Translocation and rotation of microtubules caused by multiple species of Chlamydomonas inner-arm dynein

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Summary

Dynein was extracted from outer arm-less axonemes of the mutant oda1 and fractionated by high-pressure liquid chromatography on a MonoQ column into seven distinct subspecies (named a-g). Each subspecies contained one or two heavy chains and several medium-sized and light chains; by vanadate/UV-induced photocleavage and SDS-polyacrylamide gel electrophoresis, eight distinct heavy chains were identified. Analysis of the mutant axonemes indicated that the subspecies f (containing two heavy chains) is missing in the inner-arm mutant ida1 and the subspecies a, c and d are missing in the mutant ida4. Six subspecies (all but f) supported microtubule translocation with the maximal rate ranging from 2 to 12 µm s⁻¹ and the apparent Kₘ for ATP ranging from about 10 to 100 µM. All the subspecies translocated microtubules with the plus end leading, indicating that all the inner-arm dyneins are minus end-directed motors. Five subspecies (all but b and f) displayed microtubule rotation during translocation at rates of up to about 10 Hz. Unexpectedly, the Kₘ values for ATP for translocation and rotation did not always agree; because of this, the pitch of the movement was variable with some subspecies. These observations indicate that axonemes are equipped with several inner-arm subspecies and that torque generation is a feature common to many of them.

Key words: flagella, Chlamydomonas, inner-arm dynein, microtubule translocation, in vitro motility.

Introduction

Inner and outer dynein arms, force generators in cilia and flagella, have complex structures comprising as many as 10 protein subunits. Despite their similar appearances in axoneme cross sections, the two types of arms differ significantly in molecular composition and arrangement within the axoneme. The outer arms, located every 24 nm along the length of the outer doublet microtubules, consist of single dynein arms made up of two or three high molecular mass proteins and several intermediate and low molecular mass proteins (Piperno and Luck, 1979a; Pfister et al., 1982; Pfister and Witman, 1984). The inner arms are more complex. Piperno et al. (1990) suggested that the inner arms of Chlamydomonas are composed of three subforms (I1, I2 and I3), each with two high molecular weight proteins, and the unit of the three subforms is repeated every 96 nm along the doublet. Moreover, I2 and I3 species appear to contain different heavy chains depending on their position along a flagellum (Piperno and Ramanis, 1991). These authors have identified six inner-arm heavy chains in samples fractionated by sucrose density gradient centrifugation. The arrangement of inner arms may be more complex, however, because recent electron microscope studies on Chlamydomonas axonemes suggest that the inner arms are arranged not in a single straight row, but in a staggered row or two discrete rows (Kamiya et al., 1991; Muto et al., 1991; Burgess et al., 1991). Chlamydomonas mutants missing the entire outer arms can swim slowly (Kamiya and Okamoto, 1985; Mitchell and Rosenbaum, 1985), whereas those missing both I1 and I2 inner-arm subspecies are non-motile (Kamiya et al., 1989). Hence the inner arm seems to be important and sufficient for generating the axonemal undulating movement. What, then, do the different inner-arm subspecies do in the beating mechanism? We may be able to answer this question by isolating and characterizing mutants lacking specific inner-arm subspecies. Alternatively, we may be able to obtain important information by examining the functional properties of each inner-arm subspecies in an in vitro motility assay system, as first developed by Paschal et al. (1987a) and Vale and Toyoshima (1988). In a previous study, we examined various inner-arm subspecies for the ability to translocate microtubules in vitro (Kagami et al., 1990). Ion-exchange chromatography on a MonoQ column separated axonemal dynein from an outer arm-less mutant, oda1, into seven subspecies with ATPase activities, and three of them translocated microtubules at maximal speeds of 3-5 µm s⁻¹. In addition, one subspecies apparently had the ability to rotate microtubules, as has been observed with Tetrahym -
mena 14 S ciliary dynein (Vale and Toyoshima, 1988). Independently of our study, Smith and Salé (1991) reported that two Chlamydomonas inner-arm species purified by sucrose density gradient centrifugation (a 21 S particle containing I1, and an 11 S particle containing I2 and I3) had distinct activities for translocating microtubules in a similar in vitro system.

In this study, we further characterized the molecular composition and in vitro motility properties of oda1 dyneins. We show that each of the seven subspecies of dynein (named α-g) separated by chromatography contains one or two distinct heavy chains as major components and that the total number of distinct inner-arm heavy chains within the axoneme is eight, greater than the previously suggested number of six. Furthermore, all the subspecies, except f, translocated microtubules in vitro with maximal speeds ranging from 2 to 12 µm s\(^{-1}\), and five of them caused microtubule rotation during translocation. Thus the activity of rotating microtubules appears to be a property common to many subspecies of inner-arm dynein.

Materials and methods

Strains

Chlamydomonas reinhardtii 137c (wild type), a mutant missing the entire outer dynein arm (oda1), and two types of inner-arm mutants, ida1 (idaA type) and ida4 (idaB type), were used. Isolation and characterization of these mutants have been described (Kamiya and Okamoto, 1985; Kamiya, 1988; Kamiya et al., 1991).

Dynein extraction

Wild-type and mutant cells were grown on 1.5% agar plates containing Tris-acetic acid-phosphate (TAP) medium (Gorman and Levine, 1965) under continuous illumination. Twenty plastic dishes of 800 cm\(^2\) were used. After ten days of culture, cells on the plates were scraped off and suspended in 3 l of liquid TAP medium. The cell suspension was kept aerated overnight to induce 

Separation of dynein

Crude dynein extract was fractionated using a high-pressure liquid chromatography (HPLC) system (FPLC system, Pharmacia LKB, Uppsala). Solutions for chromatography were made up in water purified with a Milli-Q system (Nihon Millipore Ltd., Tokyo) and filtered through a 0.2 µm cellulose-acetate filter (Toyo Roshi Co., Ltd., Tokyo). All the procedures for chromatography were carried out at 4°C. A MonoQ HR5/5 analytical anion exchange column (Pharmacia LKB) was equilibrated with HMDE solution containing the proteinase inhibitors as above. The diluted extract was loaded at 0.5 ml min\(^{-1}\) using a 10 ml Superloop (Pharmacia LKB). The protein was then eluted with a linear 200 to 500 mM KCl gradient for a 95 min period at a flow rate of 0.5 ml min\(^{-1}\). Fractions of 0.5 ml were collected and assayed for in vitro motility and ATPase activity within 6 h of preparation.

Electrophoresis

The composition of dynein heavy chains was analyzed by SDS-PAGE using two different methods. The first was the method of Laemmli (1970), as modified by Jarvik and Rosenbaum (1980) and Pfister et al. (1982). A 3% to 5% polyacrylamide gradient and a 3 M to 8 M urea gradient were used. The other was the method of Neville (1971) as modified by Piperno et al. (1990). A 3.6% to 5% polyacrylamide gradient was used. No urea was contained in the gel. For analyzing the intermediate chains and low molecular weight chains, a Laemmli SDS-PAGE system with a 5% to 15% acrylamide gradient was also used. All gels were stained with silver (Blum et al., 1987).

Photocleavage of heavy chains

UV-induced photocleavage of dynein heavy chains was performed essentially after Gibbons et al. (1987). Portions of MonoQ fractions containing dynein heavy chains were diluted into HMDE solution containing ATP and vanadate, to bring the final concentration of KCl to 120 mM, ATP to 50 µM, and Na\(_3\)VO\(_4\) to 2 µM. Samples were irradiated for 1 h by an EN-280L UV lamp (Spectronics, New York) placed 10 cm above them, while being kept on ice and vigorously shaken every 5 min.

Preparation of tubulin and microtubules

Tubulin was prepared from porcine brain and separated from microtubule-associated proteins by phosphocellulose chromatography (Shelanski et al., 1973). Microtubules were prepared by polymerizing tubulin in assembly buffer (80 mM PIPES, 1 mM MgCl\(_2\), 1 mM EGTA, pH 6.9) in the presence of 1 mM GTP and 10% (v/v) dimethyl sulfoxide (DMSO) at 37°C. After 30 min of incubation, polymerization was terminated, and the microtubules were stabilized by diluting the sample 100-fold into HMDE solution containing 10 µM taxol (a generous gift from Dr. Matthew Suffness at National Cancer Institute).

In vitro motility assay

Each dynein fraction was examined for the activity of translocating microtubules at 23°C according to Vale and Toyoshima (1988). A perfusion chamber (volume: about 20 µl) was made of a glass slide, a cover slip (18 mm × 18 mm), and two cover slip strips as spacers. The chamber was first filled with a dynein fraction and left standing for 2 min. Proteins that were not adsorbed were removed by perfusing the chamber with 70 µl of HMDE solution containing 500 µg ml\(^{-1}\) bovine serum albumin (BSA). Microtubule translocation was then initiated by introducing HMDE solution containing taxol-stabilized porcine microtubules (about 5 µg ml\(^{-1}\)) and ATP (100 µM) into the chamber. When the ATP concentration-dependence of the motility was examined, various concentrations of ATP and an ATP-regenerating system (70 units ml\(^{-1}\) of creatine kinase and 5 mM phosphocreatine) were
used in place of the 100 µM ATP. Salts like KCl or K-acetate were not included in the medium because microtubule translocation was more stable without them: the presence of 50 mM KCl resulted in frequent interruption of movement. Inclusion of salts, however, may result in a higher maximum instantaneous speed. Microtubule translocation was observed and recorded with a dark-field microscope (BH-2, Olympus, Tokyo), equipped with a 100 W high-pressure mercury lamp (HB-101A, Ushio Inc., Tokyo), an SIT camera (C2400-08, Hamamatsu Photonics Co., Hamamatsu), and an S-VHS video recorder (HR-S6600, JVC, Yokohama). The video image was superimposed on the monitor of a personal computer (PC-9801 VX21, NEC, Tokyo), on which the velocities of translocating microtubules were measured using a home-made program. Because the velocity of microtubule translocation by a given sample of dynein depended on the ATP concentration in a manner consistent with Michaelis-Menten kinetics, we determined the apparent Michaelis constant ($K_m$) and the maximal velocity ($V_{max}$) by measuring velocities of 10-20 microtubules at each of five different concentrations of ATP (10, 20, 50, 100 and 500 µM). A method of least-squares with Taylor expansion (Sakoda and Hiromi, 1976) was used to determine the best-fit values for the $K_m$ and $V_{max}$.

**Determination of polarity of translocation**

The polarity of microtubule translocation was assayed by the method of Vale and Toyoshima (1988). Microtubules were grown selectively from the plus ends of axonemal outer-doublet microtubules, essentially by the method of Hyman et al. (1991). Briefly, axonemes that had been extracted with a high salt solution were suspended in HMDE solution containing 10 µM taxol, and disassociated into individual outer doublets and their fragments by repeated pipetting. Microtubules were grown from the plus end of the outer doublets by mixing 1 mg ml$^{-1}$ each of native tubulin, tubulin that had been modified with N-ethylmaleimide (NEM), and the doublet tubules. This mixture was incubated at 37°C in the presence of 1 mM GTP. After 15 min of incubation, the mixture was diluted 50-fold with HMDE solution containing 10 µM taxol. The microtubules grown from the outer doublets were applied to dynein-coated slides that were prepared as described above. The polarity of movement was examined at 100 µM ATP.

**Quantification of microtubule rotation**

The rotation of translocating microtubules was observed with microtubules that had been grown from outer-doublet fragments, as above. The rotation frequency was measured on a video monitor by observing the cyclic change in the position of the curved end of the doublet. The curved outer doublets occasionally blocked rotation when the end hit the glass surface. Since microtubules did not stop translocating even when the rotation was blocked, the pitch of the rotation (the distance of translocation during a 360 degree rotation) tended to be over-estimated. Thus, to quantify the rotation frequency, we took care to select microtubule outer doublet complexes with apparently unhindered rotation; as a rule, we selected microtubules that rotated constantly for more than three successive rotations. The direction of microtubule rotation was determined by focussing the objective slightly above the surface of the glass slide, at the highest plane where the curved end of the outer doublet came into focus during rotation. The rotation direction was also inferred from the image of translocating microtubules whose rotation was hindered because the end of the curved outer doublet hit the glass surface; in such a microtubule, the doublet end was always positioned on a particular side with respect to the microtubule axis. The direction of rotation determined by varying the focusing plane always agreed with that inferred by the latter method.

**ATPase activity and protein determination**

ATPase activity in each fraction was measured using the sensitive colorimetric assay of Kodama et al. (1986). The ATP hydrolysis reaction was performed in HMDE solution containing 0.5 mM ATP and 10-fold diluted fractions. After incubation for 20 min at 23°C, the reaction was stopped by addition of 0.6 M perchloric acid. Protein concentration was determined with BioRad Bradford reagent using BSA as a standard.

**Results**

**Fractionation of odal dynein**

HPLC on a MonoQ column, as first used by Goodenough et al. (1987) in dynein fractionation, separated the high-salt extract of odal axoneme into seven distinct subspecies, a-g, containing one or two heavy chains (Fig. 1). The profile of elution was essentially the same as reported earlier (Kagami et al., 1990). However, the peaks were not as well separated as in the previous experiment; this was apparently due to the presence of Mg$^{2+}$, which was included in this experiment because it resulted in better motility.

Fractions a-g were selected for further study following SDS-PAGE analysis of all of the HPLC fractions. For analysis of the heavy chain compositions in these subspecies, we used two different SDS-PAGE systems with and without a urea gradient. In the SDS/urea-PAGE pattern, each subspecies had one or two bands of heavy chains, with six bands in total (Fig. 2A). We called these bands numbers 1-6, as in our previous study. No heavy chain bands other than these were found in any of the whole HPLC fractions. The other type of SDS-PAGE, performed after Piperno et al. (1990), also separated the total heavy chains into bands with six different mobilities (Fig. 2B). If we assume that the order of mobility in this system is the same as that of Piperno et al. (1990), the six bands should correspond to the heavy chains that they called “1α”, “2β”,

![Fig. 1. Fractionation of high-salt extract from odal axonemes by HPLC on a monoQ column. Solid line, absorbance at 280 nm. Open circles, ATPase activities (pmoles phosphate min$^{-1}$ ml$^{-1}$). Broken line, KCl concentration. a-g, peak fractions used as inner-arm subspecies.](image-url)
In the fractionated samples, the heavy chains in subspecies $a$ and $d$ had the same mobility as that of the putative "2". Those in other two subspecies, $c$ and $e$, had the same mobility as that of the putative "2". The heavy chains in subspecies $c$ and $e$, however, had different mobilities (numbers 5 and 2) in the SDS/urea-PAGE pattern. Similarly, the heavy chain in $e$ and one of the two chains in $f$ (number 2) had the same mobility in SDS/urea-PAGE but had different mobilities in SDS-PAGE performed in the absence of urea. The heavy chains in $c$, $e$ and $f$ can be thus distinguished by their different mobilities in these two PAGE systems.

For further differentiation of these heavy chains, those in each subspecies were photocleaved by UV irradiation in the presence of ATP, Mg$^{2+}$ and vanadate (Gibbons et al., 1987). SDS-PAGE analyses of irradiated and untreated samples indicated that such treatment cleaved each heavy chain into two fragments (Fig. 3; indicated by arrows), giving rise to two (all but $f$) or four bands ($f$) in the range $150-250 \times 10^3$ $M_r$. The two fragments derived from cleavage of a heavy chain differed in mobility among different subspecies. Most notably, the heavy chains in subspecies $a$ and $d$, which showed identical mobility in the above two SDS-PAGE systems, generated two pairs of fragments with different mobilities; the larger product in $a$ was smaller than that in $d$, whereas the smaller product in $a$ was larger than that in $d$.

These cleavage patterns are best accounted for by assuming an intrinsic difference in the position of the vanadate-binding site between these heavy chains. Together with SDS-PAGE analyses, the photo-cleavage experiment thus strongly suggests that the heavy chains in subspecies $a$-$g$ are different. This means that the total number of inner-arm heavy chains should be eight, greater than the previously reported number of six (Piperno et al., 1990; Piperno and Ramanis, 1991). We therefore modified the names of the heavy chains so as to distinguish those displaying identical mobilities in SDS/urea-PAGE; those contained in subspecies $a$ and $d$ were named $3a$ and $3b$, and those in $e$ and $f$ were named $2a$ and $2b$ (Table 1).

**Dynein from inner-arm mutants**

Recently we isolated two types of mutants that lack part of the inner arms (Kamiya et al., 1991). These two types (called idaA and idaB) lack different sets of two heavy-chain bands in SDS/urea-PAGE patterns, as well as discrete parts of inner-arm images in cross-section electron micrographs of the axoneme. Both types can swim, albeit more slowly than the wild type. Piperno has found that an idaA-type mutant (pf30) lacks I1, and an idaB-type (ida4) lacks I2 (Piperno et al., 1990; see also Kamiya et al., 1991).

To see which components are missing in these mutants, we examined high salt extracts from axonemes of ida1 (idaA-type; allelic with pf30) and ida4 (idaB-type) by MonoQ chromatography followed by SDS/urea-PAGE. Fig. 4 compares the elution profiles between the wild type and mutants. Unlike the profile of oda1 axoneme, the profiles of the wild type and inner-arm mutants have prominent peaks due to elution of outer-arm subparticles containing the $\gamma$ or the $\alpha$ and $\beta$ heavy chains. Among the other peaks, peak $f$ is absent in ida1 and peaks $a$, $c$ and $d$ are absent in ida4. SDS/urea-PAGE analysis of the corresponding fractions revealed that ida1 (Fig. 5, second panel) lacks heavy chains 1 and 2b, which are present in oda1 (bottom panel) in subspecies $f$. Similarly, ida4 (third panel) lacks heavy chains $3a$, $5$ and $3b$, which are present in oda1 in subspecies $a$, $c$ and $d$, respectively. (In ida4, a weak band is present at the position of heavy chain 3 in peak fractions $a$ and $d$; this is most likely the outer-arm heavy chain $\gamma$ because it did not occur in corresponding fractions from the double mutant oda1 $\times$ ida4 (data not shown).) Thus, four out of the seven subspecies were missing in either of the inner-arm mutants. Some of the remaining species, $b$, $e$ and $g$,
may constitute third inner-arm species, I3, reported by Piperno et al. (1990). However, it is possible that some of them do not actually originate from the inner arms, since in no mutant has the lack of the putative I3 components been correlated with a clear morphological defect in the inner arm.

**Light and medium-sized chain composition**

The inner-arm dynein subspecies were analyzed also for their medium and light chains (Fig. 6). SDS-PAGE showed that each MonoQ fraction of the *oda* extract contained many small and intermediate-sized peptides in addition to heavy chains (Fig. 6A), and it was thus difficult to determine which peptides are true dynein components. Repeated experiments with mutant axonemes, however, have suggested that several peptides were consistently eluted together with particular heavy chains. Table 1 summarizes such peptides.

A 97 \( \times 10^{3} \) peptide and two peptides with similar mobilities of about 140 \( \times 10^{3} \) are contained in subspecies \( f \) of *oda* (Fig. 6A) and *ida* (Fig. 6C), but are missing in *ida* that lacks I1 (Fig. 6B). Thus these peptides must be components of I1. This observation agrees with the previous report that a 97 \( \times 10^{3} \) peptide (Smith and Sale, 1991)

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**Table 1. Peptide composition of *oda* dynein**

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>a*</th>
<th>b</th>
<th>c*</th>
<th>d*</th>
<th>e</th>
<th>f†</th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner arm species‡</td>
<td>12</td>
<td>13(?)</td>
<td>12</td>
<td>12</td>
<td>12(?)</td>
<td>11</td>
<td>13(?)</td>
</tr>
<tr>
<td>Terminology, after Piperno et al. (1990)</td>
<td>3a</td>
<td>6</td>
<td>5</td>
<td>3b</td>
<td>2a</td>
<td>1, 2b</td>
<td>4</td>
</tr>
<tr>
<td>Intermediate and light chains (( M_r \times 10^{-3} ))</td>
<td>228</td>
<td>192</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(97)</td>
<td>140</td>
<td>97</td>
<td></td>
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<tr>
<td></td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>44</td>
<td>42</td>
<td></td>
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<tr>
<td></td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>38</td>
<td>28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Missing in mutant *ida*.
†Missing in mutant *oda*.
‡Tentative assignment of inner arm species (Piperno et al., 1990) to which each species belongs.
and a 140 × 10^3 M_r chain (Piperno et al., 1990) are components of II.

In extracts from both _oda1_ and _ida1_, a 42 × 10^3 M_r peptide is present in all the subspecies but _f_, a 28 × 10^3 M_r peptide is present in _a_, _c_, and _d_, and 38 × 10^3 and 44 × 10^3 M_r peptides exist in _d_. In the _ida4_ extract, the 42 × 10^3 M_r peptide is missing in fractions corresponding to _c_ and _d_ subspecies, and the 28 × 10^3, 38 × 10^3 and 44 × 10^3 M_r peptides are totally absent (Fig. 6C). In addition, the 42 × 10^3 M_r peptide present in _a_ seems to be different from those missing in _c_ and _d_, because the former occasionally had a slightly smaller mobility (data not shown). It is thus likely that a 42 × 10^3 M_r peptide is commonly missing from _a_, _c_ and _d_. On the basis of molecular mass, this peptide must be actin, which has been identified as a component of inner-arm dynein (Piperno and Luck, 1979b). The observation that actin is missing in these three fractions in _ida4_ agrees with the report that the I2 inner arm contains actin (Piperno et al., 1990). (The presence of a small amount of actin in fraction _f_ of this mutant is due to its incomplete separation from fraction _g_.)

Piperno et al. (1990) reported that an 11 S inner-arm dynein fraction, a mixture of I2 and I3 species, contains actin, and peptides of 19 × 10^3, 28 × 10^3 and 133 × 10^3 M_r. The 19 × 10^3 M_r peptide has been identified as centrin (caltractin), a calcium-binding protein (Huang et al., 1988; Salisbury et al., 1988; Piperno et al., 1990). Although partially agreeing with the report of Piperno et al. (1990), our present findings differ in several points. First, we were unable to detect 19 × 10^3 and 133 × 10^3 M_r proteins associated with any subspecies; these proteins may have been lost during chromatography. Second, we found that subspecies _d_ of _oda1_ or _ida1_ contains 38 × 10^3 and 44 × 10^3 M_r peptides that have not been previously identified as inner-arm subunits. Third, 192 × 10^3 and 228 × 10^3 M_r proteins were observed to be coeluted with heavy chain 4 in many independent gels. These chains thus appear to be components of subspecies _g_, although a definite conclusion must await further studies using samples purified further.

**Microtubule translocation in vitro**

We next examined the fractionated _oda1_ dynein for the activity of translocating microtubules in vitro. Each MonoQ fraction was introduced into the observation chamber and left standing to effect adsorption of dynein onto the glass surface. The chamber was then perfused with a buffer solution containing 0.5 mg ml⁻¹ of BSA. The use of BSA increased the number of microtubules displaying translocation, possibly because it prevented microtubules from sticking to the glass surface directly, or facilitated adsorption of dynein in the right orientation (see Howard et al., 1989).

When microtubules and ATP were introduced into a chamber that had been perfused with fractions containing any one of the inner-arm heavy chains, microtubules became stuck to the glass surface. In addition, they underwent active translocation except when subspecies _f_ was used. The number of subspecies displaying a microtubule-translocating activity thus increased from the three in the previous study (Kagami et al., 1990) to six; this is probably because of the improvement in protocols for fractionation and motility assay. When dynein fractions from _ida4_ lacking _a_, _c_ and _d_ subspecies were examined in place of _oda1_ fractions, subspecies _b_, _e_ and _g_ displayed motility whereas the other fractions did not (data not shown). This indicates that the motility observed with _b_ and _e_ was not caused by the presence of small amounts of other sub species in these fractions. Judging from the purity of each fraction and the velocity profile in Fig. 7 (see below), it seems highly unlikely that the microtubule translocating activity observed with any one of the six subspecies is due to contamination by other subspecies.

Fig. 7 shows the velocity of microtubule translocation caused by each _oda1_ fraction in the presence of 100 µM ATP. The velocity profile has several peaks coinciding with the elution profile except for that of subspecies _f_. When the concentration of the dynein heavy chain was low, as in fractions 19-21, 31 or 38-40, microtubules tended to float in the medium, resulting in lower gross translational speeds. With fractions 26 and 32-37, microtubules did not become adsorbed to the glass surface.

The velocity of microtubule translocation caused by each subspecies varied with the ATP concentration in a manner consistent with Michaelis-Menten kinetics; in each case, double reciprocal plots of these values yielded a fairly straight line (for example, see Fig. 9A and C). Table 2 summarizes the maximal velocity (V_max) and the apparent Michaelis constant (K_M) for each species, estimated from
data obtained in three independent experiments using different preparations of sample. These data show that the velocity and $K_m$ values are reasonably reproducible with subspecies $b$, $c$, $d$ and $g$ but somewhat variable with $a$ and $e$. The variability with the latter subspecies appeared to have originated mainly from their lower stability than those of other species; upon storage, they lost the activities for microtubule translocation more quickly than other sub-
species. It is clear, however, that different subspecies differed in \( K_m \) values for ATP as well as in maximal velocities. These results suggest a functional heterogeneity among different inner-arm subspecies.

To determine the direction of the microtubule translocation, we used microtubules grown exclusively at the plus ends of outer-doublet fragments. All the dynein subspecies that displayed motility were found to translocate microtubules with their plus ends leading and the doublet tubules at the minus end trailing. Hence, all these dyneins are minus-end-directed motors, as has been reported with other kinds of dynein.

**Rotation of microtubules**

In the previous study (Kagami et al., 1990), we observed that the subspecies \( g \) caused both translocation and rotation of microtubules, as has been observed with *Tetrahymena* 14 S dynein (Vale and Toyoshima, 1988). Rotation was evident when the microtubule had a kink or a small particle attached. While examining the polarity of translocation using microtubules grown from curved outer doublet fragments, we found that rotation actually occurred with five dynein subspecies, \( a, c, d, e, \) and \( g \), but not with subspecies \( b \) and \( f \). The direction of microtubule rotation was always clockwise when viewed from the minus end of the outer doublet, i.e., the same as that of the rotation observed with *Tetrahymena* 14 S dynein.

The microtubule-rotating activities of subspecies \( c \) and \( g \) were stable, and we were able to measure the rotation frequency at various ATP concentrations over a period of about 1 h. In subspecies \( c \), the rotation frequency increased in an almost linear fashion with the translocation velocity (Fig. 8A). In subspecies \( g \), however, the rotation frequency was not simply proportional to the translocation velocity (Fig. 8C). Hence, the pitch of the movement is almost constant with subspecies \( c \) (0.4-0.5 \( \mu \)m; Fig. 8B) but not with subspecies \( g \) (Fig. 8D). In the latter case, the pitch increases linearly from about 0.2 to 0.5 \( \mu \)m, with increasing translocation velocity (Fig. 8D). The pitch varies because the apparent \( K_m \) for ATP for translocation and rotation differs: 31.5 (± 0.4-0.5 \( \mu \)m) \( K_m \) for translocation velocity and 10.6 (± 2.1) \( K_m \) for rotation frequency (Fig. 9C and D). In contrast, the two kinds of movements caused by the \( c \) subspecies had almost identical \( K_m \) values: 79.4 (± 11.5) \( K_m \) and 67.4 (± 9.1) \( K_m \) (Fig. 9A and B).

It was possible that the variable pitch observed with subspecies \( g \) was somehow caused by its incomplete separation from subspecies \( f \). To check this possibility, we examined subspecies \( g \) fractionated from dynein of the mutant ida1 that lacks subspecies \( f \). The subspecies \( g \) from ida1, however, displayed the same tendency as observed with that from oda1; i.e., the pitch of the movement by this subspecies also varied between 0.2 and 0.6 \( \mu \)m (data not shown). In this case, measurements on 100 translocating microtubules yielded \( K_m \) values of 45.8 (± 6.8) \( K_m \) for translocation and 5.4 (± 1.3) \( K_m \) for rotation. These observations indicate that the variable pitch in microtubule translocation is caused by an intrinsic property of subspecies \( g \).

### Table 2. Microtubule translocation caused by different oda1 dynein subspecies

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>( a )</th>
<th>( b )</th>
<th>( c )</th>
<th>( d )</th>
<th>( e )</th>
<th>( f )</th>
<th>( g )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Velocity (s.d.) at 100 ( \mu )M ATP (( \mu )m s(^{-1} ))</td>
<td>5.7 (0.2)</td>
<td>2.5 (1.2)</td>
<td>6.7 (1.4)</td>
<td>5.7 (1.5)</td>
<td>4.3 (1.1)</td>
<td>–</td>
<td>3.9 (0.8)</td>
</tr>
<tr>
<td>( V_{max} ) (s.d.) (( \mu )m s(^{-1} ))</td>
<td>7.6 (2.3)</td>
<td>2.4 (0.1)</td>
<td>12.1 (2.7)</td>
<td>8.0 (0.1)</td>
<td>6.0 (0.3)</td>
<td>–</td>
<td>5.4 (0.1)</td>
</tr>
<tr>
<td>( K_m ) (s.d.) (( \mu )M ATP)</td>
<td>51 (24)</td>
<td>8 (5)</td>
<td>70 (18)</td>
<td>45 (8)</td>
<td>28 (7)</td>
<td>–</td>
<td>31 (5)</td>
</tr>
<tr>
<td>Rotation</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Data from three independent experiments with different preparations of dynein are listed. \( K_m \) and \( V_{max} \) were obtained as best-fit values for Michaelis-Menten kinetic constants for the ATP-dependent variation of the velocity of microtubule translocation. Velocities of 10–20 microtubules were measured at each of 10, 20, 50, 100 and 500 \( \mu \)M ATP.
Fig. 8. Relationship between microtubule rotation and translocation induced by subspecies c (A and B) and subspecies g (C and D) of oda1. ATP concentration was varied between 10 µM to 200 µM. A and C show the relationship between rotation frequency and translocation velocity. Solid lines are the theoretical hyperbolic curves calculated from the $K_m$ and $V_{max}$ values obtained from a least-square analysis of data (see Fig. 9 legend). B and D show the relationship between the pitch of movement (translocation distance during one rotation) and the velocity of translocation. Solid lines are the theoretical linear lines calculated from the best-fit $K_m$ and $V_{max}$ values as used above. Each point represents a measurement on a single microtubule. Assay conditions are described in Materials and methods.

Fig. 9. Double reciprocal plots of the translocation velocity (A and C) and rotation frequency (B) against ATP concentrations. A and B, subspecies c; C and D, subspecies g. Data were from the experiments shown in Fig. 8. Each point is an average of 10-20 measurements with a standard deviation. The $K_m$ and $V_{max}$ were determined as best-fit values by a least-square analysis of all the data assuming Michaelis-Menten kinetics; the $K_m$ and $V_{max}$ in c are 79.4 (± s.d. 11.5) µM (ATP) and 4.4 (± 0.1) µm s$^{-1}$ for translocation and 67.4 (±9.1) µM and 3.4 (± 0.1) Hz for rotation; those in g are 31.5 (± 5.3) µM and 3.4 (± 0.1) µm s$^{-1}$ for translocation and 10.6 (± 2.1) µM and 7.9 (± 0.1) Hz for rotation. The linear lines in A-D are linear regression lines for the five average data points at different ATP concentrations. The apparent $K_m$ values yielded by these lines are somewhat different from the above best-fit values: 104 µM(A); 102 µM(B); 28 µM(C); and 11 µM(D).

Discussion

Presence of eight discrete inner-arm heavy chains

We have shown that MonoQ chromatography can separate the axonemal dynein from oda1 into seven subspecies (a-g) each with one or two major heavy chains. SDS-PAGE, performed in the presence and absence of a urea gradient, and vanadate/UV-induced photocleavage, revealed the presence of eight different heavy chains. Piperno et al. (1990) have reported that their SDS-PAGE system, on which one of our SDS-PAGE systems was based, resolved six inner-arm heavy chains, and called them “1α”, “1β”, “2”, “2′”, “3”, and “3′”. These data and those from electron microscopy on mutant axonemes have led them to suggest that Chlamydomonas inner arm dynein comprises three distinct species I1 (containing “1α” and “1β”), I2 (containing “2” and “2′”) and I3 (containing “3” and “3′”) (see Introduction). Piperno has further found that an ida1 allele pf50 lacks “1α” and “1β” whereas ida4 lacks heavy chains “2′” and has a reduced amount of “2” (Piperno et al., 1990; personal communication cited by Kamiya et al., 1991). Our present findings that ida1 lacks subspecies f and ida4 lacks subspecies a, c, d agree with his findings since subspecies f contains the putative “1α” and “1β”, while a and d contain the putative “2′” (3a and 3b, in our terminology) and c contains the putative “2” (5). Our analyses, however, suggest that “2′” may correspond to two different heavy chains 3a and 3b, and “2” may correspond to 2a and 5. Since subspecies containing these different heavy chains occurred constantly in many independent chromatography runs except when ida4 was used, we believe that these heterologous heavy chains are present in native axonemes.

The three subspecies a, c and d missing in ida4 are similar to each other in that they have similar light chain compositions. Piperno and Ramanis (1991) have recently suggested that the inner-arm dyneins differ in composition depending on the position along the axonemal length; although both “2” and “2′” heavy chains constitute inner arm I2 and both “3” and “3′” constitute I3. “2′” occurs only in the distal portion and “3′” only in the proximal portion. By analogy, the two “2′” heavy chains in a and d (3a and 3b) may occur at different positions within an axoneme. Together with the “2′” heavy chain in c (5), both of the “2′” chains may constitute the I2 group. The subspecies e also has a putative “2′” heavy chain, and thus may be regarded as belonging to I2. In agreement with this idea, we have recently isolated a new inner-arm mutant (ida5) that lacks subspecies e in addition to lacking a, c and d (T. Kato, O. K. and R. K., unpublished result); this indicates that the
subspecies e is related to other subspecies that apparently constitute I2. Taken together, our present findings suggest that the composition and arrangement of the inner arm I2 is more complex than has been suggested.

Different inner arm subspecies translocate microtubules at different speeds

The present study has demonstrated that all but the f subspecies of the oda1 dynein have the activity to cause microtubule translocation in vitro. The translocation was found to proceed always with the same polarity such that the plus end of a microtubule was leading. Thus the inner-arm dynein subspecies that displayed in vitro motility are minus end-directed motors, as are the outer arm dynein of sea urchin sperm (Paschal et al., 1987b) and Tetrahymena (Vale and Toyoshima, 1988) and cytoplasmic dynein (Paschal et al., 1987a). This polarity agrees also with that in the sliding of outer doublets in outer arm-depleted axonemes (Fox and Sale, 1987). We cannot, however, rule out the possibility that some inner-arm subspecies may translocate microtubules in the opposite direction under certain conditions, as demonstrated with a dynein-like motor protein from Reticulomyxa (Schliwa et al., 1991).

The maximal velocities of translocation ranged from 2 \( \mu \text{m s}^{-1} \) of subspecies b to 12 \( \mu \text{m s}^{-1} \) of subspecies c. The latter maximal velocity is higher than the maximal sliding velocity measured in disintegrating oda1 axoneme (about 5.3 \( \mu \text{m s}^{-1} \); Kurimoto and Kamiya, 1991), and also higher than the one estimated from the waveform of beating oda1 axoneme (4.5-7 \( \mu \text{m s}^{-1} \), calculated from Brokaw and Kamiya, 1987; see Kurimoto and Kamiya, 1991). Recently, we have measured the velocities of outer doublet sliding in disintegrating axonemes of the wild type and mutants lacking either outer arms or part of the inner arms, and reached a conclusion that inner and outer arms are motors that differ intrinsically in their maximal speeds; the outer arm apparently causes five times faster sliding than the inner arm under load-free conditions (Kurimoto and Kamiya, 1991). This result is puzzling, because it is not easy to understand how two kinds of motors with different speeds can work together efficiently within an axoneme. The present finding that different inner-arm subspecies differ greatly in intrinsic speeds poses the same puzzling question for inner-arm dyneins.

Among the subspecies that displayed microtubule translocation, b has characteristics somewhat different from those of other subspecies. It has the lowest \( V_{\text{max}} \) (about 2 \( \mu \text{m s}^{-1} \)) and \( K_m \) (10 \( \mu \text{M ATP} \)) values among all the motile subspecies (Table 2), and it is the only subspecies without the microtubule rotating activity. These observations suggest that subspecies b may perform a unique function in axonemal movement. Piperno and Ramanis (1991) found that the heavy chain “3”, corresponding to the subspecies b heavy chain, was missing in short (< 6 \( \mu \text{m} \)) flagella. Furthermore, they showed that it is located in the proximal portion of a flagellum, and suggested that the heavy chain “3” is involved in the initiation of bend formation that is necessary for the axonemal beat. Although this hypothesis needs to be examined further, it is interesting that such a hypothetical “bend initiator” has distinct in vitro motility characteristics.

Subspecies f, corresponding to I1 dynein, did not display microtubule translocation in this and previous studies (Kagami et al., 1990). Smith and Sale (1991), on the other hand, reported that I1 purified by sucrose density gradient centrifugation caused microtubule translocation at a low velocity (0.76 \( \mu \text{m s}^{-1} \) in 1 mM ATP) in an in vitro assay system. The reason for the difference between their result and ours is not clear. It may be that the I1 dynein purified by ion-exchange chromatography lacks some components essential for its activity. Alternatively, the I1 dynein obtained by sucrose density gradient centrifugation may contain other species of dynein. It is also possible that the poor motility in subspecies f is simply due to its inappropriate adsorption to the glass surface. The situation with subspecies f is similar to that of the Chlamydomonas outer-arm dynein; both dynein species usually do not display good motility in vitro while their absence in vivo leads to reduction of motility, as can be seen from mutants missing them (Brokaw and Kamiya, 1987; Kamiya et al., 1989). Interestingly, all the inner-arm subspecies that displayed good in vitro motility contain actin as a light chain, while subspecies f and outer-arm dynein do not. Whether this correlation is a mere coincidence must await further studies.

Torque generation by inner-arm dyneins

We have found that all but the b and f subspecies of solubilized oda1 dyneins have activities for rotating microtubules as well as translocating them. Torque generation thus appears to be a property common to many subspecies of inner-arm dynein. Microtubule rotation by dynein was first observed with Tetrahymena 14 S dynein, although its location within the axoneme was not clear (Vale and Toyoshima, 1988). Judging from the similar in vitro behavior, the 14 S dynein may also be an inner-arm dynein.

Unexpectedly, subspecies g displayed different ATP-concentration dependencies for rotation and translocation, whereas subspecies c showed similar dependencies for the two kinds of movements. Because of these features, the rotation frequency and the translocation speed were proportional to each other in c, but not in g; in other words, the movement caused by c had a constant pitch (0.4-0.5 \( \mu \text{m} \)), as observed with Tetrahymena 14 S dynein (pitch about 0.54 \( \mu \text{m} \); Vale and Toyoshima, 1988), but the movement by g had a variable pitch ranging between 0.2 and 0.5 \( \mu \text{m} \). This behavior was also observed in the same subspecies from the mutant ida1 that lacks subspecies f; the pitch was observed to vary between 0.2 and 0.6 \( \mu \text{m} \).

Since the sample of subspecies g of oda1 contained only one kind of heavy chain except for a small amount of heavy chains of f which did not display in vitro motility by itself, and that of ida1 contained almost no other heavy chains (Fig. 5), it is likely that the presence of distinct \( K_m \) values for rotation and translocation is due to the nature of a single motor protein of g. One possible explanation is that this dynein subspecies has two distinct activities of translocating and rotating microtubules with more than one \( K_m \) for ATP. This idea is not entirely unlikely since the recently determined primary structure of an outer-arm heavy chain indicates it has four or five ATP binding sites (Gibbons et al., 1991; Ogawa, 1991). A second, probably more plausi-
ble, explanation is that the occurrence of distinct $K_m$ values is due to the effect of dynein arms that bind strongly to microtubules. Like myosin bound to actin in the absence of nucleotides, dynein becomes strongly bound to microtubules when no ATP or ADP plus $P_i$ is bound to it (Johnston, 1983), and such a strong (“rigor”) cross-bridge works as a drag that retards the translocation and rotation of microtubules. The degree of retardation may differ for the two kinds of movements if the strength of the “rigor” cross-bridges differs depending on the direction of applied force. Because the number of dynein arms in the strongly bound state varies with the ATP concentration, a change in the concentration should affect the translocation and rotation to different extents, resulting in different apparent $K_m$ values. If this is true, the difference in $K_m$ must reflect the mechanical anisotropy in the dynein cross-bridges.

The significance of the torque generation by inner-arm dynein in the axonemal beating mechanism is unknown. Since our studies on inner-arm mutants have indicated that the inner arms play an important role in causing the axoneme to beat, and since torque generation is a property common to many inner-arm subpecies, torque generation may play a crucial role in the beating mechanism. Simple geometric considerations suggest that the torque produced by inner arms has the function of modulating the position of an outer doublet relative to the adjacent one. In particular, the torque may change the distance between outer arms and adjacent microtubules. Such modulation in position may well control the interaction of outer arms or some inner arms with the outer doublet.

The presence of heterologous inner-arm species with different mechano-chemical properties may be important for an axonemal beat that must require controlled generation of force. Further studies correlating the in vitro motility of various dynein species and the motility of mutant axonemes lacking them should make clear how different dynein species cooperate in producing the axonemal beat.

This study has been supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan (01657001, 02239101, 03223101).

References


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(Received 29 April 1992 - Accepted 10 August 1992)