αβ, β and γ subparticles were found to have an activity to translocate microtubules. The optimal conditions for motility varied with the species of subparticles. The αβ subparticle translocated microtubules with a high probability (up to 80% of the total microtubules attached to the surface) on the silicone-coated glass surface. Use of HCl-washed glass slides resulted in lower probability of translocation (less than 10% of the total microtubules). Preincubation with tubulin was not needed for the translocation by the αβ subparticle. The velocity of microtubule translocation, 4.6±1.0 μm/second, was unchanged before and after the preincubation (Fig. 6).

With the β subparticle, use of the glass slides washed with HCl, preincubation with tubulin dimer, and suppression of Brownian motion with methylcellulose were all found crucial for microtubule translocation. Microtubules readily became

Table 1. Microtubule translocation under various conditions

Glass surface	Silicone coated		Washed with HCl	
Tubulin treatment	+	–	+	–
αβ subparticle	++	++	+	+
β subparticle	–	–	+*	–†
γ subparticle	++	–†	–‡	–†

*The β subparticle induced microtubule translocation only in the presence of methylcellulose.

†Microtubules were stuck to the glass surface that had been treated with dynein subparticle.

‡Microtubules were attached to the glass surface that had been treated with dynein subparticles in the absence of ATP, and detached upon addition of ATP.

++: more than 60% of microtubules were translocated.

+: 1-10% of microtubules were translocated.

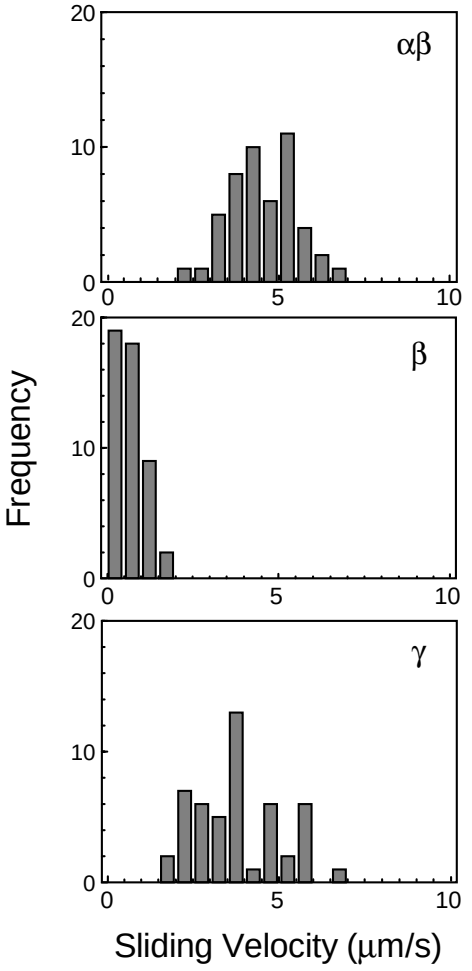

Fig. 6. Velocity distribution in microtubule translocation caused by the αβ, β and γ subparticles (n=50). Conditions for motility assays shown are the optimal ones for each subparticle. Conditions for the αβ subparticle: silicone coated glass slide, HMDE solution plus 50 mM K-acetate and 1 mM ATP, preincubated with 0.1 mg/ml tubulin. For the β subparticle: HCl-washed glass slide, HMDE solution plus 0.2% methylcellulose and 1 mM ATP, preincubated with 0.1 mg/ml tubulin. For the γ subparticle: silicone-coated glass slide, HMDE solution plus 1 mM ATP, preincubated with 0.1 mg/ml tubulin. Concentration of each subparticle was 0.1 mg/ml. Temperature: 23.0±1.0°C. The average velocity and standard deviation for each subparticle are given in the text.

attached to HCl-washed glass slides, but not silicone-coated slides, to which the β subparticles were adsorbed. However, they did not dissociate from the surface upon addition of ATP. We suspected that this ATP-insensitive binding is due to the interaction between the microtubules and the ATP-independent binding site of the β subparticles, because we observed that microtubules did not attach to the glass surface when the glass surface was coated with BSA, in place of the β subparticles. In the hope of blocking the putative ATP-insensitive microtubule binding, we incubated the β subparticles with tubulin dimer before perfusion into the observation chamber. This procedure was in fact found effective in reducing ATP-insensitive binding; upon addition of ATP most of the microtubules were detached from the surface. In this case, a small number of microtubules showed slow translocation (~0.1 μm/second) before the detachment. To improve the percentage and speed of microtubule translocation, we next added 0.2% methylcellulose to the ATP solution to suppress Brownian movement of microtubules, and found that this in fact resulted in frequent observation of translocation. The translocation was slow (0.7 μm/second on average; Fig. 6) and uni-directional.

The γ subparticle translocated microtubules only after it was preincubated with tubulin dimer and adsorbed onto silicone-coated glass slides. With glass slides washed with HCl and not coated with silicone, microtubules became attached to the surface to which the γ subparticles had been adsorbed, in an ATP-dependent manner. In this case, however, no microtubules displayed translocation upon addition of ATP. Microtubule translocation was observed when the γ subparticle was preincubated with tubulin dimer and was adsorbed on the silicone-coated glass surface. The velocity was 3.8±1.2 μm/second (Fig. 6).

As stated above, the β and γ subparticles translocated microtubules only when they were preincubated with tubulin dimer. This preincubation had to be ≥15 minutes. After insufficient preincubation, neither the β subparticle nor the γ subparticle translocated microtubules efficiently. This suggests that the effect of the preincubation is due to some specific interaction between dynein subparticles and tubulin dimers.

Fig. 7 shows the ATP-dependence of the velocity of translocation by the three kinds of subparticles. All increase with the ATP concentration and saturated at >1 mM ATP. With the αβ and γ subparticles, the velocity varied in a Michaelis-Menten fashion. Double reciprocal plots (not shown) indicate that the K_m for ATP and Vmax are 110 μM and 5.0 μm/second for the αβ subparticle, and 45 μM and 4.0 μm/second for the γ subparticle. The velocity in the β subparticles did not conform to Michaelis-Menten kinetics. Its maximal velocity appeared to be lower than 1 μm/second.

Effect of K-acetate concentration

We also examined the dependence of the microtubule translocation on the K-acetate concentration (Fig. 8). In previous experiments examining the sliding disintegration of *Chlamydomonas* axonemes, 50 mM K-acetate was found to be optimal for induction of microtubule sliding at high velocity (as high as the velocity of microtubule sliding in beating axonemes) (Kurimoto and Kamiya, 1991). In our in vitro motility assay experiment, increased K-acetate concentrations tended to prevent microtubules from attaching to the glass surface to which one of the three subparticles had been adsorbed. The concentrations of K-acetate that inhibited the attachment of microtubules on the surface differed with subparticles. Namely, the αβ subparticles held microtubules in the presence of up to 75 mM K-acetate, whereas the γ subparticles held microtubules only at ≤25 mM K-acetate, and the β subparticles were unable to hold them even in the absence of K-acetate.

To promote attachment of microtubules to the dynein-adsorbed glass surface, we followed the method of Uyeda et al. (1990), and added 0.2% methylcellulose to the reactivation buffer. In these samples, microtubule gliding was observed at

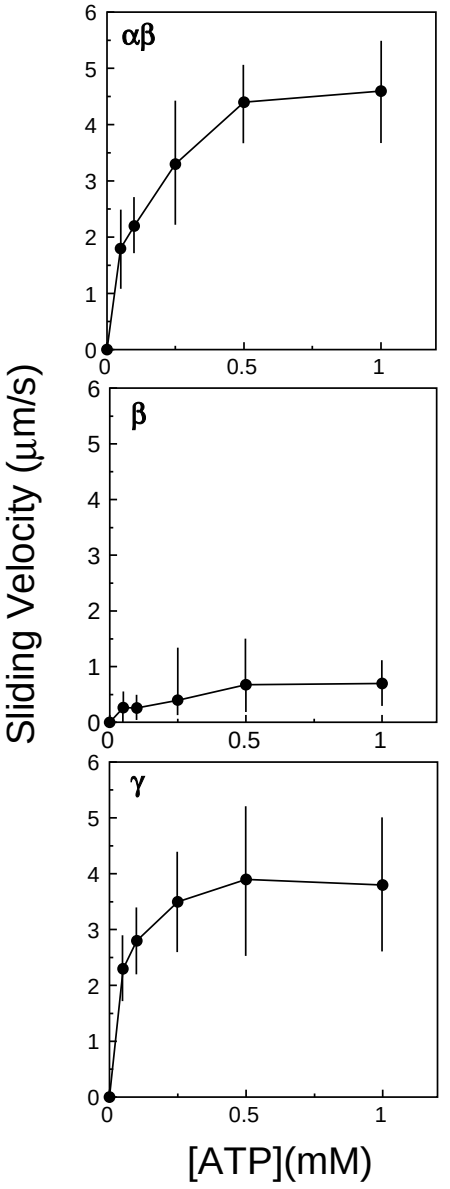

Fig. 7. ATP concentration dependence of microtubule translocation by the αβ, β and γ subparticles. Various concentrations of ATP and an ATP regeneration system (70 units/ml creatine phosphokinase and 5 mM phosphocreatine) were used. Conditions for motility assays were the same as described in legend to Fig. 6. Twenty-five translocating microtubules were measured at each concentration (error bars indicate the standard deviations).

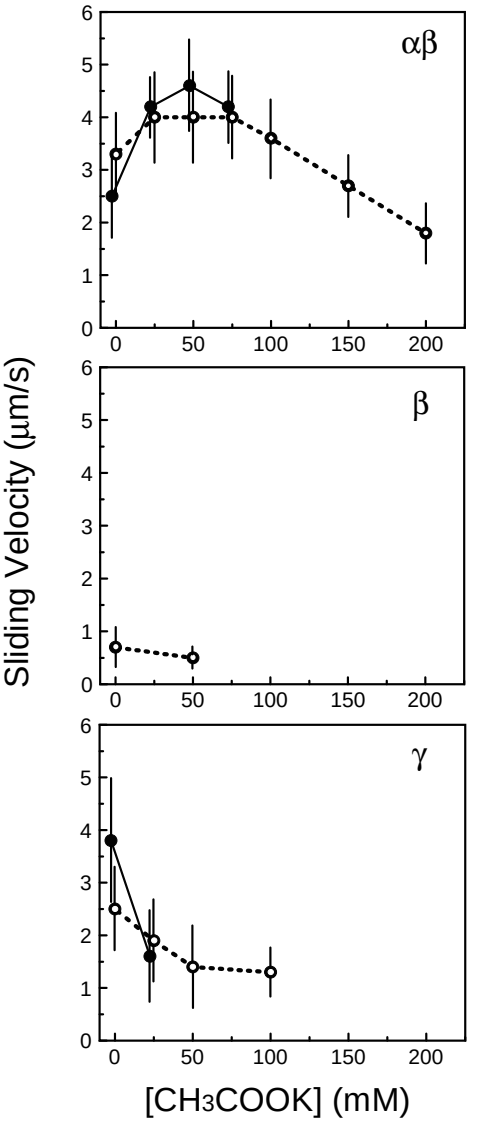

Fig. 8. Dependence of the translocation velocity on the potassium acetate concentration. Various concentrations of potassium acetate were added to HMDE solution plus 1 mM ATP with or without methylcellulose. (●) Without methylcellulose. (○) With methylcellulose. Twenty-five translocating microtubules were measured at each concentration (error bars indicate the standard deviations). Temperature: 23.0±1.0°C.

higher K-acetate concentrations (Fig. 8, open circle and broken line). Approximate concentrations of K-acetate up to which microtubule translocation was observed were 200 mM for the αβ subparticle, 100 mM for the γ subparticle, and 50 mM for the β subparticle. The velocity of microtubule translocation was essentially unchanged whether or not methylcellulose was present. In the β and γ subparticles, the velocity monotonically decreased with K-acetate concentration. In the αβ subparticle, the velocity showed a biphasic dependence, the maximum being at about 50 mM.

DISCUSSION

In this study, we have assayed the αβ, β and γ outer arm dynein subparticles of *Chlamydomonas* for their ability to bind and translocate microtubules in vitro. As described below, the use of *oda11* mutant lacking the α heavy chain and the improvement in the conditions for motility assay allowed us to detect the striking functional difference among different subparticles.

Isolation of outer arm dynein subparticles

The method we employed for isolation of dynein subparticles

took advantage of the dissociation of *Chlamydomonas* dynein into the αβ and γ subparticles upon high-salt extraction (Piperno and Luck, 1979; Pfister et al., 1982). Furthermore, the use of the mutant *oda11* lacking the α heavy chain enabled us to isolate the β subparticle. As an extension of the present study, we will also be able to purify and assay other outer arm subparticles. For example, if we use the mutant *oda4-s7* that has a truncated β heavy chain (Sakakibara et al., 1993), we will be able to isolate a dynein subparticle that contains the α heavy chain but not a functional β heavy chain. Also, Takada et al. (1992) have found that *Chlamydomonas* outer arm dynein, if isolated in the presence of Mg^{2+}, remains to form a three-headed particle containing all of the α, β and γ heavy chains. So, we may also be able to assay microtubule translocation by the three-headed particle. Comparison of the motility profiles of these outer arm preparations will be an interesting future subject.

Purification of dynein subparticles by MonoQ anion-exchange chromatography is rapid and provides good separation. An important advantage is that the γ subparticle of the outer-arm and inner-arm dynein subspecies are clearly separated (Goodenough et al., 1987). The separation of the γ subparticle from the inner subparticles has been difficult by sucrose density gradient centrifugation, because some inner arm subspecies, like the γ subparticle, sediment at 11-12S (Piperno and Luck, 1979). However, anion-exchange chromatography may have resulted in loss of minor components of dynein subparticles. For example, the γ subparticle used in this study has lost the 18 kDa light chain, which has been shown to be associated with the γ heavy chain and have a Ca^{2+}-binding activity (King and Patel-King, 1995). Though the γ subparticle maintained motor activity in our assays, it might have lost some functions such as Ca^{2+} regulations.

Binding of dynein subparticles to microtubules and tubulin

We have shown that each of the αβ, β and γ subparticles has both ATP-sensitive and ATP-insensitive binding sites for microtubules. A 78 kDa intermediate chain associated with the β heavy chain has been shown to bind to microtubules (King et al., 1991; Patel-King et al., 1995). Thus this intermediate chain may well be the site for the ATP-insensitive microtubule binding in αβ and β subparticles. In addition, the heavy chains of the three subparticles may also constitute part of the ATP-insensitive microtubule binding sites, since the α and β heavy chains of sea urchin sperm have been shown to bind to microtubules in an ATP-insensitive manner (Moss et al., 1992b; Mocz and Gibbons, 1993).

We have also shown that preincubation with tubulin dimer was necessary to induce microtubule translocation with both β and γ subparticles. We speculate that dimeric tubulin facilitated microtubule translocation by blocking the ATP-insensitive microtubule binding sites, which otherwise would act as drag against the active movement.

In vitro motility

We have shown that all of the αβ, β and the γ subparticles have the activity for microtubule translocation, but optimal conditions for microtubule translocation differed with different subparticles. With the αβ and γ subparticles, microtubule translocation was most readily observed on a silicone-coated glass surface. This is in contrast to the inner arm dynein of *Chlamydomonas* which showed good motility on a cleaned uncoated glass surface rather than a silicone-coated glass surface (Kagami and Kamiya, 1992; H. Sakakibara, unpublished observation). Since the αβ and γ subparticles did not show good motility on uncoated glass surface even at high protein concentrations, we speculate that the surface conditions may affect the orientation of dynein particles adsorbed on the surface.

Recently, Wilkerson and Witman (1995) also reported that both the αβ and the γ subparticles have motor activities. In their experiments, however, the αβ and γ subparticles translocated microtubules only at low ATP concentrations (<0.1 mM) and higher concentrations of ATP inhibited motility. In contrast, we have shown that all of the αβ, β and γ subparticles translocated microtubules at 1 mM ATP. A possible reason for this discrepancy may be the difference in the assay conditions, especially those of the glass surface; Wilkerson and Witman used cleaned glass slides, whereas we used silicone-coated glass slides. Since lower ATP concentrations have been known to favor microtubule sliding in axonemal disintegration (Lindemann and Gibbons, 1975; Tanaka and Miki-Noumura, 1988; Kurimoto and Kamiya, 1991), it is conceivable that axonemal dyneins may have an intrinsic tendency to display better motility at lower ATP concentrations, and this tendency may stand out under less favorable experimental conditions.

During sliding disintegration of the *Chlamydomonas* axoneme, the presence of 50 mM K-acetate was found to increase the sliding velocity (Kurimoto and Kamiya, 1991). In contrast, the use of K-acetate results in unstable microtubule translocation in in vitro motility assays of the inner arm dyneins (Kagami and Kamiya, 1992). In this study, we showed that the αβ subparticle translocated microtubules fastest at about 50 mM K-acetate but the β and γ subparticles did not hold microtubules stably and, consequently, displayed poor motility. The β and γ subparticles, however, were found to translocate microtubules at 50 mM K-acetate when the Brownian motion was suppressed by addition of methylcellulose. These dynein particles may well generate force efficiently in the axoneme, where the outer doublet microtubules are always located in the vicinity of these dyneins.

The three kinds of subparticles differed in the velocity of microtubule translocation as well as the optimal conditions for microtubule translocation. The maximal velocities were 5.0 μm/second with the αβ subparticle, 0.7 μm/second with the β subparticle, and 4.0 μm/second with the γ subparticle. These movements were significantly lower than that of the microtubule sliding in disintegrating axoneme, where velocities of 15-20 μm/second have been observed (Kurimoto and Kamiya, 1991). Since the sliding velocity in mutant axonemes that lack the outer arm was as low as 5 μm/second, Kurimoto and Kamiya (1991) suggested that the high velocity in axonemal disintegration was solely caused by the outer arm dynein. Velocities higher than 10 μm/second have actually been observed with the outer arm dynein of sea urchin sperm (Moss et al., 1992a) and *Tetrahymena* (Mimori and Miki-Noumura, 1994; Vale and Toyoshima, 1989). In the present in vitro motility study, however, the translocation velocity was not as high as the expected values. It may be that the high speed

was not realized because the experimental conditions, such as the conditions of the glass surface, were not optimal. Also, it may well be due to the random attachment of the dynein particles on the glass surface, as in the case of in vitro motility experiments with actin and myosin: randomly attached myosin molecules have been found to translocate actin filaments more slowly than those attached in a uni-directionally oriented manner (Iwane et al., 1997). Alternatively, it is also possible that the outer arm dyneins can translocate microtubules at a high velocity of 15-20 μm/second only when the three heavy chains cooperate, or when they cooperate with inner arm dyneins.

Differentiation in functional properties of different heavy chains

Among the three dynein subparticles studied here, the β subparticle is distinguished from the other two in its slow speed of translocation and different dependence on the K-acetate concentration. Since serious mutations in the β heavy chain have been found to cause an almost total loss of the activity of outer arm dynein (Sakakibara et al., 1993), the β heavy chain is considered essential to the function of outer dynein arm. The slow velocity observed in the present study prompted us to speculate that the β heavy chain may have a function more important than mere generation of force. For example, it may well participate in the regulation of force production of entire outer arms; this idea is supported by the fact that mutants with special types of mutations in the β heavy chain can function as a suppressor of the paralyzed phenotype of mutants defective in the central pair/radial spoke system (Huang et al., 1982; Porter et al., 1994).

The differentiation of functional properties among heavy chains is peculiar to axonemal dyneins (see Asai, 1995; Gibbons, 1995; Kamiya, 1995), while two heavy chains of cytoplasmic dynein are identical (Mikami et al., 1993; Neely et al., 1990). Studies with inner arm dynein subspecies indicate that different dyneins translocate microtubules at significantly different speeds of 1-12 μm/second (Kagami and Kamiya, 1992; Smith and Sale, 1991). It is puzzling that the axoneme equipped with multiple motor proteins with different intrinsic speeds can produce efficient movement (Kagami and Kamiya, 1992). The finding in this study that the three subspecies of outer arm dyneins translocate microtubules at different speeds also poses the question as to how different motors cooperate in the axonemal beating. Sliding between dyneins and microtubules should be highly regulated in the axonemal beating. Presence of motor proteins with various functional properties must be advantageous for the axonemal function. The reason for this remains to be elucidated.

For a further understanding of the function of each species of outer arm heavy chain, analysis of the force-velocity relationship of each dynein subparticle, as performed in disintegrating axonemes (Kamimura and Takahashi, 1981; Oiwa and Takahashi,1988) should be important. Detailed knowledge of the force production properties of all kinds of dynein heavy chains may enable us to envisage the mechanism for the coordination between different dyneins.

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