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INTERACTIONS OF *TETRAHYMENA* DYNEIN WITH MICROTUBULE PROTEIN

TUBULIN-INDUCED STIMULATION OF DYNEIN ATPase ACTIVITY

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SUMMARY

The ATPase (EC 3.6.1.3) activity of 30 S dynein from *Tetrahymena* cilia was remarkably stimulated by porcine brain tubulin at pH 10. The activity increased with increasing concentration of tubulin until the molar ratio of tubulin dimer to 30 S dynein reached approx. 10. The optimum of the ATPase activity of 30 S dynein in the presence of tubulin was 1–2 mM for $MgCl_2$ and 2 mM for $CaCl_2$. Increasing ionic strength gradually inhibited the stimulation effects of tubulin. Activation energies of 30 S dynein in the presence and absence of tubulin were almost the same. At the temperatures beyond 25 °C stimulation effects of tubulin disappeared. ATP was a specific substrate even in the presence of tubulin. In kinetic investigations parallel reciprocal plots were observed in a constant ratio of divalent cations to ATP of 2, indicating that tubulin was less tightly bound to 30 S dynein in the presence of ATP than in the absence. The similar results were obtained at pH 8.2. 14 S dynein and the 12 S fragment which have poor ability to recombine with outer fibers were also activated with brain tubulin.

INTRODUCTION

Since the success in extracting dynein ATPase (EC 3.6.1.3) from *Tetrahymena* cilia [1–3], dynein or dynein-like protein has also been found in sea urchin flagella [4–6], mitotic apparatus of sea urchin eggs [7], sea-urchin egg cortices [8], *Chlamydomonas* flagella [9], axostyles of gut protozoa [10] and tubulin fractions from brain [11, 12]. In all the cases, dynein occurs in the structures associated with microtubules which are concerned with cell motility. The motive force of the motility is probably

Abbreviations: EGTA, ethyleneglycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morphorino)-ethanesulfonic acid; CAPS, cyclohexylaminopropanesulfonic acid; MOPS, 3-(*N*-morphorine)propanesulfonic acid; TAPS, *N*-tris-(hydroxymethyl)methyl-3-aminopropanesulfonic acid; EPPS, *N*-2-hydroxyethylpiperazine-*N'*-propanesulfonic acid.

generated through interactions between dynein and tubulin using ATP as energy source. Concerning cilia and flagella, evidence for these interactions has been presented on several points. Sliding can be caused between outer doublets in the axoneme by brief tryptic digestion and addition of ATP [13–15]. The profiles of ATP usage in reactivated cilia and flagella are in good agreement with the characteristics of dynein [16–21]. The movement and ATPase activity of demembranated spermatozoa are inhibited by the addition of anti-dynein antiserum [22]. Lack of dynein arms is observed in immotile human spermatozoa and bronchial cilia from persons with no mucociliary transport [23, 24]. Swelling of flagella caused by ATP is observed by measuring pellet weights [25–27]. Addition of ATP to a suspension of isolated cilia or a mixture of outer fibers and 30 S dynein causes a decrease in turbidity [28–30]. Furthermore, solubilized dynein can be bound to outer doublets in the presence of Mg^{2+} or Ca^{2+} [3, 5, 6, 30–32].

The ATPase activity of dynein is believed to couple with microtubules or tubulin during flagellar and ciliary movement. Dephosphorylation of ATP by glycerinated spermatozoa was shown to depend on the movement of flagella [33–35]. In addition to the evidence that free and outer fiber-bound dyneins show different enzymatic properties [6], Otokawa [36] first showed an increase in the ATPase activity of 30-S dynein from *Tetrahymena* caused by addition of ciliary tubulin prepared from crude dynein fraction. Fragment A, a tryptic fragment of flagellar dynein from sea-urchin spermatozoa was also activated by B-tubulin or A-tubulin [37].

In the present paper porcine brain tubulin was used to investigate stimulation effects of tubulin on the ATPase activity of 30-S dynein from *Tetrahymena* because of its easy preparation and solubility. Kinetic analysis of activation was also performed.

MATERIALS AND METHODS

Preparation of dynein

Dynein was extracted from ciliary axonemes of *Tetrahymena pyriformis*, strain W, as previously described [31]. 30 S and 14 S dynein were partially purified by zonal centrifugation. The 12-S fragment was prepared from 30 S dynein by incubation with trypsin (trypsin: dynein ratio of 1 : 20 by weight, 25 °C for 20 min) and separation by zonal centrifugation [38].

Enzymes were stored at −20 °C in 50 % (v/v) glycerol containing 0.1 mM EDTA, 0.1 mM dithiothreitol and 10 mM Tris · HCl (pH 8.2), and diluted with buffer just before use.

Preparation of tubulin

Tubulin was obtained from porcine brain by temperature-dependent cycles of polymerization and depolymerization [39] using a reassembly buffer (50 mM KCl, 0.5 mM $MgSO_4$, 1 mM EGTA and 5 mM MES/KOH, pH 6.8) containing 1 mM ATP according to Kuriyama [40] and stored in the reassembly buffer at −80 °C. This tubulin fraction was used throughout the following experiments unless otherwise specified.

In some experiments the tubulin fraction prepared by two cycles of polymerization and depolymerization was subjected to gel filtration on Bio-Gel A-5m [41] or

to column chromatography on either DEAE-Sephadex A-50 [42] or phosphocellulose [43] to purify tubulin dimer.

(1) *Gel filtration.* The tubulin fraction (39.1 mg protein, 6 ml) was applied on a Bio-Gel A-5m column (2.6 × 12.5 cm) which was equillibrated with the reassembly buffer containing 0.1 mM GTP. The flow rate was 15.8 ml/cm^2 per h, and of 3.7 ml fractions were collected. The tubulin dimer peak fraction was used for the stimulation assay.

(2) *DEAE-Sephadex A-50 column.* The tubulin fraction (19.5 mg protein) was applied on a DEAE-Sephadex A-50 column (1.2 × 7 cm) equilibrated with 0.1 M MES/KOH (pH 6.8) containing 0.1 mM $MgSO_4$ and 0.1 mM GTP. Tubulin dimer was eluted by stepwise at 0.8 M KCl and desalted by dialysis against the reassembly buffer containing 0.1 mM GTP and 4 M glycerol.

(3) *Phosphocellulose column.* Using the tubulin fraction containing 26.1 mg protein on a 1.2 × 8 cm column, tubulin dimer was eluted as non-adsorbed fraction with the reassembly buffer containing 0.2 mM GTP. In all the above cases, the tubulin dimer fractions were free of high molecular weight components as judged on SDS-polyacrylamide gel electrophoresis.

ATPase assay

In the standard assay, the reaction medium contained 1 mM $MgCl_2$, 1 mM ATP and either 25 mM Tris · HCl (pH 8.2) or 30 mM CAPS/KOH (pH 10.0). Reactions were initiated by the addition of ATP and terminated by adding trichloroacetic acid at a final concentration of 5 % (w/v). Incubation temperature was 25 °C. Liberated inorganic phosphate was determined by the procedure of Fiske and SubbaRow [44] after precipitating the protein, if necessary.

The ATPase activity of dynein was also assayed in the presence of tubulin. Tubulin fraction used in the present experiments showed slight ATPase activity, which was subtracted from the total activity of the mixture of dynein and tubulin. The compositions of the reaction medium, especially buffer and divalent cations, varied from one experiment to another. Details are mentioned later in the text.

In kinetic experiments the reactions were stopped by direct addition of sulfuric acid and then ammonium molybdate and 1-amino-2-naphthol-4-sulfonic acid were added sequentially.

Stock solutions of dynein and tubulin were diluted with the buffer involved in the assay medium to make up the desired protein concentrations just before use.

Protein determination

Protein was determined by the method of Lowry et al. [45] with bovine serum albumin as a standard.

Sucrose gradient centrifugation

Zonal centrifugation was carried out as previously described [31]. Briefly, 5–20 % sucrose gradient was made in 10 mM Tris-HCl (pH 8.2), 0.1 mM EDTA and 0.1 mM dithiothreitol. A Hitachi RPS 25-2 rotor was used at 24 000 rev./min for 20 h in a Hitachi 65P ultracentrifuge.

SDS-gel electrophoresis

Electrophoresis on SDS-polyacrylamide gels was carried out principally as

previously described [31, 32]. The mixture of 5 % (w/v) acrylamide and 0.17 % (w/v) bisacrylamide was polymerized in 20 mM Tris · glycine (pH 8.2) and 0.05 % (w/v) SDS with ammonium persulfate as catalyst. Proteins were reduced with β-mercaptoethanol in 8 M urea. After electrophoresis, proteins were stained with 0.005 % Coomassie brilliant blue in 40 % (v/v) methanol and 10 % (v/v) acetic acid and destained in 7 % (v/v) acetic acid.

Reagents

Tris, CAPS, MOPS and TAPS were purchased from Nakarai Chemicals; MES, EPPS and EGTA from Dojindo Laboratories; all nucleotides except for GTP from Kyowa Hakko Kogyo; GTP, dithiothreitol, trypsin (bovine pancreas, Type III) and soybean trypsin inhibitor (Type I-S) from Sigma; bovine albumin (Fraction V) from Iwai Kagaku Yakuhin; DEAE-Sephadex A-50 from Pharmacia; Bio-Gel A-5m from Bio-Rad Laboratories; and phosphocellulose from Serva Feinbiochemica. Other chemicals were all analytical grade reagents available commercially.

RESULTS

ATPase stimulation of 30 S dynein by brain tubulin

The ATPase activity of 30 S dynein increased up to 35 % at pH 8.2 with increasing concentrations of brain tubulin. The stimulation rate was comparable

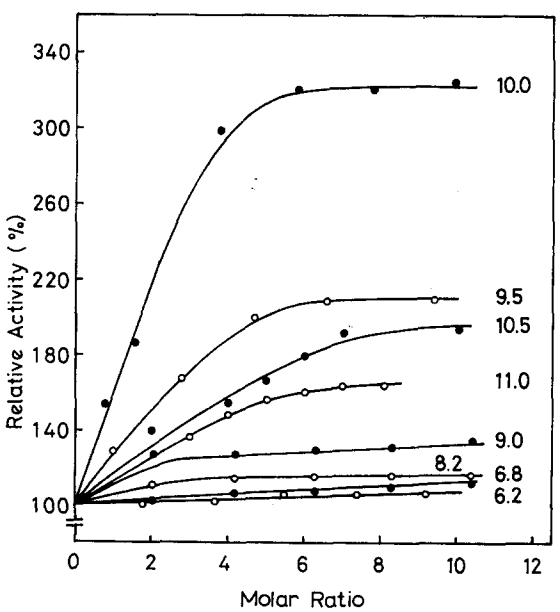

Fig. 1. Effects of brain tubulin on the ATPase activity of 30 S dynein at various pH values. The assay medium (1 ml) consisted of 5 mM $MgCl_2$, 30 mM buffer, 10.6–44.6 μg 30 S dynein, 1 mM ATP and a varying concentration of brain tubulin. The reaction was carried out at 25 °C for 15–20 min. Figures shown beside the curves indicate the pH value of the assay medium. The buffers used were MES (pH 6.2, 6.8), EPPS (pH 8.2), TAPS (pH 9.0, 9.5), and CAPS (pH 10.0, 10.5, 11.0). The pH of each buffer was adjusted by KOH. The unit of molar ratio shown on the abscissa represents the ratio of 110 000 g tubulin dimer to 560 000 g 30 S dynein subunit.

to that of ciliary tubulin (30–50 %, calculated from the figure and table presented by Otokawa, ref. 36). However, low stimulation made it difficult to investigate further analysis of dynein-tubulin interaction in respect of dynein ATPase activity. Experimental conditions, especially pH, were examined for optical stimulation of dynein ATPase. With varying tubulin concentration, stimulation of 30 S dynein ATPase was assayed at several pHs (Fig. 1). Stimulation rate increased with rising pH and showed a maximum at pH 10.0. pHs higher than 10 lowered stimulation rate, although it was still much larger than that at pH 8.2. Neutral and acidic pHs gave little effect on stimulation of dynein. The rate of stimulation occassionally varied from one assay to another probably because of the difference in tubulin preparations. However, stimulation rate was always maximum at pH 10.0. Upon increasing the concentration of tubulin, the ATPase activity of 30 S dynein reached a plateau, that is, 30 S dynein seemed to be saturated with tubulin.

Fig. 1 shows stimulation of 30 S dynein ATPase by tubulin as a function of pH and tubulin concentration. As the stimulation of ATPase was represented as relative activities, absolute values of activities were not clear. To clarify stimulation profile at various pHs, tubulin concentration was kept constant in a tubulin to dynein molar ratio of 10, at which dynein showed a maximum activity at pH 10.0, and ATPase activity was assayed as a function of pH (Fig. 2a). In the absence of tubulin, 30 S dynein ATPase activity had an optimum at pH 9.5. A remarkable activation was induced by tubulin at pH 10. At pHs below 9, the activity was stimulated a little by

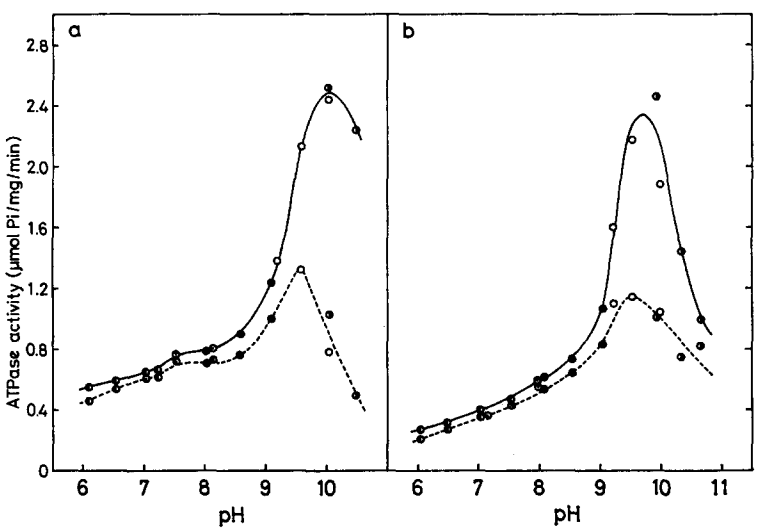

Fig. 2. pH profiles of 30 S dynein in the presence and absence of brain tubulin. (a) Mg^{2+}-activated ATPase. The assay medium (1 ml) consisted of 5 mM $MgCl_2$, 30 mM buffer, 1 mM ATP and 21.2 μg 30 S dynein with or without 42.6 μg brain tubulin. The reaction was carried out at 25 °C for 15 min. The molar ratio of brain tubulin dimer to 30 S dynein subunit was 10.2 (b) Ca^{2+}-activated ATPase. 1 mM $CaCl_2$ was added to the assay medium instead of 5 mM $MgCl_2$. The other conditions were the same as described in (a). The solid and dotted lines indicate the ATPase activity of 30 S dynein in the presence and absence of brain tubulin, respectively. The buffers used were MES (◐), MOPS (○), TAPS (●), a mixture of TAPS and CAPS (○), and CAPS (◑). pH was adjusted by KOH.

addition of tubulin. The results were fundamentally parallel to those in Fig. 1. The Ca-activated ATPase also showed a similar profile with a maximum of stimulation shifting lower pH (Fig. 2b). In the following experiments, ATPase assay was mainly performed at pH 10.0 (30 mM CAPS/KOH) and in a molar ratio of tubulin dimer (M_r 110 000, ref. 40) to 30-S dynein subunit (M_r 560 000, ref. 46) of about 10. Upon calculation of the molar ratio, it was assumed that tubulin and dynein fractions contained no impurities.

Some properties of ATPase stimulation

In the above experiments on pH profiles, ATPase activity was measured at adequate concentrations of Mg^{2+} or Ca^{2+}. Here, in the presence of tubulin, the effects of divalent cations on the activity of dynein were examined by varying concentrations of the ions. As shown in Fig. 3, Mg^{2+} strikingly enhanced the tubulin-induced stimulation of dynein ATPase at concentrations of 1–2 mM. Beyond that concentration, gradual decrease in stimulation was observed until 50 mM, although high level of activation was still maintained. On the other hand, the Ca-ATPase of dynein was also stimulated by tubulin at almost the same rate over a wide range of Ca concentrations, representing the concentration dependency in a fashion similar to the control. From now on, tubulin-induced ATPase stimulation was performed with Mg^{2+} and Ca^{2+} at a concentration of 1 mM each.

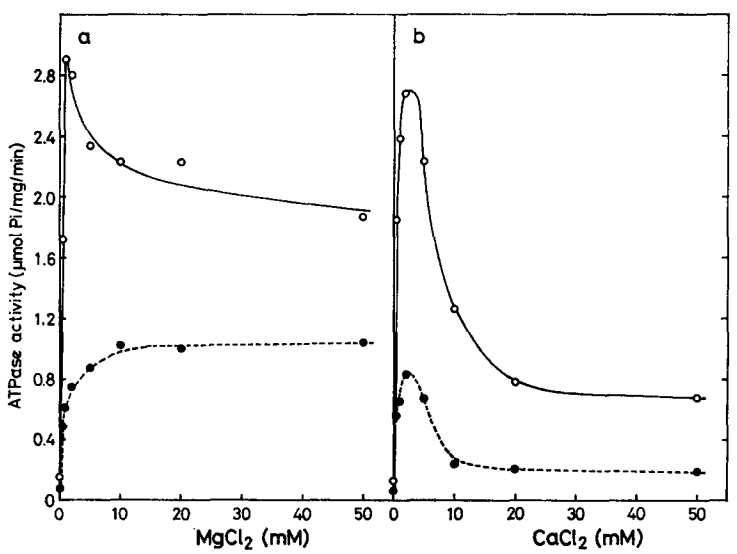

Fig. 3. Effects of divalent cations on the ATPase activity of 30 S dynein in the presence and absence of brain tubulin. (a) Effects of $MgCl_2$. The assay medium (1 ml) consisted of a varying concentration of $MgCl_2$, 30 mM CAPS/KOH (pH 10.0), 1 mM ATP and 14.1 μg 30 S dynein in the presence or absence of 28.4 μg brain tubulin. The reaction was carried out at 25 °C for 15 min. The molar ratio of brain tubulin dimer to 30 S dynein subunit was 10.3. (b) Effects of $CaCl_2$. The assay medium (1 ml) consisted of a varying concentration of $CaCl_2$, 30 mM CAPS/KOH (pH 10.0), 1 mM ATP and 14.2 μg 30 S dynein in the presence or absence of 28.4 μg brain tubulin. The reaction was carried out at 25 °C for 5 min in the presence of tubulin and for 15 min in the absence of tubulin. The molar ratio of brain tubulin dimer to 30 S dynein subunit was 10.2. ○, ATPase activity of 30 S dynein in the presence of tubulin; ●, ATPase activity of 30 S dynein in the absence of tubulin.

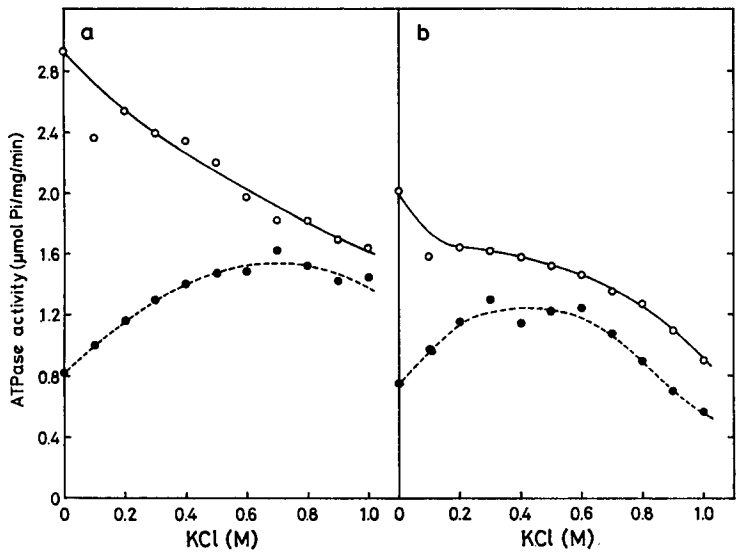

Fig. 4. Effects of KCl on the ATPase activity of 30 S dynein in the presence and absence of brain tubulin. (a) Mg^{2+}-activated ATPase. The assay medium (1 ml) consisted of 1 mM $MgCl_2$, 30 mM CAPS/KOH (pH 10.0), 1 mM ATP, 14.1 μg 30 S dynein with or without 28.4 μg brain tubulin, and a varying concentration of KCl. The reaction was carried out at 25 °C for 15 min. The molar ratio of brain tubulin dimer to 30 S dynein subunit was 10.3. (b) Ca^{2+}-activated ATPase. 1 mM $CaCl_2$ was used instead of $MgCl_2$. The other conditions were the same as described in (a). ○–○, the ATPase activity of 30 S dynein in the presence of brain tubulin; ● --- ●, ATPase activity of 30 S dynein in the absence of brain tubulin.

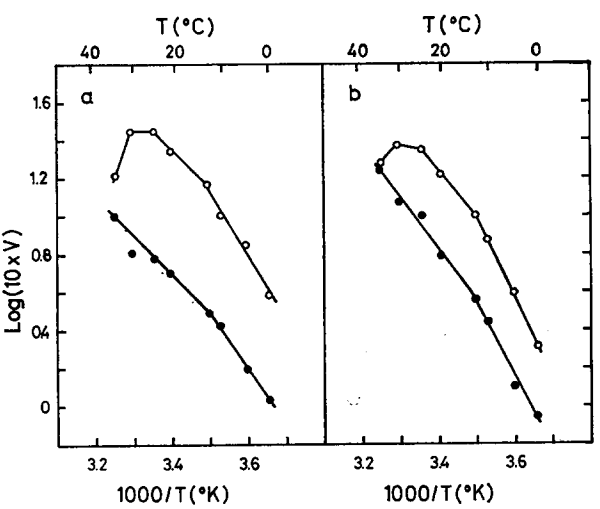

Fig. 5. Arrhenius plots of the log of the ATPase activity of 30 S dynein vs. the reciprocal of the absolute temperature. (a) Mg^{2+}-activated ATPase and (b) Ca^{2+}-activated ATPase. The assay medium (1 ml) consisted of 1 mM divalent cations, 30 mM CAPS/KOH (pH 10.0), 1 mM ATP and 14.1 μg 30 S dynein in the presence or absence of 28.4 μg brain tubulin. The reaction was carried out at varying temperature for 15 min. The molar ratio of brain tubulin dimer to 30 S dynein subunit was 10.3. V indicates the specific activity of 30 S dynein (μmol P_i/mg protein per min). ○, in the presence of brain tubulin; ●, in the absence of brain tubulin.

In order to investigate ionic interaction between dynein and tubulin, ATPase stimulation was assayed under varying conditions of ionic strength. For both Mg- and Ca-ATPase, increasing the KCl concentration gradually lowered stimulation (Fig. 4). Especially Mg-ATPase decreased to the level of the control at 1 mM KCl.

The activation energy of dynein was estimated in the presence and absence of tubulin by assaying at several temperatures (Fig. 5). In the absence of tubulin, the Mg-activated ATPase (the control) showed two lines intersecting at 12 °C. The activation energies were estimated to be 9.1 and 13.5 kcal per mol of dynein subunit in the temperature ranges above and below 12 °C, respectively. In the presence of tubulin the plots were parallel to those of the control at temperatures below 25 °C, and the activation energies were 9.2 and 16.4 kcal per mol of dynein subunit in the temperature ranges above and below 12 °C, respectively. At temperatures beyond 25 °C, stimulation by tubulin decreased or disappeared and the activation energy could not be calculated. This curious tendency can possibly be ascribed to the conformational change of tubulin at higher pHs and temperatures [47]. Similar results were also obtained with Ca-ATPase. The activation energies in the absence of tubulin were estimated to be 8.2 and 17.9 kcal per mol of dynein subunit, similarly intersecting at 12 °C, and 11.6 and 19.4 kcal in the presence of tubulin.

The tubulin-induced stimulation of dynein was examined by substituting several nucleotides for ATP as substrates (Table I). All the nucleotides tested were hydrolysed more in the presence of tubulin than in the absence. However, the specificity of 30-S dynein for ATP did not change in the presence of tubulin.

The tubulin fraction prepared by one cycle of polymerization and depolymerization contained high molecular weight components and revealed slight ATPase activity. It has been reported that dynein-like protein is associated with microtubules reassembled in vitro [11, 12]. In order to clarify that tubulin itself stimulated the ATPase activity of dynein, excluding the possibility that the increase of ATPase activity in the mixture of dynein and tubulin fractions was due to activation of an

TABLE I

SUBSTRATE SPECIFICITIES OF 30-S DYNEIN IN THE PRESENCE AND ABSENCE OF TUBULIN

The assay medium (1 ml) consisted of 1 mM $MgCl_2$, 30 mM CAPS/KOH (pH 10.0), 1 mM nucleotide indicated and 14.1 μg 30 S dynein with or without 28.4 μg brain tubulin. Reaction was carried out at 25 °C for 15 min. The molar ratio of brain tubulin dimer to 30 S dynein subunit was 10.3. Specific activities were expressed as μmol P_i/mg protein per min.

Substrate	Activity			
	+ tubulin		− tubulin	
	Specific	Relative	Specific	Relative
ATP	2.541	100	0.546	100
ADP	0.432	17	0.144	26
GTP	0.097	4	0.027	5
ITP	0.073	3	0.036	7
CTP	0.301	12	0.130	24
UTP	0.097	4	0.045	8

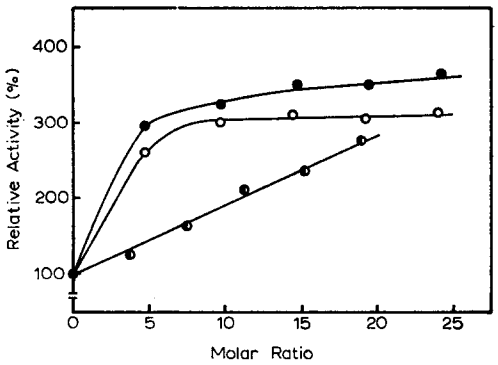

Fig. 6. Effects of purified tubulin dimer on the ATPase activity of 30 S dynein. The assay medium (1 ml) consisted of 1 mM $MgCl_2$, 30 mM CAPS/KOH (pH 10.0), 18.0 μg 30 S dynein, 1 mM ATP and a varying concentration of brain tubulin dimer which was purified by column chromatography on Bio-Gel A-5m (●), DEAE-Sephadex A-50 (◐), or phosphocellulose (○). The reaction was carried out at 25 °C for 20 min.

ATPase involved in the tubulin fraction, tubulin dimer fractions purified by Bio-Gel A-5m, phosphocellulose or DEAE-Sephadex column chromatography were subjected to the stimulation assay (Fig. 6). In fact, tubulin dimer fractions obtained by column chromatography on Bio-Gel A-5m and phosphocellulose stimulated the ATPase activity of dynein in the same magnitude as observed with the tubulin fraction. In the case of the tubulin dimer fraction purified by column chromatography on DEAE-Sephadex A-50, a larger amount of tubulin was required for stimulation of dynein ATPase. Furthermore, tubulin dimers gel-filtered in 0.6 M KCl also showed a stimulation rate of approx. 70 % with a tubulin:dynein molar ratio of 7.5. These seemed to be due to partial denaturation of tubulin by elution at a high salt concentration.

Kinetics of ATPase stimulation

The mechanism of ATPase stimulation by tubulin was investigated using kinetic experiments.

The apparent Michaelis constant was estimated from the Lineweaver-Burk plot by keeping Mg^{2+} constant at 1 mM and the tubulin:dynein molar ratio constant at 10. In the presence and absence of tubulin, the apparent K_m values for ATP at pH 10 were 0.227 and 0.014 mM, respectively (Fig. 7a). The presence of tubulin in the assay medium increased both the values of K_m and maximum reaction velocity. The K_m values of Ca-ATPase were similarly examined at a Ca concentration of 1 mM (Fig. 7b). Two reciprocal plots intersected at the same point on the abscissa meaning no change of K_m in the presence and absence of tubulin. The K_m was estimated to be 0.117 mM.

The effects of divalent cations on the stimulation of dynein ATPase by tubulin (Fig. 3) suggested that either Mg^{2+} or Ca^{2+} directly affected the ATPase activity of dynein and did not take positive part in stimulating, for example, the binding of dynein to tubulin. Then, ATPase activity was assayed by keeping the concentration ratio of Mg^{2+} to ATP constant all over varying concentration ratio of ATP. Fig. 8

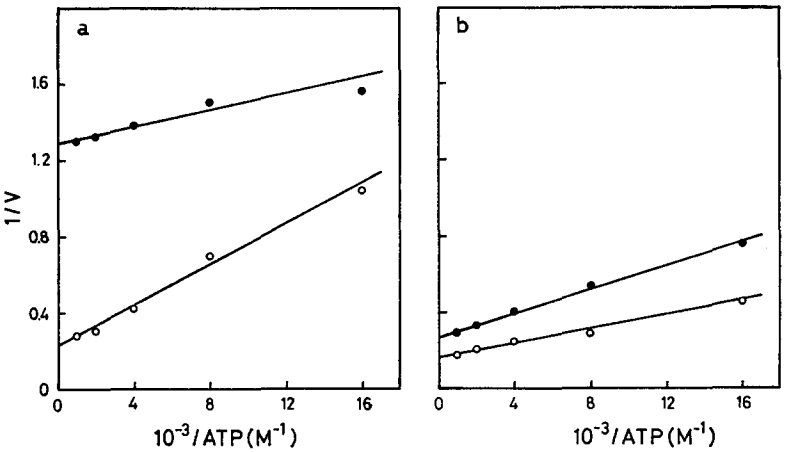

Fig. 7. Lineweaver-Burk plots of the ATPase activity of 30 S dynein in the presence and absence of brain tubulin at a constant concentration of divalent cations. (a) Mg^{2+}-activated ATPase and (b) Ca^{2+}-activated ATPase. The assay medium (1 ml) consisted of 1 mM divalent cations, 30 mM CAPS/KOH (pH 10.0), 14.1 μg 30 S dynein with or without 28.4 μg brain tubulin and a varying concentration of ATP. Reaction was performed at 25 °C. The molar ratio of brain tubulin dimer to 30 S dynein subunit was 10.3. Reaction velocity (V) is in arbitrary units. ●, in the presence of brain tubulin; ○, in the absence of brain tubulin.

illustrates three reciprocal plots obtained by ATPase assays at different molar ratios of tubulin to dynein: the upper line at a molar ratio of zero, namely, in the absence of tubulin (the control), the middle line at 1.0, and the lower at 10.1. Parallelism in reciprocal plots means that the maximum reaction velocity increases at the rate equal to the increasing rate of apparent Michaelis constant with rising concentration of tubulin. The K_m for dynein alone was 0.078 mM. When Ca^{2+} was substituted for Mg^{2+}, parallel plots were also obtained. The K_m for the control was 0.109 mM.

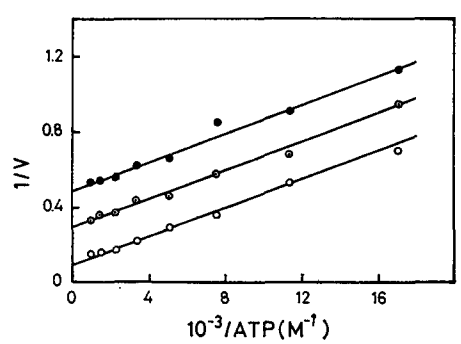

Fig. 8. Lineweaver-Burk plots of the ATPase activity of 30 S dynein in the presence of brain tubulin in a constant ratio of Mg^{2+} to ATP. The assay medium (1 ml) consisted of 30 mM CAPS/KOH (pH 10.0), 14.1 μg 30 S dynein, a varying concentration of brain tubulin and a varying concentration of ATP. The molar ratio of $MgCl_2$ to ATP was 2. Reaction was carried out at 25 °C. ●, in the absence of brain tubulin; ⊙, in the presence of 2.8 μg brain tubulin (the molar ratio of brain tubulin dimer to 30 S dynein subunit was 1.0); ○, in the presence of 28.4 μg brain tubulin (the molar ratio was 10.3).

At pH 8.2, the kinetics similarly gave parallel reciprocal plots and the apparent K_m for the control was 0.016 mM. Therefore, the interaction of tubulin with dynein on ATPase stimulation seems to be independent of pH and divalent cations may be needed only for the formation of metal · ATP complex.

ATPase stimulation of other dynein

The result that the ATPase activity of 30 S dynein was stimulated by brain tubulin led to the design of experiments demonstrating tubulin-induced stimulation of both 14 S dynein and the 12 S ATPase fragment which was produced from 30 S dynein by tryptic digestion [38]. Similar to 30 S dynein, the ATPase activities of 14 S dynein and the 12 S ATPase fragment were stimulated by brain tubulin, producing the same profile as obtained with 30 S dynein at pH 8.2.

DISCUSSION

It was shown that brain tubulin was capable of activating the ATPase activity of 30-S dynein from *Tetrahymena* cilia similar to the result previously reported with axonemal tubulin [36]. The tubulin-induced activation was also observed with 14 S dynein and the 12 S ATPase fragment obtained from tryptic digestion of 30 S dynein. It has been known that 14 S dynein is less active than 30 S dynein in its interactions with microtubules, such as binding [3, 30, 32] and turbidity change [28, 30]. Furthermore, the 12 S fragment has no ability to associate with outer fibers [38]. These results suggest that the activation of dynein ATPase does not involve the binding site at which dynein recombines with outer fibers. Such an activation of dynein ATPase activity seems to be common to tubulin from different origins. This may suggest that an interaction between cytoplasmic tubulin and dynein probably takes part in cell motility such as transport of cytoplasmic components or chromosome migration within the mitotic apparatus, considering various evidence demonstrating cytoplasmic dynein [7, 8, 11, 12]. It is an intriguing problem as to whether cytoplasmic dynein is (similarly) activated by tubulin as is ciliary or flagellar dynein.

The concentration of brain tubulin necessary for activation is lower than that of ciliary tubulin. Brain tubulin used in this study has the ability to polymerize into microtubules just before dilution for the assay, while axonemal tubulin must largely be denatured during solubilization by prolonged dialysis against Tris/EDTA or during thermal treatment [48]. If outer fibers are solubilized by sonication into tubulin dimers possessing polymerizing ability [49], activation might be much stimulated at low concentrations of the tubulin. This kind of experiment combining dynein and tubulin from various materials could provide much information on their interaction.

In order to investigate the mechanism of activation, kinetic analysis was performed. With 1 mM Mg^{2+} both the apparent K_m and maximum reaction velocity increase in the presence of tubulin, whereas when assayed with 1 mM Ca^{2+}, the apparent K_m does not alter in spite of increase in maximum reaction velocity. It is intriguing that the effects of these divalent cations are greatly different irrespective of that both Mg- and Ca-ATPase of 30 S dynein are activated with tubulin at almost the same rate. Since metal · ATP complex has been shown as substrate for dynein from flagellar axonemes of sea-urchin sperm [50], kinetic measurements were carried

out by keeping the ratio of divalent cations to ATP constant (in this experiment, the mol/mol ratio was always 2). Lineweaver-Burk plots were made assuming that all the added ATP formed complexes with metals, without calculation of the concentration of free ATP and metal · ATP complexes because of lack of data about the dissociation constant of metal · ATP complexes at pH 10. The assumption was incorrect but might be valid enough at the concentrations of ATP added. In all the cases for Mg-ATPase at either pH 10 or 8.2 and for Ca-ATPase at pH 10, parallel lines were observed in the reciprocal plots. Therefore, the activation mechanism can be well explained according to Frieden [51]. His paper concerns treatment of data for enzymes, the activities of which are influenced by substances bound specifically to sites other than enzymatically active sites. Here, tubulin can be regarded as such a substance which is usually defined as a modifier. We can assume that there is only one kind of modifier site in 30 S dynein but there would be several binding sites on the modifier. They are assumed to be independent and identical and thus indistinguishable kinetically, i.e. 30 S dynein is composed of several subunits having ATPase activity all of which are thought to be identical. Binding of tubulin to one of the subunits gives no influence to the other subunits in the binding of tubulin or ATP. Frieden describes the mechanism as follows.

1. $E+S \rightleftharpoons ES$ $\qquad ES \xrightarrow{k_5} E+P$
2. $E+M \rightleftharpoons EM$
3. $ES+M \rightleftharpoons EMS$
4. $EM+S \rightleftharpoons EMS$ $\qquad EMS \xrightarrow{k_6} EM+P$

In these equations, S, E, and M are ATP, 30 S dynein (or the subunit) and tubulin, respectively. Rapid equilibrium treatment produces a general form of the equation for the above mechanism.

$$\frac{v_0}{[E]_0} = \frac{k_5(1+k_6 M/k_5 K_3)/(1+M/K_3)}{1+\frac{K_1}{[S]}\left\{\frac{1+M/K_2}{1+M/K_3}\right\}} \tag{1}$$

In Eqn. 1, K_1 to K_4 are dissociation constants describing steps 1 to 4 (i.e. $K_1 = [E][S]/[ES]$) and k_5 and k_6 are the rate constants for breakdown of the ES and EMS complexes, respectively. The number of cases in which reciprocal plots are parallel is limited to two. (i) In essential activation, there is no enzymatic activity in the absence of modifier; the modifier being indispensable for the activity. As a specific case of essential activation with special parameters, parallel lines are obtained. However, this case is distinguished from the other in that the enzyme shows no activity in the absence of the modifier. (ii) If K_2/K_3 is equal to k_5/k_6, Eqn. 1 becomes

$$\frac{v_0}{[E]_0} = \frac{k_5(1+k_6 M/k_5 K_3)/(1+M/K_3)}{1+\frac{K_1}{[S]}\left\{\frac{1+k_6 M/k_5 K_3}{1+M/K_3}\right\}} = \frac{a \cdot k_5}{1+\frac{a \cdot K_1}{[S]}} \tag{2}$$

The apparent K_m and maximum velocity are both multiplied by the same factor a. Reciprocal plots in the presence of the modifier will always be parallel to the plot in

the absence of the modifier. The plot illustrated in Fig. 8 exactly corresponds to case (ii). Activation is expressed as $k_6 > k_5$, resulting in K_3 being larger than K_2. This means that tubulin, the modifier, must be less tightly bound to dynein in the presence of ATP than in the absence of ATP. Since the parallelism of reciprocal plots can also be observed at pH 8.2, the mechanism of association and dissociation of tubulin with dynein would function in the presence of ATP over a wide pH range. This speculated mechanism strongly suggests the binding and dissociation of dynein with tubulin during the cyclic beating of ciliary and flagellar movement. In the mechanism as mentioned above, it is assumed that one molecule of tubulin dimer binds to the subunit of 30 S dynein. This assumption should be checked in further investigations. Moreover, the analyses on turnover of the binding and interaction of dynein with microtubules instead of solubilized tubulin also remain for future study.

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REFERENCES

1 Gibbons, I. R. (1963) Proc. Natl. Acad. Sci. U.S. 50, 1002–1010
2 Gibbons, I. R. and Rowe, A. J. (1965) Science 149, 424–426
3 Gibbons, I. R. (1965) Arch. Biol. (Liège) 76, 317–352
4 Mohri, H., Hasegawa, S., Yamamoto, M. and Murakami, S. (1969) Sci. Papers Coll. Gen. Educ. Univ. Tokyo 19, 195–217
5 Ogawa, K. and Mohri, H. (1972) Biochim. Biophys. Acta 256, 142–155
6 Hayashi, M. and Higashi-Fujime, S. (1972) Biochemistry 11, 2977–2982
7 Weisenberg, R. and Taylor, W. (1968) Exp. Cell Res. 53, 372–384
8 Mabuchi, I. (1973) Biochim. Biophys. Acta 297, 317–332
9 Watanabe, T. and Flavin, M. (1973) Biochem. Biophys. Res. Commun. 52, 195–201
10 Mooseker, M. S. and Tilney, L. G. (1973) J. Cell Biol. 56, 13–26
11 Burns, R. G. and Pollard, T. D. (1974) FEBS Lett. 40, 274–280
12 Gaskin, F., Kramer, S. B., Cantor, C. R., Adelstein, R. and Shelanski, M. L. (1974) FEBS Lett. 40, 281–286
13 Summers, K. E. and Gibbons, I. R. (1971) Proc. Natl. Acad. Sci. 68, 3092–3096
14 Summers, K. (1974) J. Cell Biol. 60, 321–324
15 Lindemann, C. B. and Gibbons, I. R. (1975) J. Cell Biol. 65, 147–162
16 Gibbons, I. R. (1958) J. Cell Biol. 25, 400–402
17 Brokaw, C. J. (1967) Science 156, 76–78
18 Gibbons, B. H. and Gibbons, I. R. (1972) J. Cell Biol. 54, 75–97
19 Brokaw, C. J. and Gibbons, I. R. (1973) J. Cell Sci. 13, 1–10
20 Gibbons, B. H. and Gibbons, I. R. (1973) J. Cell Sci. 13, 337–357
21 Saavedra, S. and Renaud, F. (1975) Exp. Cell Res. 90, 439–443
22 Okuno, M., Ogawa, K. and Mohri, H. (1976) Biochem. Biophys. Res. Commun. 68, 901–906
23 Afzelius, B. A., Eliasson, R., Johnsen, Ø. and Lindholmer, C. (1975) J. Cell Biol. 66, 225–232
24 Afzelius, B. A. (1976) Science 193, 317–319
25 Tibbs, T. (1965) Biochem. J. 96, 340–346
26 Raff, E. C. and Blum, J. J. (1966) J. Cell Biol. 31, 445–453
27 Blum, J. J. and Hayes, A. (1974) Arch. Biochem. Biophys. 161, 239–247
28 Gibbons, I. R. (1965) J. Cell Biol. 26, 707–712
29 Raff, E. C. and Blum, J. J. (1969) J. Biol. Chem. 244, 366–376

30 Shimizu, T. (1975) J. Biochem. 78, 41–49
31 Hoshino, M. (1974) Biochim. Biophys. Acta 351, 142–154
32 Hoshino, M. (1975) Biochim. Biophys. Acta 403, 544–553
33 Brokaw, C. J. and Benedict, B. (1968) Arch. Biochem. Biophys. 125, 770–778
34 Brokaw, C. J. and Benedict, B. (1968) J. Gen. Physiol. 52, 283–299
35 Brokaw, C. J. and Benedict, B. (1971) Arch. Biochem. Biophys. 142, 91–100
36 Otokawa, M. (1972) Biochim. Biophys. Acta 275, 464–466
37 Ogawa, K. (1973) Biochim. Biophys. Acta 293, 514–525
38 Hoshino, M. (1977) Biochim. Biophys. Acta 492, 70–82
39 Shelanski, M. L., Gaskin, F. and Cantor, C. R. (1973) Proc. Natl. Acad. Sci. 70, 765–768
40 Kuriyama, R. (1975) J. Biochem. 77, 23–31
41 Haga, T. and Kurokawa, M. (1975) Biochim. Biophys. Acta 392, 335–345
42 Murphy, D. B. and Borisy, G. G. (1975) Proc. Natl. Acad. Sci. 72, 2696–2700
43 Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y. and Kirschner, M. W. (1975) Proc. Natl. Acad. Sci. U.S. 72, 1858–1862
44 Fiske, C. H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400
45 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
46 Mabuchi, I. and Shimizu, T. (1974) J. Biochem. 76, 991–999
47 Ventilla, M., Cantor, C. R. and Shelanski, M. (1972) Biochemistry 11, 1554–1561
48 Stephens, R. E. (1970) J. Mol. Biol. 47, 353–363
49 Kuriyama R. (1976) J. Biochem. 80, 153–165
50 Hayashi, M. (1974) Arch. Biochem. Biophys. 165, 288–296
51 Frieden, C. (1964) J. Biol. Chem. 239, 3522–3531