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C/A dynein isolated from sea urchin sperm flagellar axonemes

Enzymatic properties and interaction with microtubules

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SUMMARY

C/A dynein is a novel dynein isolated from sea urchin sperm flagellar axonemes. It is composed of C and A heavy chains and some additional lower molecular mass polypeptide chains. The characterization of ATPase activity and the interaction of this dynein with microtubules polymerized from calf brain tubulin were investigated in this study. The ATPase activity of C/A dynein (0.3-0.4 μmol P_i/min per mg) was about one half that of outer arm 21 S dynein (0.6-0.8 μmol P_i/min per mg) at 25°C. Vanadate inhibited the ATPase activity with a half-maximal inhibition at 1 μM. C/A dynein adsorbed to the glass surface was able to translocate the microtubules towards its plus end. The velocity of the microtubule movement in the presence of 1 mM ATP was 4.0 to 4.5 μm/s at 22°C. C/A dynein binds to and bundles the microtubules even in the presence of ATP. Cross-bridges were found between adjacent microtubules in the bundle with an axial periodicity of about 24 nm. The ATPase activity of C/A dynein was enhanced up to several-fold by the microtubules at concentration as low as 1 mg/ml. On the other hand, 21 S dynein bound to the microtubules with 24 nm axial periodicity only in the absence of ATP. Its ATPase activity was not activated by the microtubules. From these results, it is concluded that the manner of interaction with microtubules of C/A dynein is different from that of the outer arm dynein.

Key words: dynein, inner arm, outer arm, microtubule, sperm, flagella, sea urchin

INTRODUCTION

In the cilia and flagella, the outer and inner arms cause active sliding between the outer doublet microtubules (Gibbons, 1981). Both arms are composed of force-generating protein assemblies called dyneins. Dyneins are complex proteins comprising several high molecular mass polypeptides (heavy chain) of more than 400 kDa and several additional polypeptides called intermediate and light chains (Witman, 1989). The outer arms appear to be homogeneous in the structure: only one type of dynein molecule (arms) is arranged on the outer doublet microtubules with 24 nm periodicity (Goodenough and Heuser, 1989). The biochemical and structural properties of outer arm dynein have been well characterized. The outer arm consists of two or three heavy chains, two or three intermediate chains and several light chains (Johnson, 1985; Witman, 1989). The molecule appears as a bouquet-like structure in which globular head-like domains of the heavy chains are connected by stalk-like domains as seen by electron microscopy (Johnson and Wall, 1983; Witman et al., 1983; Goodenough and Heuser, 1984; Sale et al., 1985). The outer arm dynein is able to rebind to the outer doublet microtubules at the original position (Mabuchi et al., 1976; Gibbons and Fronk, 1979; Sakakibara and Kamiya, 1989). In sea urchin sperm flagella, the rebinding of the outer arm dynein, called 21 S dynein, causes recovery in both the beat frequency (Gibbons and Gibbons, 1979) and the rate of sliding of outer doublet microtubules (Yano and Miki-Noumura, 1981) in the outer arm-depleted axonemes. The outer arm dynein also binds to purified bovine brain microtubules (Haimo et al., 1979; Porter and Johnson, 1983; Moss et al., 1992). Furthermore, outer arm dynein attached to the glass surface allows gliding movement of purified microtubules (Paschal et al., 1987a; Vale and Yano-Toyoshima, 1988).

The inner arms in the *Chlamydomonas* flagellar axoneme are considered to be heterogeneous in structure and molecular composition. At least three types of inner arms, refered to as I1, I2 and I3, are arranged to form a triplet set, which repeats every 96 nm along each outer doublet microtubule (Piperno et al., 1990). Recently, it has been reported that the heavy chain composition of I1 remains the same along the entire length of the axoneme, while those of I2 and I3, respectively, differ in proximal and distal regions (Piperno and Ramanis, 1991). Furthermore, the inner arm row on the outer doublet microtubules is shown to be staggered, suggesting a complex nature of the inner arms (Kamiya et al., 1991; Muto et al., 1991). On the other hand, the inner arms in cilia or flagella from organisms such as *Tetrahymena* or sea urchin sperm have not been studied well either biochemically or structurally.

The functional role of each dynein arm in ciliary or flagellar movement has not been clearly established. It has been revealed that removal of the outer arms from sea urchin sperm flagellar axonemes results in a 50% reduction in the beat frequency (Gibbons and Gibbons, 1973; Fox and Sale, 1987)

or sliding velocity (Yano and Miki-Noumura, 1981). From these results, it was considered that the functions of the inner and outer arms may be equivalent. On the other hand, it has been suggested from studies with *Chlamydomonas* mutants that activities of these dynein arms differ significantly. The sliding velocity of outer doublet microtubules in axonemes lacking outer arms was significantly less than one-half of the velocity observed in axonemes of wild-type cells (Okagaki and Kamiya, 1986; Brokaw and Kamiya, 1987). Furthermore, mutant cells lacking the outer arms were motile, while those lacking the large part of the inner arms were non-motile (Mitchell and Rosenbaum, 1985; Kamiya and Okamoto, 1985; Brokaw and Kamiya, 1987; Kamiya et al., 1989, 1991). To understand better the role of the inner arms in flagellar or ciliary bending, it is desirable to isolate and characterize individual inner arm dyneins in vitro. Recently, *Chlamydomonas* inner arm dynein I1 has been isolated and its biochemical and physiological characterizations have been reported (Smith and Sale, 1991, 1992). We reported in the preceding paper (Yokota and Mabuchi, 1994) isolation of a novel dynein from the sea urchin sperm flagella, which contains C and A heavy chains. This dynein (C/A dynein) is not a component of outer arms but may be a component of inner arms. In this study, we investigate the enzymatic properties and interaction with microtubules of C/A dynein. The properties of C/A dynein are distinct from those of outer arm 21 S dynein.

MATERIALS AND METHODS

Materials

The following biochemicals were purchased: ATP from Yamasa Shoyu Co., Tokyo, phosphoenolpyruvate from Boehringer Mannheim Crop., Tokyo, and pyruvate kinase from Oriental Yeast Co., Ltd., Tokyo. Taxol was a gift from Dr Matthew Suffness (National Cancer Institute, Bethesda, MD).

Preparation of 21 S dynein, C/A dynein and tubulin

21 S dynein and C/A dynein were prepared from sea urchin (*Anthocidaris crassispina*) sperm flagella as described in the preceding paper (Yokota and Mabuchi, 1994).

Tubulin was prepared from bovine brain by two cycles of the temperature-dependent polymerization-depolymerization in the presence of glycerol, and freed from microtubule-associated proteins by DEAE-Sephacel (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) column chromatography as described by Murata et al. (1992). Microtubules were prepared by polymerizing tubulin in assembly buffer containing 50 mM PIPES (pH 6.9), 0.5 mM $MgSO_4$, 1 mM EGTA and 1 mM GTP at 36°C, for 30 minutes. Polymerized microtubules were subsequently stabilized by the addition of 10 μM taxol.

Measurement of dynein ATPase activity

The ATPase activity of 21 S dynein or C/A dynein as a function of ATP concentration was measured at 25°C in assay medium containing 0.15 M KCl, 4 mM $MgSO_4$, 0.5 mM EDTA, 1 mM phosphoenolpyruvate, 50 μg/ml pyruvate kinase, 0.01 to 1 mM ATP and 20 mM Tris-HCl (pH 8.0), and the amount of pyruvate liberated was measured according to the method of Reynard et al. (1961).

The ATPase activity of dynein in the presence of taxol-stabilized microtubules was assayed at 20°C. The assay medium contained 2 mM $MgSO_4$, 1 mM EGTA, 1 mM ATP, 10 μM taxol and 50 mM Tris-HCl (pH 8.0). The amount of P_i liberated was determined by the method of Fiske and SubbaRow (1925).

Cosedimentation analysis of dynein and microtubules

The Dynein fraction was mixed with taxol-stabilized microtubules in a solution containing 2 mM $MgSO_4$, 1 mM EGTA, 10 μM taxol and 50 mM Tris-HCl (pH 8.0) and kept standing for 10 minutes at room temperature. As a control, the dynein fraction alone was treated in the same manner. The samples were centrifuged at 20,000 ***g*** for 20 minutes at room temperature. Supernatants and pellets were analyzed by gel electrophoresis. The amount of dynein bound to the microtubules was measured by densitometry of a Coomassie Brilliant Blue-stained SDS-gel of the pellet fractions.

The mixture of the dynein fraction and microtubules was examined by negative staining or thin-section electron microscopy. For thin-section electron microscopy, the pellet obtained by centrifugation of the mixture was fixed with 8% (w/v) tannic acid and 2.5% (v/v) glutaraldehyde dissolved in 50 mM sodium phosphate buffer (pH 6.8) for 1 hour at room temperature. It was then postfixed with 1% OsO_4 in 0.1 M sodium cacodylate (pH 7.4) for 1 hour on ice. After rinsing in distilled water, the sample was dehydrated in a graded concentration series of ethanol and embedded in Spurr (Polyscience Inc., Warrington, PA). Thin sections were examined in a JEM 100B electron microscope (JEOL, Tokyo, Japan). Negatively stained specimens were prepared as described in the preceding paper (Yokota and Mabuchi, 1994).

In vitro motility assay

The microtubule motility assay was performed according to Vale and Yano-Toyoshima (1988). A flow chamber with a volume of 12-15 μl was made from a glass slide and a coverslip separated from each other by two strips of sticky tape. A 20 μl sample of 50-80 μg/ml dynein fraction was introduced into the flow chamber and dynein molecules were adsorbed onto the glass surface for 1-2 minutes at room temperature. The flow chamber was then perfused with 60 μl of a solution of 0.5% (w/v) bovine serum albumin (BSA), 2 mM $MgSO_4$, 1 mM EGTA, 2 mM DTT and 20 mM Tris-HCl (pH 8.0). After incubation for 1 minute, 90 μl of the standard assay solution consisting of 20 μg/ml taxol-stabilized microtubules, 1 mM ATP, 0.15 M potassium acetate, 2 mM $MgSO_4$, 1 mM EGTA, 2 mM DTT and 20 mM Tris-HCl (pH 8.0), was applied. Movement of the microtubules was viewed under a Nikon Optiphot microscope equipped with a dark-field condenser (Nikon, Tokyo, Japan). Images were recorded on video tapes with a high-sensitivity television camera (C2400-08 SIT camera, Hamamatsu Photonics KK, Japan) and a video recorder (Maclord NV, FS70, National Co., Tokyo), and were processed with Argus 10 image processor (Hamamatsu Photonics KK, Hamamatsu, Japan).

In order to determine the directionality of the microtubule movement, the microtubules were polymerized selectively from the plus ends of axonemes or outer doublet microtubules prepared from *Tetrahymena* cilia according to the method of Vale and Yano-Toyoshima (1988). A 4 mg/ml sample of brain tubulin solution was incubated with 1 mM *N*-ethylmaleimide (NEM) on ice for 5 minutes. After addition of 10 mM DTT, the tubulin solution was kept on ice for a further 15 minutes. A 4 mg/ml sample of *Tetrahymena* ciliary axonemes (a kind gift from Dr Yoko Yano-Toyoshima) were incubated with an equimolar mixture of non-treated and NEM-treated tubulin (total concentration, 1 mg/ml) in the presence of l mM GTP for 12 minutes at 37°C. Polymerized microtubules were stabilized by dilution with a solution of 30 μM taxol, 100 mM potassium acetate, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM GTP and 80 mM PIPES-NaOH (pH 6.8), and used in the in vitro motility assay.

Other methods

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed according to the method of Laemmli (1970). Gels were scanned with a dual wavelength chromatoscanner CS-910 (Shimadzu Seisakusho, Ltd., Kyoto, Japan).

Salt/Triton X-100-treated axonemes (ST-axonemes) were prepared from sea urchin sperm flagella (*Anthocidaris crassispina*) as described previously (Yokota et al., 1987). In brief, flagellar axonemes were first isolated in the absence of detergent. The axonemes were extracted with a solution containing 0.6 M NaCl and then with a solution containing both 0.1% Triton X-100 and 0.6 M NaCl. These extractions remove almost all arm structures from the axonemes.

RESULTS

Dynein ATPase activity and effect of vanadate on the ATPase activity

ATPase activity of C/A dynein was usually 0.3 to 0.4 μmol P_i/min per mg at 1 mM ATP at 25°C, which was about half that of 21 S dynein (0.6 to 0.8 μmol P_i/min per mg). Fig. 1A shows Lineweaver-Burk plots of the ATPase activities of C/A dynein and 21 S dynein. Both plots showed the downward bend at low concentrations of ATP as reported for outer arm dynein ATPase (Shimizu, 1981; Yano-Toyoshima, 1985). At concentrations of ATP below 100 μM, the V_{max} and K_m of C/A dynein or those of 21 S dynein were 0.20 μmol P_i/min per mg and 19 μM or 0.53 μmol P_i/min per mg and 4.5 μM, respectively. On the other hand, the V_{max} and K_m of C/A dynein or those of 21 S dynein for high concentrations of ATP (above 100 μM), were 0.47 μmol P_i/min per ml and 179 μM or 0.86 μmol P_i/min per mg and 71 μM, respectively.

Vanadate is well known to inhibit dynein ATPase activity (Gibbons et al., 1978; Shimizu and Johnson, 1983). Fig. 1B shows the effect of vanadate on the ATPase activities of C/A dynein and 21 S dynein. The ATPase activities of both dyneins were inhibited effectively in a similar manner. The half-maximal inhibition of the ATPase activity was seen at about 1 μM vanadate in each case.

In vitro microtubule motility

It has been revealed that dynein, attached to a glass surface, that has been isolated from various sources is capable of translocating microtubules (Paschal et al., 1987a,b; Vale and Yano-Toyoshima, 1988; Kagami et al., 1990; Smith and Sale, 1991). C/A dynein also possesses this activity: brain microtubules glide along the glass surface in the direction of their long axis. The velocity of the microtubule gliding induced by C/A dynein or 21 S dynein was dependent on pH, potassium acetate concentration and ATP concentration in the assay medium. Fig. 2A shows the pH dependency of the gliding velocity of microtubules. The gliding velocity on the C/A dynein-coated glass surface was increased gradually with increasing pH to 9.0. On the other hand, 21 S dynein allowed a marked pH dependency: no movement was observed at pH 6.5 while high velocities were obtained at alkaline pH values. In addition, the velocities on either C/A dynein- or 21 S dynein-coated glass surfaces were not affected by Tris buffer concentrations between 10 and 50 mM.

The gliding velocity of microtubules on the C/A dynein-coated glass surface showed a broad peak between 0.05 and 0.2 M potassium acetate (Fig. 2B). Above 0.3 M potassium acetate, few microtubules were detected on the dynein-coated glass surface, indicating that the interaction between C/A dynein and microtubules was abolished at a high ionic strength. On the other hand, in the case of 21 S dynein, the velocity showed rather a sharp peak around 0.15 to 0.2 M potassium acetate (Fig. 2B).

Fig. 2C shows the dependency on ATP concentration. The gliding velocity with either C/A dynein or 21 S dynein saturated at 100 μM ATP with a maximal velocity of about 4.0 μm/s and an apparent K_m for ATP of 20 to 40 μM.

To determine the directionality of the microtubule translocation caused by C/A dynein, axonemes from which brain microtubules were polymerized selectively from the plus end were applied to glass coated with C/A dynein. The microtubules glided towards its plus end (Fig. 3). The axonemes did not rotate during the translocation, in contrast to the case of *Tetrahymena* 14 S dynein (Vale and Yano-Toyoshima, 1988).

Binding of dynein to microtubules

Both C/A dynein and 21 S dynein rebound to the outer doublet microtubules in ST-axonemes in which almost all arm structures had been removed (data not shown). The maximal binding of C/A dynein or 21 S dynein to ST-axonemes was observed at dynein:ST-axonemes weight ratios of about 1:5 to 8 or 1:13 to 15, respectively.

The binding of dyneins to microtubules was investigated in detail using microtubules polymerized from calf brain tubulin. Both C/A dynein and 21 S dynein bound to brain microtubules in the absence of ATP in the co-sedimentation assay (Fig. 4Ab,Bb), while the dyneins themselves did not sediment under the same conditions (Fig. 4Aa,Ba). About 70% of the 21 S

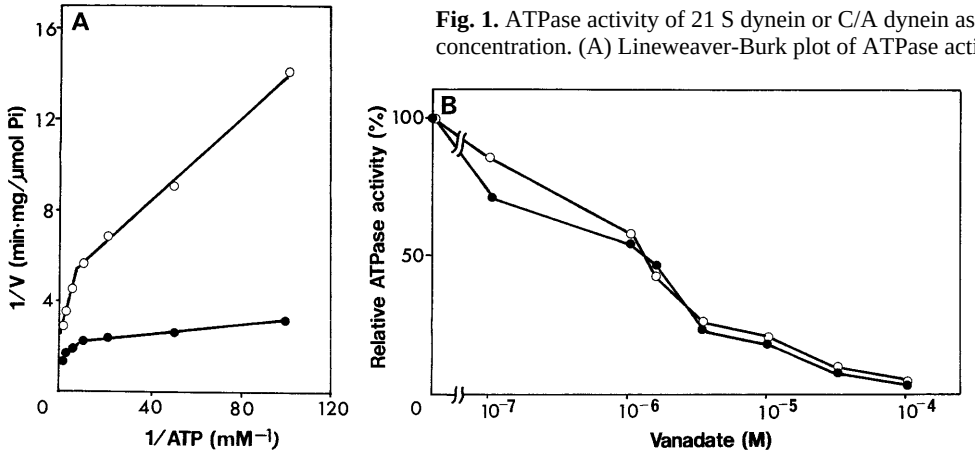

Fig. 1. ATPase activity of 21 S dynein or C/A dynein as a function of ATP or vanadate concentration. (A) Lineweaver-Burk plot of ATPase activity of 21 S dynein (filled symbols) or C/A dynein (open symbols). ATPase activity was measured at 25°C as described in Materials and Methods. (B) Effect of vanadate on the ATPase activity of 21 S dynein (filled symbols) or C/A dynein (open symbols). ATPase activity was measured at 25°C in assay medium consisting of 0.15 M KCl, 4 mM $MgSO_4$, 0.5 mM EDTA, 1 mM ATP and 20 mM Tris-HCl (pH 8.0). Activities relative to those in the absence of vanadate were plotted.

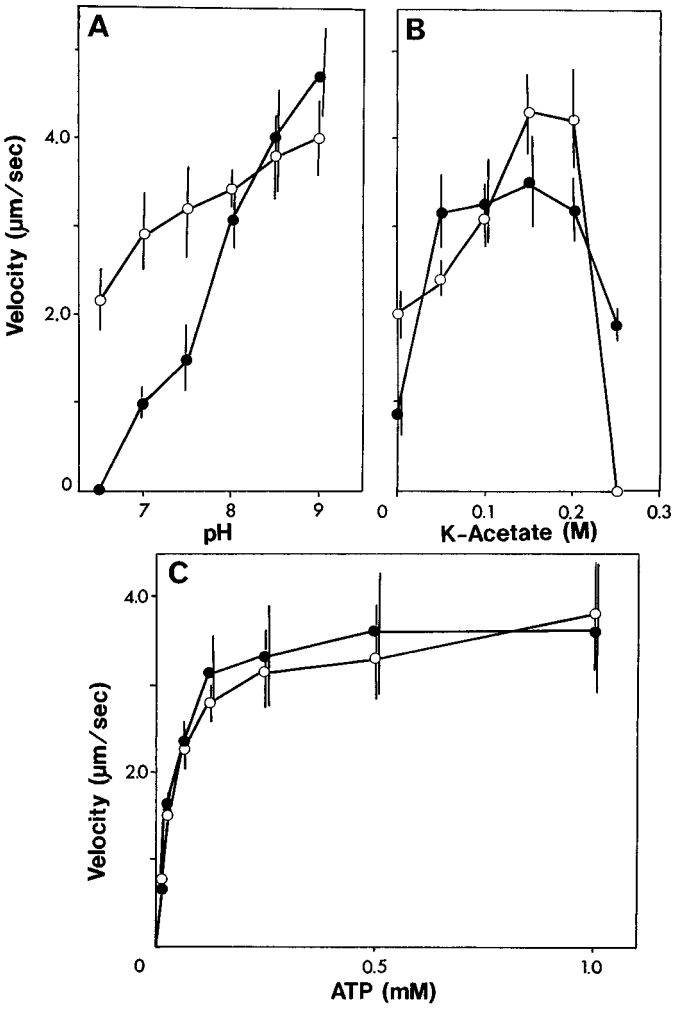

Fig. 2. Gliding of microtubules on a dynein-coated glass surface. Filled symbols and open symbols indicate results for 21 S dynein and C/A dynein, respectively. (A) Effect of pH on the gliding velocity. The gliding velocity was measured as described in Materials and Methods in assay medium consisting of 0.15 M potassium acetate, 2 mM $MgSO_4$, 1 mM EGTA, 2 mM DTT, 1 mM ATP and 20 mM MOPS-NaOH (pH 6.5-7.5) or Tris-HCl (pH 8.0-9.0). (B) Effect of potassium acetate concentration. The assay medium consisted of 2 mM $MgSO_4$, 1 mM EGTA, 2 mM DTT, 1 mM ATP and 20 mM Tris-HCl (pH 8.0). (C) Effect of ATP concentration on the gliding velocity. The assay medium consisted of 0.15 M potassium acetate, 2 mM $MgSO_4$, 1 mM EGTA, 2 mM DTT, 1 mM phosphoenolpyruvate, 50 μg/ml pyruvate kinase and 20 mM Tris-HCl (pH 8.0).

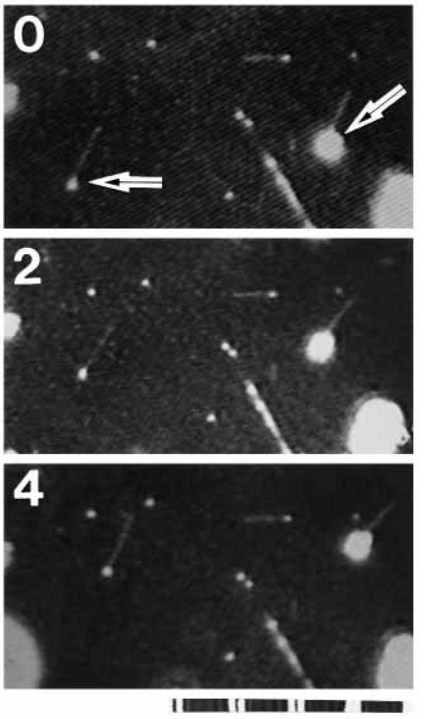

Fig. 3. Directionality of microtubule translocation induced by C/A dynein. This sequence of images obtained by dark-field microscopy shows gliding of two axonemal fragments with microtubules polymerized from the plus end (arrows). The time (in seconds) is indicated at the left. The scale represents 10 μm.

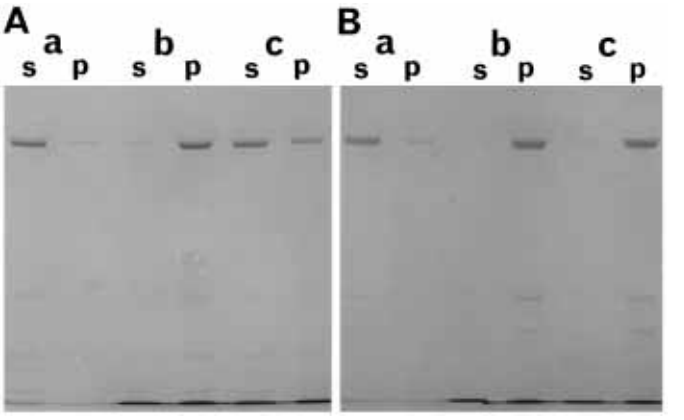

Fig. 4. Binding of 21 S dynein or C/A dynein to purified brain microtubules. Samples of 40 μg/ml 21 S dynein (A) or C/A dynein (B) were mixed with 0.6 mg/ml microtubules in 2 mM $MgSO_4$, 1 mM EGTA, 10 μM taxol and 50 mM Tris-HCl (pH 8.0) and kept standing at room temperature for 10 minutes. Then ATP and vanadate (final concentrations of 1 mM and 50 μM, respectively) were added (c). After centrifugation, the supernatant (s) and the pellet (p) were electrophoresed on a 5% polyacrylamide gel. Lanes a, supernatant (s) and pellet (p) of 21 S dynein or C/A dynein alone. Lanes b, mixture of dynein and microtubules in the absence of ATP and vanadate. Tubulins migrated at dye front.

dynein were recovered in the supernatant fraction (Fig. 4Ac) upon addition of ATP and 50 μM vanadate. In contrast, almost all C/A dynein remained in the pellet fraction under these conditions. A similar result was obtained in the presence of both ATP and an ATP regeneration system (1 mM ATP, 1 mM phosphoenolpyruvate and 50 μg/ml pyruvate kinase) but in the absence of vanadate (data not shown). Therefore, the binding of C/A dynein to the microtubules is ATP-insensitive.

To visualize the interaction of the 21 S dynein or C/A dynein to microtubules, the mixture of dynein and microtubules was examined by negative staining (Fig. 5) or thin-section (Fig. 6) electron microscopy. In the absence of dyneins, single microtubules dispersed on the film (Fig. 5A,B). The microtubules mixed with 21 S dynein were arranged into sheet-like struc-

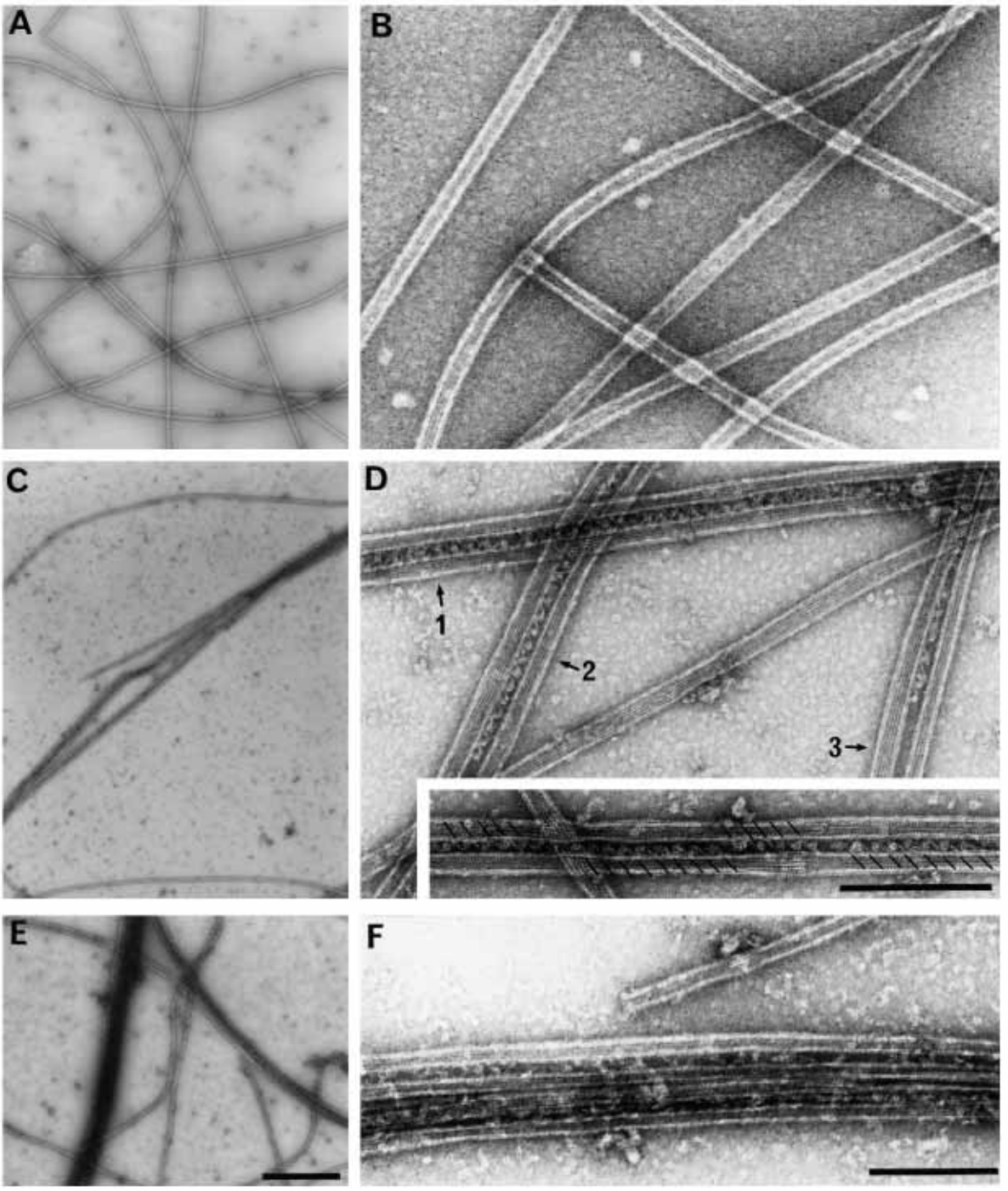

Fig. 5. Negative staining electron micrographs of a mixture of 21 S dynein or C/A dynein and microtubules. Samples of 70 μg/ml 21 S dynein (C and D) or C/A dynein (E and F) were mixed with 120 μg/ml microtubules and kept standing at room temperature for 10 minutes. Samples were then stained negatively with 2% uranyl acetate. (A and B) The microtubules alone. (A,C and E) Low-power images. Bar, 1.0 μm. (B,D and F) High-power images. Bar, 0.2 μm. Projections on both microtubules in a pair are superimposed in region 1. Projections on only one microtubule are seen in region 2. No projection is seen between the microtubules in region 3. Inset in D: the paired microtubules possess opposite polarity as seen by the attachment of the dynein projections, indicated by small bars that are arranged to be parallel to the stalk region of the attached dynein molecules.

tures (Figs 5C and 6A). The 21 S dynein molecules were clearly identified as projections attached to the microtubules with a longitudinal periodicity of 24 nm (Fig. 5D). The projections were restricted to the region between paired microtubules and were rarely found on the surface of single microtubules. Furthermore, these projections showed a patchy distribution between the paired microtubules: the periodical projections were observed in one region, while no projections

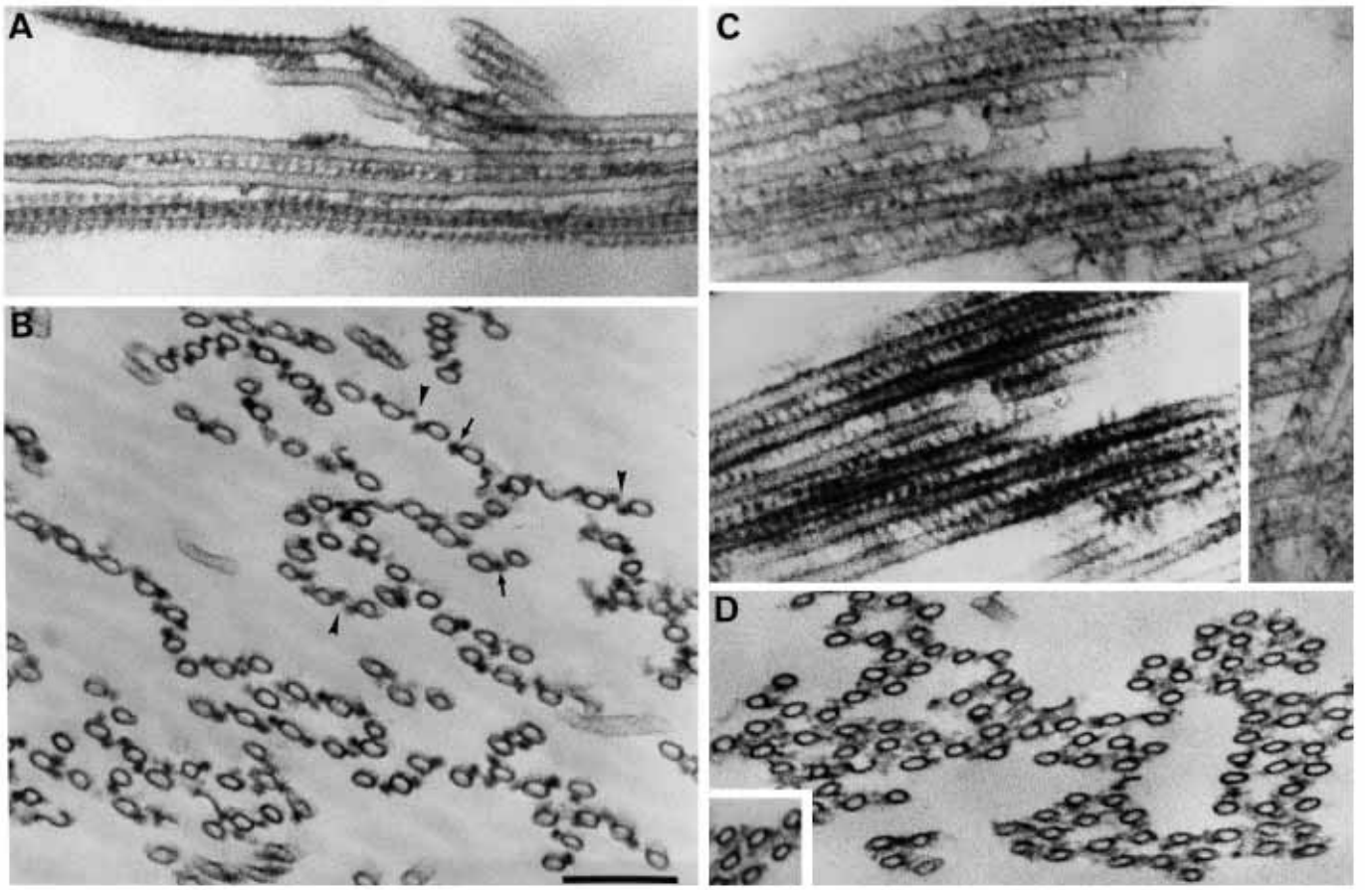

Fig. 6. Thin-section electron micrographs of a mixture of 21 S dynein or C/A dynein with microtubules. Samples of 40 μg/ml 21 S dynein (A and B) or C/A dynein (C and D) were mixed with 100 μg/ml microtubules and kept standing at room temperature for 10 minutes. After centrifugation, the pellet fraction was fixed, embedded and examined by thin-section electron microscopy as described in Materials and Methods. (A and C) Longitudinal sections. (B and D) Transverse sections. Arrowheads in (B) indicate two projections between microtubules, while arrows indicate one projection between adjacent microtubules. Inset in (C): a superimposed image of the region on the left obtained after translation along the length of microtubules showing the axial periodicity of the projections. Inset in (D): a typical microtubule possessing three arms. They are arranged at about 120° around the microtubule axis. Bar, 0.2 μm.

were seen in the other region. Sometimes, the projections extended from each microtubule were superimposed to form a cross-stitch pattern between the paired microtubules (Fig. 5D). From the morphology of the projections, it was found without exception that each 21 S dynein molecule was arranged in the same direction on one microtubule of a pair of microtubules, while on the other microtubule they were arranged in the opposite direction (Fig. 5D, inset). Therefore, the paired microtubules possessed opposite polarity. In thin section, microtubules usually had one or two projections (Fig. 6B). In the latter case, the projections were seen on the opposite sides of the microtubule wall. The sheets seemed to be formed through interaction between the projections on adjacent microtubules (Fig. 6B, arrowheads) and/or between the projection and the adjacent microtubule wall (Fig. 6B, arrows).

On the other hand, the microtubules were packed into bundles in the presence of C/A dynein (Fig. 5E,F and Fig. 6C,D). With increasing concentrations of C/A dynein, the bundles became thicker. Cross-bridges were found between the microtubules (Fig. 6D). There were two or three projections on a microtubule and these were arranged at an angle around 120° when viewed from the axial direction of the microtubule (Fig. 6D and inset). Axial periodicity of the cross-bridges was not shown clearly by either negative staining (Fig. 5F) or thin-section electron microscopy (Fig. 6C). However, a periodicity of 24 nm became apparent when two images of a single thin-section electron micrograph were superimposed after translation along the length of the microtubules (Fig. 6C, inset).

ATPase activity of C/A dynein stimulated by microtubules

Fig. 7 shows the ATPase activities of 21 S dynein and C/A dynein in the presence of microtubules. The ATPase activity of 21 S dynein was not affected by the addition of microtubules up to 1.0 mg/ml. On the other hand, the ATPase activity of C/A dynein was stimulated up to 6- to 7-fold in the presence of microtubules. The double-reciprocal plot of ATPase activity and concentration of microtubules shows a linear relationship (Fig. 7B). The V_{max} for the ATPase activity of C/A dynein activated by microtubules and the K_m for microtubules were 1.20 μmol P_i/min per mg and 37 μg/ml, respectively. On the

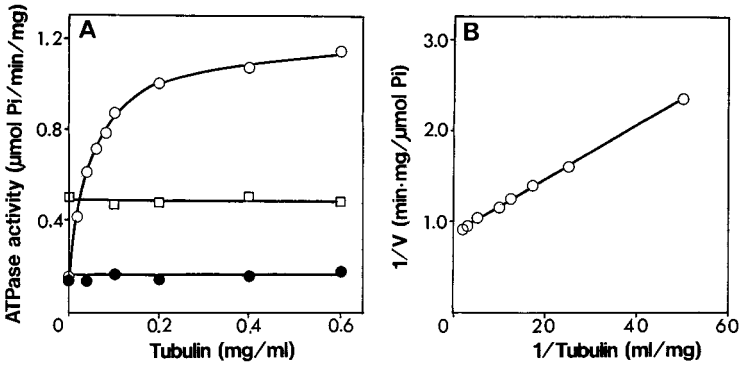

Fig. 7. ATPase activity of 21 S dynein or C/A dynein in the presence of microtubules. The ATPase activity was assayed at 20°C in a medium consisting of 2 mM $MgSO_4$, 1 mM EGTA, 1 mM ATP, 10 μM taxol and 50 mM Tris-HCl (pH 8.0). (A) ATPase activity of 21 S dynein (□) and C/A dynein (○) in the presence of microtubules. Filled symbols (●) show the ATPase activity of C/A dynein in the presence of unpolymerized tubulin instead of microtubules. (B) Double-reciprocal plot of ATPase activity of C/A dynein and microtubule (tubulin) concentration.

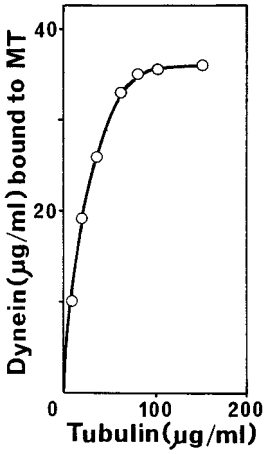

Fig. 8. Binding of C/A dynein to microtubules as a function of microtubule concentration. A 40 μg/ml sample of C/A dynein was mixed with microtubules in 2 mM $MgSO_4$, 1 mM EGTA, 10 μM taxol and 50 mM Tris-HCl (pH 8.0) and kept standing at room temperature for 10 minutes. After centrifugation, the supernatant and the pellet were electrophoresed on a 5% polyacrylamide gel. The amount of C/A dynein bound to the microtubules was measured as described in Materials and Methods and plotted versus tubulin concentration.

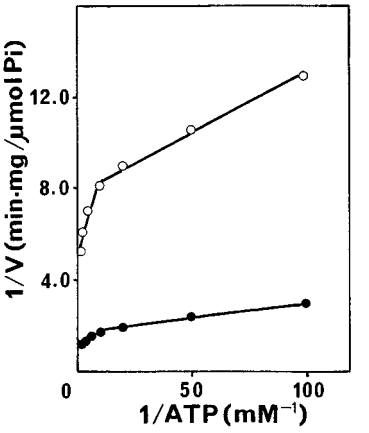

Fig. 9. The Lineweaver-Burk plot of ATPase activity of C/A dynein in the absence or presence of microtubules. ATPase activity was measured at 20°C as described in Materials and Methods, except that the assay medium contained 1 mM phosphoenolpyruvate and 50 μg/ml pyruvate kinase. Open symbols or filled symbols indicate ATPase activity in the absence or presence of 0.3 mg/ml microtubules, respectively.

other hand, unpolymerized tubulin did not show any apparent effect on the C/A dynein ATPase activity (Fig. 7).

To investigate the relation between the microtubule-activated ATPase activity and the microtubule-binding activity of C/A dynein, the amount of C/A dynein bound to the microtubules was measured as a function of microtubule concentration by the co-sedimentation assay. Fig. 8 shows that the amount of C/A dynein bound to the microtubules was dependent on the microtubule concentration. The amount bound reached a plateau level at a tubulin:dynein weight ratio of about 2.2 to 2.8:1. The concentration of tubulin required to attain 50% binding of C/A dynein, namely the apparent K_m for microtubules, was 29 μg/ml, which is in good agreement with the value obtained from the ATPase activation experiment.

Fig. 9 shows the dependency of the microtubule-activated ATPase activity of C/A dynein on the ATP concentration. The extent of activation of C/A dynein ATPase by microtubules was not affected by the ATP concentration. The Lineweaver-Burk plot of the ATPase activity of C/A dynein in the presence of microtubules also showed the biphasic feature as seen in the absence of microtubules (Fig. 9). At low ATP concentrations, the V_{max} and K_m of C/A dynein ATPase activity in the absence or presence of microtubules were 0.13 μmol P_i/min per mg and 6.8 μM or 0.59 μmol P_i/min per mg and 7.7 μM, respectively. On the other hand, the V_{max} and K_m in the absence or presence of microtubules for high ATP concentrations was 0.21 μmol P_i/min per mg and 100 μM or 1.1 μmol P_i/min per mg and 100 μM, respectively.

The extent of activation of C/A dynein ATPase by microtubules was dependent on both potassium acetate concentration and pH (Fig. 10). With increasing the potassium acetate concentration, the activity of C/A dynein alone increased gradually. The activation by microtubules, however, was observed below 0.1 M potassium acetate, but was not detected above 0.15 M. With increasing pH, the activities of both C/A

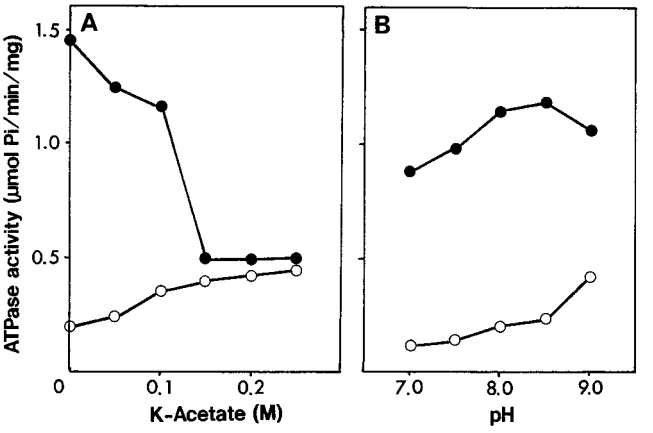

Fig. 10. ATPase activity of C/A dynein as a function of potassium acetate concentration or pH. Open symbols or filled symbols show ATPase activity in the absence or presence of 0.3 mg/ml microtubules, respectively. (A) Effect of potassium acetate concentration. The assay medium contained 2 mM $MgSO_4$, 1 mM EGTA, 1 mM ATP, 10 μM taxol, 50 mM Tris-HCl (pH 8.0) and various concentrations of potassium acetate. (B) Effect of pH. The assay medium contained 2 mM $MgSO_4$, 1 mM EGTA, 1 mM ATP, 10 μM taxol and 50 mM Tris-HCl.

dynein alone and that with microtubules increased, and the extent of the activation was also enhanced.

DISCUSSION

C/A dynein has biochemical properties characteristic of the axonemal or cytoplasmic dyneins reported previously. Its ATPase activity is inhibited by vanadate at low concentrations like other dyneins (see Gibbons, 1989, for a review). C/A dynein binds to axonemal microtubules as well as to microtubules polymerized from calf brain tubulin. C/A dynein induces microtubule gliding in vitro from its minus end toward its plus end. This directionality is the same as that of microtubule sliding in the axonemes (Sale and Satir, 1977; Fox and Sale, 1987).

The velocity of microtubule gliding on C/A dynein and the apparent K_m for ATP, 3.5 to 4.5 μm/s and 20 to 40 μM, respectively, are similar to the values for 21 S dynein. It should be noted that the gliding velocity is comparable to the velocity of sliding of outer doublet microtubules in reactivated or disintegrating axonemes of sea urchin sperm flagella depleted of outer arms, that is 6 to 8 μm/s (Yano and Miki-Noumura, 1981; Fox and Sale, 1987). Thus, the velocity of microtubule gliding produced by C/A dynein in an in vitro motility assay is comparable to that induced by inner arm dyneins in the axonemes.

From the co-sedimentation assay and electron microscopic observations, it is revealed that both 21 S dynein and C/A dynein bind to and cross-link the microtubules polymerized from brain tubulin. The binding properties of *A. crassispina* 21 S dynein to the microtubules are similar to those reported previously for outer arm dyneins. (1) The binding is largely ATP-sensitive as reported for outer arm dyneins isolated from *Tetrahymena* cilia (Porter and Johnson, 1983) and *Strongylocentrotus purpuratus* sperm flagella (Moss et al., 1992). (2) 21 S dynein forms projections on the microtubules with 24 nm periodicity as reported for the outer arm dyneins isolated from *Hemicentrotus pulcherrimus* (Hisanaga and Hirokawa, 1987) or *S. purpuratus* (Moss et al., 1992) sperm flagella, *Chlamydomonas* flagella (Haimo et al., 1979) or *Tetrahymena* cilia (Porter and Johnson, 1983). (3) 21 S dynein bound to microtubules in a cooperative manner (Haimo et al., 1979; Moss et al., 1992). One of our observations was unique: the microtubules paired by cross-linking possessed opposite polarity, while similar experiments reported by others showed the same polarity for paired microtubules (Haimo et al., 1979; Moss et al., 1992). The reason for this discrepancy is not clear at present.

We found that the binding of C/A dynein to microtubules is insensitive to ATP. This property is distinct from that of the outer arm dyneins. The manner of binding of C/A dynein to the microtubules as seen by electron microscopy is also different from that of 21 S dynein. C/A dynein forms microtubule bundles instead of sheets. The cross-bridges were found to be more irregular in both axial and transverse views, although an axial periodicity of about 24 nm was found in a superimposed electron micrograph. It is not clear whether the binding to the microtubules occurred in a cooperative manner or not. Similar irregular binding has been obtained for the inner arm dynein subtype I1 of *Chlamydomonas* flagella (Smith and Sale, 1991). Since C/A dynein or subtype I1 may represent only one component of inner arm dyneins, an interaction of C/A dynein or subtype I1 with other inner arm dyneins may be required for clear periodic binding. It is also possible that the outer doublet microtubules have certain structures or adaptor proteins recognized by each inner arm dynein to ensure regular binding.

The ATPase activity of C/A dynein is activated by brain microtubules, while that of 21 S dynein was not. On the other hand, 21 S dynein is activated by microtubules when it is in flagellar axonemes (Gibbons and Fronk, 1979; Yokota et al., 1987). Somewhat similar observations have been reported for *Tetrahymena* ciliary outer arm 22 S dynein. This dynein is activated by brain microtubules with a K_m value for the microtubules as high as at least 25 mg/ml (Omoto and Johnson, 1986), that is 1,000 times higher than that obtained for C/A dynein. However, the ATPase activity of 22 S dynein is highly activated by brain microtubules when it is chemically cross-linked to microtubules (Shimizu et al., 1989). These findings suggest that dynein ATPase activity is well activated by microtubules when it is firmly attached to microtubules in the presence of ATP. Therefore, it is reasonable that the C/A dynein ATPase activity is enhanced by brain microtubules, since its binding to microtubules is ATP-insensitive, in contrast to sperm flagellar 21 S dynein (this study) or *Tetrahymena* ciliary 22 S dynein (Porter and Johnson, 1983).

The activation by microtubules of C/A dynein ATPase activity was well observed at low ionic strength but was abolished by 0.15 M potassium acetate. The same held true for the activation of 21 S dynein ATPase activity in axonemes (Yokota et al., 1987). However, ionic strength showed an opposite effect on microtubule gliding activity on a dynein-coated glass surface: the microtubule gliding velocity on either 21 S or C/A dynein was optimal at 0.1 to 0.2 M potassium acetate. In addition, microtubule gliding was observed on 21 S dynein under similar conditions where no ATPase activation was observed. Therefore, the ATPase activity stimulated by microtubules may not be fully coupled to the microtubule gliding activity.

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