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Activation of the Dynein Adenosinetriphosphatase by Cross-Linking to Microtubules†

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ABSTRACT: The microtubule–dynein complex consisting of 22S dynein from *Tetrahymena* cilia and MAP-free microtubules was subjected to treatment with various concentrations of 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC), a zero-length cross-linker, at 28 °C for 1 h. Following cross-linking of the microtubule–dynein complex, nearly all of the ATPase activity cosedimented with the microtubules in the presence of ATP. Electron microscopic observation by negative staining revealed that, following treatment with 1 mM EDC, the complex did not dissociate in the presence of ATP, although the dynein decoration pattern was disordered. The complex treated with 3 mM EDC exhibited normal microtubule–dynein patterns even after the addition of ATP. The ATPase activity of the microtubule–dynein complex was enhanced about 30-fold by the treatment with 1–3 mM EDC. These results indicate that the ATPase activation was caused by the close proximity of the dynein ATPase sites to the microtubules and provide further support for the functional interaction of all three dynein heads with the microtubule. The maximal specific activity was 12 μmol min^{-1} (mg of dynein)$^{-1}$, corresponding to a turnover rate of 150 s^{-1}, which may be the rate-limiting step at infinite microtubule concentration and may represent the maximum rate of force production in the axoneme.

Axonemal dynein from cilia or flagella couples the hydrolysis of ATP to produce a force for microtubule sliding [for reviews, see Gibbons (1981) and Johnson (1985)]. Recent work has demonstrated a related cytoplasmic dynein in numerous cell types (Hisanaga & Sakai, 1983; Asai & Wilson, 1985; Paschal & Vallee, 1987; Pratt, 1986). Depending on the source of its isolation, dynein is composed of 2 or 3 polypeptides with a molecular weight of 400 000 or more (Mabuchi & Shimizu, 1974; Bell, 1983; Lee-Eiford et al., 1986), 2 or 3 intermediate chains, and 8–10 low molecular weight chains. *Tetrahymena* dynein, used in this study, has three globular heads connected to a common base by three strands and a net molecular weight of 1.9 million (Johnson & Wall, 1983). This dynein adenosine-5′-triphosphatase (ATPase)[1] has been shown to have an ATPase mechanism similar to that of actomyosin (Johnson, 1985) and serves as the paradigm for understanding the ATPase mechanism of all dyneins.

One of the characteristics of dynein is its ATP-sensitive binding to microtubules (Haimo et al., 1979; Porter & Johnson, 1983a,b; Omoto & Johnson, 1986). This microtubule–dynein complex constitutes a simple model system for cilia or flagella, as actomyosin is for muscle. Omoto and Johnson (1986) have demonstrated activation of the dynein ATPase by a high concentration of the microtubules free from microtubule-associated proteins (MAPs) under physiological conditions. Their study indicated that activation of the dynein ATPase required a high concentration of the MAP-free microtubules which could be rationalized in terms of the very high local concentration of microtubules in the axoneme. ATPase activation was also observed at low microtubule concentrations if the ATP concentration was sufficiently low to allow the dynein to remain transiently tethered to the microtubule by one of the three heads. For the current work, we reasoned that if dynein could be cross-linked to the mi-

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[1] Abbreviations: ATPase, adenosine-5′-triphosphatase; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MAP(s), microtubule-associated protein(s); PIPES, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate.

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crotubules, we should expect a much larger ATPase activation.

Chemical cross-linking with a zero-length cross-linker, EDC, has been proven to be very useful in investigating the nature of protein–protein interactions. Myosin subfragment 1 (S1) was shown to have at least two sites for the direct contact with actin although the acto–S1 complex is formed with a stoichiometry of one S1 per actin monomer (Sutoh, 1982a; Greene, 1984; Chen et al., 1985a,b) and the contact regions have been located to a narrow range of the polypeptides (Sutoh, 1982b, 1983). Furthermore, acto–S1 cross-linked by EDC no longer dissociates upon addition of ATP although the ATPase activity is comparable to that at infinite actin concentration (Weeds & Taylor, 1975; Mornet et al., 1981). This result also indicates that myosin S1 could hydrolyze ATP while being attached to F-actin (Mornet et al., 1981). Chemical cross-linking with EDC has also been useful to define the actin contact sites of several actin binding proteins like depactin (Sutoh & Mabuchi, 1984), fragmin (Sutoh & Hatano, 1986), and plasma gelsolin (Harris, 1985).

Here, we investigated the effects of EDC on the microtubule–dynein complex. The data indicate that dynein could be covalently bound to the microtubules by the EDC treatment such that the complex became insensitive to ATP-induced dissociation and showed a large enhancement of ATPase activity.

Materials and Methods

Reagents. EDC was from Sigma. Taxol was a generous gift from the National Cancer Institute (Bethesda, MD). Other reagents were of analytical grade, as described (Shimizu & Johnson, 1983a,b). Water was distilled and deionized by the MilliQ system (Millipore).

Protein Purification. Dynein was prepared from cilia of *Tetrahymena thermophila* strain B-255 by the method described previously (Porter & Johnson, 1983a). In this study, only 22S dynein was used, and the term "dynein" will refer to 22S dynein.

Tubulin was purified by chromatography on DEAE-Sephacel (Omoto & Johnson, 1986). The purified MAP-free tubulin was polymerized to form microtubules at 35 °C by inclusion of taxol at a concentration equivalent to that of tubulin dimer, or by addition of 7% dimethyl sulfoxide. The microtubules thus obtained were incubated with dynein at 28 °C to form the microtubule–dynein complex. The final protein concentrations were 200 and 400 μg/mL for microtubules and dynein, respectively. Polyacrylamide gel electrophoretic patterns of the proteins are seen in Figure 2.

Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard.

EDC Treatment. EDC was dissolved in 100 mM HEPES–NaOH (pH 7.4), and 1 volume of this solution was mixed with 9 volumes of the microtubule–dynein complex suspension in 50 mM PIPES–NaOH (pH 7.0) and 4 mM $MgCl_2$ to start the reaction. The EDC concentration described herein refers to that during the reaction of EDC with the proteins. After 1-h incubation at 28 °C, the reaction was terminated by the addition of a small molar excess of 2-mercaptoethanol over EDC (Sutoh, 1982a). The resultant mixture was left standing at room temperature and used within 2–3 h, unless otherwise noted.

ATPase Assay. The ATPase assay was carried out in the assay solution consisting of 50 mM HEPES–NaOH (pH 7.4), 4 mM $MgCl_2$, and 1 mM ATP. The reaction was started by adding the proteins and allowed to proceed at 28 °C for 3–10 min. Then, 0.5 M trichloroacetic acid (final concentration) was added to stop the reaction, and the protein precipitates, if formed, were removed by centrifugation. Inorganic phosphate was quantitated by the method of Fiske and SubbaRow (1925).

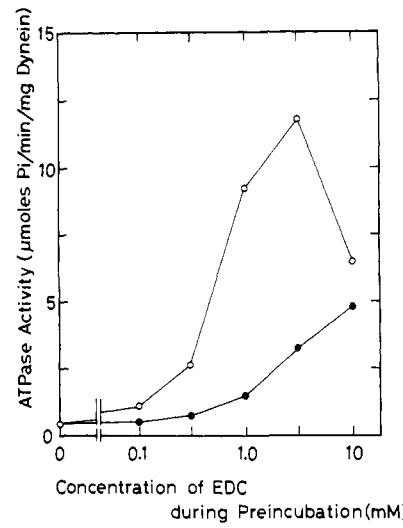

FIGURE 1: Effect of EDC on the ATPase activity of dynein or the microtubule–dynein complex. The microtubules were polymerized from MAP-free bovine brain tubulin in the presence of equimolar taxol (per tubulin) at 35 °C in 50 mM PIPES–NaOH (pH 7.0) and 4 mM $MgCl_2$. The microtubules (200 μg/mL) were incubated with dynein (400 μg/mL) to form the microtubule–dynein complex at 28 °C. The resultant suspension was then reacted with EDC at various concentrations as described under Materials and Methods. Results obtained with microtubule–dynein complex (○) are compared to a parallel sample of dynein treated in the absence of the microtubules (●).

SDS–Polyacrylamide Gel Electrophoresis. Sample proteins were denatured by boiling in the presence of 0.1% SDS and 5% 2-mercaptoethanol. Samples were electrophoresed on 5–15% polyacrylamide gradient gel and then stained with Coomassie Brilliant Blue as described previously (Porter & Johnson, 1983a). In order to investigate the dynein heavy-chain region, a 3–5% polyacrylamide gradient gel of 40 cm long was employed (not shown).

Electron Microscopy. The microtubule–dynein complex was prepared as described above, diluted 5-fold with buffer, placed on a carbon- and formvar-coated grid, and then stained with uranyl acetate as described (Porter & Johnson, 1983a). Micrographs were obtained with a Philips EM 300 operating at 60 kV.

Results

Effect of EDC on the ATPase Activity of Dynein. The EDC treatment of the microtubule–dynein complex remarkably enhanced the ATPase activity (Figure 1). The maximal activity was observed at 1–3 mM EDC under the present experimental conditions. The magnitude of enhancement was about 30-fold, the highest enhancement ever observed for the dynein ATPase. The maximal specific activity was 12 μmol min^{-1} (mg of dynein)$^{-1}$, which is comparable to that of the ATPase of fragment A (16 μmol of P_i mg^{-1} min^{-1}), a proteolytic fragment of sea urchin outerarm dynein (Ogawa, 1975).

The ATPase activity of dynein alone was activated by the EDC treatment, although to a much lesser extent (Figure 1). The activation of dynein ATPase by chemical modification has been known for more than 10 years (Blum & Hayes, 1974; Shimizu & Kimura, 1974; Gibbons & Fronk, 1979). The microtubules had negligible ATPase activity [0.01–0.05 nmol min^{-1} (mg of protein)$^{-1}$], which was not affected by the EDC treatment. Thus, the large activation by the EDC treatment

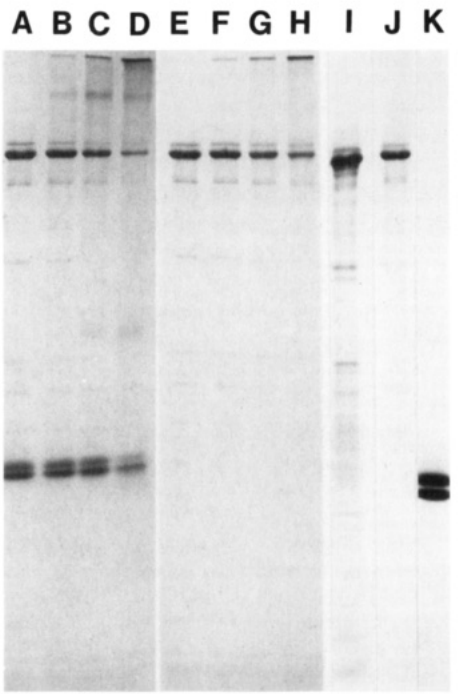

FIGURE 2: Electrophoretic analysis of the EDC-treated microtubule–dynein complex. The treatment of the microtubule–dynein complex or dynein alone was done as described in the legend to Figure 1. After termination of the reaction by the addition of 2-mercaptoethanol, the samples were boiled for 5 min in the presence of 0.1% SDS and 5% 2-mercaptoethanol and then electrophoresed on a 5–15% polyacrylamide gradient gel. (A–D) The microtubule–dynein complex treated with 0, 0.3, 1.0, and 3.0 mM EDC; (E–H) dynein treated with 0, 0.3, 1.0, and 3.0 mM EDC; (I) crude dynein fraction before sucrose density gradient centrifugation; (J) dynein; (K) MAP-free tubulin.

was brought about through the interaction of dynein with the microtubules.

Electrophoretic analysis of the microtubule–dynein complex after EDC treatment is shown in Figure 2. Upon increasing the EDC concentration, the density of tubulin and dynein high molecular weight polypeptide bands decreased while several bands with higher apparent molecular weight appeared. Some staining was seen even on top of the stacking gel as a result of the EDC cross-linking of polypeptides.

Cosedimentation of Dynein with Microtubules in the Presence of ATP. ATP induces rapid and complete dissociation of the microtubule–dynein complex (Porter & Johnson, 1983b; Johnson, 1985). To examine the effect of the EDC treatment on the cross-linking of dynein with the microtubules, another set of samples treated with EDC in the same manner as above were quickly centrifuged before and after the addition of 2 mM ATP.

The ATPase activities before centrifugation and of the supernatant and the pellet after centrifugation are shown in Figure 3. The ATPase activity in the supernatant remained almost constant up to 1 mM EDC and then decreased upon further increase in EDC concentration. On the other hand, the activity of the pellet almost paralleled that of the uncentrifuged sample, indicating that most of the ATPase activity was attributable to that of the cross-linked M·D complex. The sum of the activities of the supernatant and pellet was nearly equal to the activity of the uncentrifuged sample.

Dynein treated with EDC in the absence of microtubules was also centrifuged, but the ATPase activity in the supernatant was the same as that before centrifugation; thus, dynein alone reacted with EDC did not sediment, and no precipitates were observed.

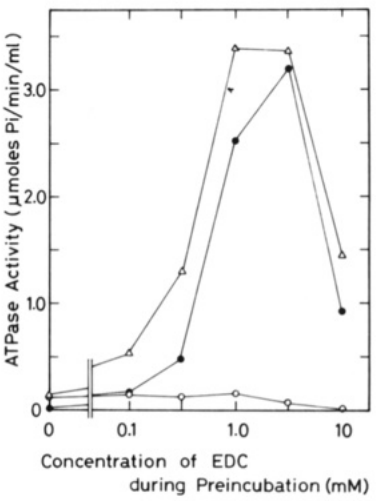

FIGURE 3: Effect of EDC on the cosedimentation of ATPase activity with microtubules in the presence of ATP. The EDC treatment of the microtubule–dynein complex was carried out in the same manner as described in the legend to Figure 1. After the addition of 2-mercaptoethanol to stop further reaction by EDC, the solution was mixed with 2 mM ATP final concentration and centrifuged for 1 min in an Airfuge (Beckman Instruments) to sediment the microtubules and material attached to them. The supernatant was recovered and saved, and the pellet was suspended in the same buffer solution containing 2 mM ATP and centrifuged again. The resultant pellet was resuspended in the ATP-free fresh buffer solution and saved. The ATPase activities of the supernatant (○) and the pellet (●) thus obtained as well as the uncentrifuged sample (△) were determined.

These results demonstrate that the activation of the dynein ATPase was directly correlated with the degree of covalent cross-linking of the dynein to the microtubules. To further explore this dependence, the supernatant and pellet from the experiment described in Figure 3 were examined by gel electrophoresis to get the results shown in Figure 4.

Prior to treatment with EDC, most of the dynein heavy chains were released into the supernatant by the addition of ATP as described previously (Porter & Johnson, 1983a). Following EDC treatment, dynein heavy chains were seen to cosediment with microtubules in the presence of ATP. The fraction of dynein heavy chains which cosedimented with microtubules increased with EDC concentration in parallel to the partitioning of the ATPase activities as described above.

Even in the presence of taxol, some tubulin bands appeared in the supernatant, which may indicate that some tubulin disassembled after ATP addition. Reaction with EDC apparently stabilized the microtubules to reduce their disassembly into the supernatant, either by tubulin–tubulin cross-linking or by dynein–tubulin cross-linking.

Analysis of the Cross-Linked Microtubule–Dynein Complex. The microtubule–dynein complex treated with 1–3 mM EDC was insensitive to ATP-induced dissociation. To further quantitate this reaction, we used turbidity to monitor the microtubule–dynein complex. As reported previously (Porter & Johnson, 1983a; Shimizu & Furusawa, 1986), ATP of sufficient concentration induced an abrupt turbidity decrease of the suspension. With increasing EDC concentrations, the ATP-induced turbidity change decreased in a dose-dependent manner (Table I). At 1 mM EDC or more, the turbidity change was negligible, indicating that the binding became ATP-insensitive, consistent with the sedimentation experiments. It should be noted that the further addition of vanadate to the suspension which had been already mixed with ATP did not change the turbidity significantly.

Next, we checked the state of the microtubule–dynein complex after addition of ATP by electron microscopy. At

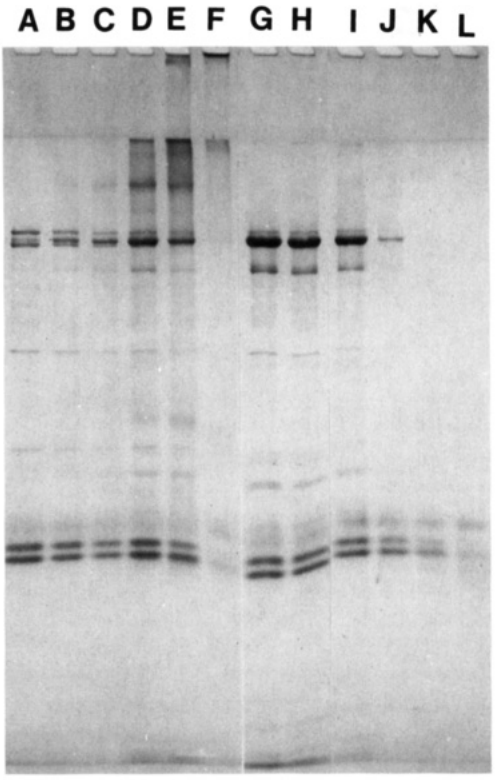

FIGURE 4: Electrophoretic analysis of the supernatant and the pellet after EDC treatment. The supernatant and the pellet whose ATPase activities are shown in Figure 4 were subjected to electrophoresis on a 5–15% polyacrylamide gradient gel in the presence of SDS. (A–F) The pellet of the microtubule–dynein complex treated with 0, 0.1, 0.3, 1.0, 3.0, or 10.0 mM EDC, respectively; (G–L) the supernatant of the microtubule–dynein complex treated with 0, 0.1, 0.3, 1.0, 3.0, or 10.0 mM EDC, respectively.

Table I: Effect of ATP on the Cross-Linked Microtubule–Dynein Complex[a]

EDC concn (mM)	turbidity drop	EDC concn (mM)	turbidity drop
0	0.173	1.0	<0.01
0.1	0.158	3.0	<0.01
0.3	0.096	10.0	0.009

[a] The microtubule–dynein complex was made and treated with various concentrations of EDC as described under Materials and Methods. After the reaction was stopped by 2-mercaptoethanol, a portion of the suspension was transferred into a cuvette, and the absorbance at 429 nm due to turbidity before and after addition of a small volume of ATP solution was recorded. The absorbance value before the addition of ATP was about 0.3.

0 mM EDC, the normal microtubule–dynein complexes and naked microtubules with a number of dynein molecules on the background were seen before and after ATP addition, respectively (Figure 5a,b), as already reported (Porter & Johnson, 1983a). At 0.3 mM EDC after addition of ATP, still we saw almost naked microtubules (Figure 5c), indicating most dynein molecules were not bound to microtubules although a small number of dynein molecules were in close proximity to the microtubules.

At 1 mM EDC, the situation changed. Before ATP addition, the electron microscopic image was the same as that at 0 mM EDC (Figure 5d). However, after addition of ATP, most dynein molecules lay near the microtubule walls (Figure 5e), and the periodic pattern of the dynein decoration along the microtubules was no longer maintained; rather, the decoration pattern was disordered. This disordering can be seen more clearly with the image taken after addition of ATP and vanadate (Figure 5f). The images appeared as if there was a partial dissociation of dynein molecules that remained tethered to the microtubule by one or more heads (Shimizu & Johnson, 1983b).

At 3 mM EDC, even after the addition of ATP and ATP plus vanadate, the decoration pattern appeared regular (Figure 5g–h). This seems to indicate that the dynein molecules were cross-linked via multiple points of attachment to the microtubules such that we could not see even traces of partial dissociation. At 10 mM EDC, the electron microscopic images were indistinguishable from those at 3 mM EDC (not shown).

Cold Stability of the Cross-Linked Microtubule–Dynein Complex. Dynein is known to stabilize otherwise unstable microtubules by binding to the microtubule lattice (Porter & Johnson, 1983a,b). Treatment of the complex with EDC would be expected to further stabilize it against cold-induced disassembly. To examine this, MAP-free tubulin was polymerized by the presence of 7% dimethyl sulfoxide but in the absence of taxol and was used to form the microtubule–dynein complex as above. The EDC treatment of the complex and the quenching the reaction with 2-mercaptoethanol were performed in the same manner as above. Half was left standing at 25 °C while the other half was placed on ice for 20 h.

The ATPase activity after standing either at 25 °C or on ice was almost the same as that before standing (data not shown). The electron microscopic observations of the samples left at 0 °C showed that the one treated with up to 0.3 mM EDC had a few microtubule–dynein complexes with large number of dynein molecules on the background (data not shown). The one treated with 1 mM EDC had more microtubule–dynein complexes, but the pattern was distorted while the one with 3 mM EDC was shown to be more normally looking complexes with few free dynein molecules. The EDC treatment conferred the microtubule–dynein complex resistance against cold depolymerization.

Discussion

The sedimentation experiments, electrophoretic analysis, and electron microscopic observations indicate that dynein molecules were cross-linked covalently to microtubules by the EDC treatment. In the acto–S1 system, the cross-linked products could be identified on polyacrylamide gels (Mornet et al., 1981; Sutoh, 1982a). In contrast, dynein from *Tetrahymena* cilia is a much larger molecule consisting of at least three very large heavy chains, two to three intermediate, and four to six light chains (Johnson, 1985) so that cross-linking of dynein and tubulin could not be ascertained directly by analysis of electrophoretic mobility even on a 40-cm-long gel (data not shown). Nonetheless, the insensitivity toward ATP suggests the dynein became covalently attached to the microtubules.

One should expect that there are direct and specific contacts between dynein polypeptides and tubulin subunits which might be preferentially cross-linked, because EDC provides a zero-length cross-link. This study therefore provides the method of choice for defining the tubulin–dynein contacts.

The 1 mM EDC treatment under the present experimental condition brought about 30-fold activation of the microtubule–dynein ATPase activity, with negligible effect on the free dynein activity. The sedimentation experiment showed that most dynein molecules were bound to the microtubules, although the electron microscopic images indicated that the dynein became disordered upon the addition of ATP (Figure 5e,f).

The dynein molecule is shown to have three heads connected to a common base by thin strands: each of the three heads

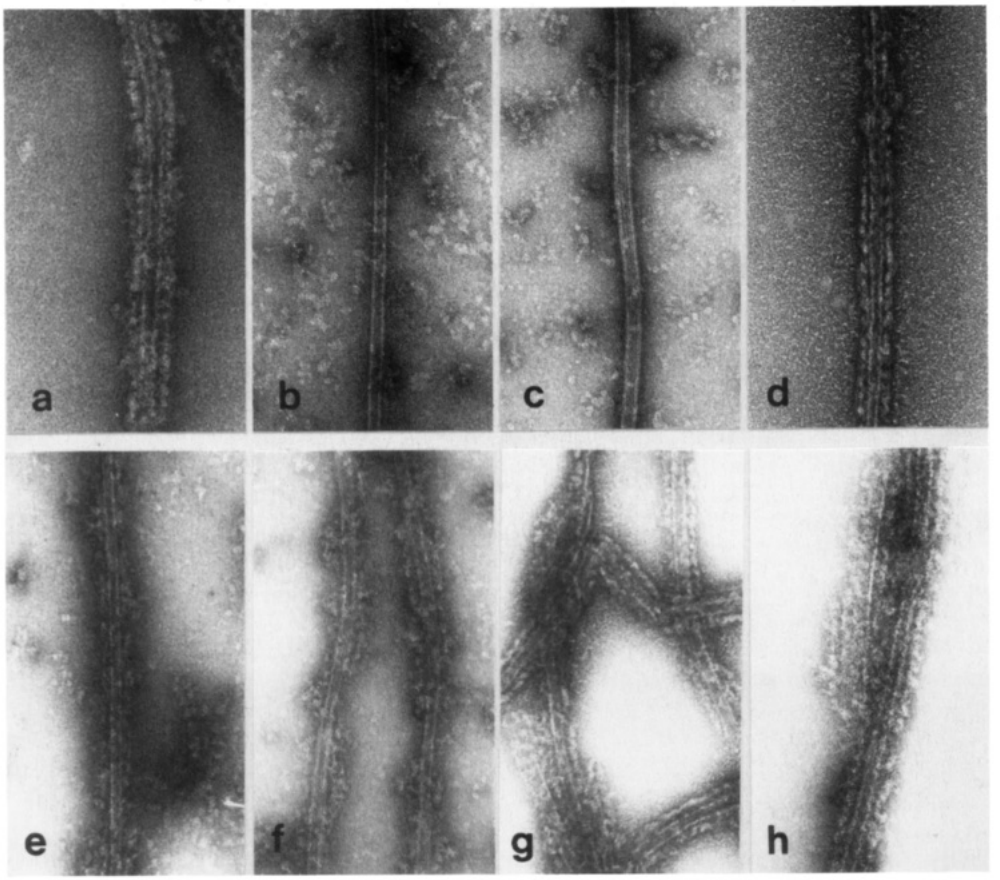

FIGURE 5: Electron microscopic images of the microtubule–dynein complex treated with EDC in the presence or absence of ATP. The microtubule–dynein complex was treated with various concentrations of EDC in the same manner as described in the legend to Figure 1. The samples were diluted 5-fold with fresh buffer and added with 2 mM ATP final concentration with or without 0.1 mM vanadate. The samples were negatively stained with uranyl acetate: (a) treated with no EDC and before addition of ATP; (b) no EDC, after addition of ATP; (c) 0.3 mM EDC, after addition of ATP; (d) 1.0 mM EDC before addition of ATP; (e) 1.0 mM EDC after addition of ATP; (f) 1.0 mM EDC after addition of ATP plus vanadate; (g) 3.0 mM EDC, before addition of ATP; (h) 3.0 mM EDC, after the addition of ATP plus vanadate. Magnification: 81300×. The bar in the figure represents 200 nm.

is considered to interact with different tubulin molecules in the microtubule wall in an ATP-sensitive manner (Johnson & Wall, 1983). The distorted pattern of the cross-linked microtubule–dynein complex seen in the presence of ATP suggested that ATP caused dissociation of the heads not covalently bound to tubulin while the entire molecule remained covalently bound to the microtubules by one or two of the heads. Accordingly, the free heads would be constrained to remain near the microtubule and would be fully activated. Thus, the present observation may provide additional support for the interaction of all three dynein heads with the microtubule (Johnson, 1983; Shimizu & Johnson, 1983b).

At higher concentrations of EDC, the heads were likely to be rigidly bound to the microtubules by multiple cross-links. Therefore, ATP could not distort the regular decoration pattern of microtubules by dynein molecules.

When we modeled the dissociation of dynein from the microtubules (Shimizu & Johnson, 1983b; Johnson et al., 1983), we predicted that the dynein molecule with its one or two head(s) attached to the microtubule would have the same light-scattering property as that attached with all three heads and that only the molecule whose all three heads dissociated would contribute to the light-scattering change. In the present work, the distortion of the periodic decoration of the microtubules by dynein molecules after the addition of ATP, especially with vanadate, did not contribute to the turbidity change. Therefore, we conclude that the intensity of light scattering by the microtubule–dynein complex did not depend upon the configuration of dynein molecules as long as they were attached to the microtubules by at least one head, thus supporting our earlier assumption.

The maximum ATPase activity was about 150 s^{-1}. This means that every step of the dynein ATPase pathway must have a rate constant equal to or greater than 150 s^{-1}. Previously (Johnson, 1983; Shimizu & Johnson, 1983a; Omoto & Johnson, 1986), we estimated the hydrolysis rate and products release rate to be 60 and 2–6 s^{-1}, respectively. More recent measurements using more pure dynein preparations and a newer quench-flow apparatus have indicated that the rate of ATP hydrolysis at the active site is 130–150 s^{-1}. In the present study, the steady-state rate of the dynein ATPase was about 4 s^{-1}. The microtubule-induced activation should be the result of enhancing the product release rate.

The present study indicates that the ATP hydrolysis occurred without the dissociation of dynein from microtubules. In the case of the acto–S1 system, a similar observation has been interpreted to mean that the S1 is capable of hydrolyzing ATP without dissociating from actin; that is, the reaction sequence is not strictly ordered with acto–S1 dissociation preceding ATP hydrolysis (Mornet et al., 1981). However, one should exercise caution in reaching such a conclusion because it is reasonable to suppose that the important protein–protein bonds between actin and S1 are broken after ATP binding even though a single covalent linkage holds the S1 near the actin.

In the present study, we cannot conclude that hydrolysis occurs without dissociation because of the multiple dynein heads. The observed activation could be accounted for by the

dissociation and rebinding of individual heads due to dynein molecules tethered to the microtubule by one or two heads. The decrease in ATPase activity at higher EDC concentrations may be due to the complete cross-linking of all three heads, implying that hydrolysis is inhibited by binding of the dynein heads to the microtubule. This conclusion is supported by our analysis of the effects of microtubules on the phosphate–water oxygen exchange reactions (Holzbaur & Johnson, 1989a,b). Alternatively, the results may suggest a more subtle conformational coupling required for ATP turnover. At low levels of cross-linking, the dynein may be free to proceed through multiple conformational states during ATP turnover even though it is covalently attached to the microtubule such that higher levels of cross-linking may restrict these conformational changes and inhibit ATP turnover. A third, less interesting possibility is that the chemical modification blocks the ATPase site at high levels of EDC. Nonetheless, the data demonstrate a high level of ATPase activation by microtubules and may serve to define the maximal ATP turnover rate which limits the rate of force production in the cilium.

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