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A microtubule-activated ATPase from sea urchin eggs, distinct from cytoplasmic dynein and kinesin

(mitosis/cell motility/GTPase/microtubule-associated protein/taxol)

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ABSTRACT **We report an ATPase activity, present in sea urchin egg cytosol, that is activated by microtubules. The activity sediments at 10 S in sucrose gradients and is clearly distinct from activities at 12 S and 20 S due to cytoplasmic dynein. Potent activation of the ATPase is observed when endogenous egg tubulin is induced to assemble with taxol or when exogenous taxol-stabilized pure brain tubulin microtubules or flagellar outer-doublet microtubules are added. No activation by tubulin subunits or taxol alone is detectable. In contrast to flagellar or cytoplasmic dynein, the microtubule-activated enzyme is unaffected by vanadate or by nonionic detergents and hydrolyzes GTP in addition to ATP. In contrast to kinesin, it cosediments with microtubules in the presence or absence of ATP. The microtubule-activated enzyme may have a role in microtubule-based motility.**

The mitotic spindle has long been understood to represent the machinery for the separation of chromosomes during mitosis. The major structural components of the spindle are microtubules, but their specific role in mitotic movement is not understood. The presence of known mechanochemical enzymes in the spindle has been reported (3, 35), but their functional significance has been disputed by other evidence (refs. 36 and 37; see also below).

We have approached the question of the molecular composition of the spindle by purifying microtubules from the sea urchin egg, a cell in which the microtubules are devoted primarily to a role in mitosis. Because self-assembly of microtubules does not occur in cytosolic extracts of sea urchin eggs, the assembly-promoting drug taxol was used to help in microtubule purification (1, 2). In addition to tubulin, a number of other proteins were identified as components of the purified microtubule preparations. Use of monoclonal antibodies to the non-tubulin proteins has shown (1, 2) so far that five of these proteins are spindle components. It was also found that these proteins are abundant in the unfertilized egg, indicating that the microtubule-associated components of the spindle are stockpiled prior to fertilization. Thus, the egg appears to be a very promising system in which to search for further structural and functional components of the spindle.

The egg cytoplasm contains an abundant ATPase known as "cytoplasmic dynein." This enzyme has received considerable attention because it was found in preparations of isolated mitotic spindles (3) and because some of its pharmacological characteristics were consistent with those reported for reactivated mitotic spindle models (4, 5). Cytoplasmic dynein has enzymological characteristics very similar to those of ciliary and flagellar axonemal dynein (6–9). Rather than serving a role in mitosis, it may, therefore, be a precursor for the cilia that form in the blastula-stage sea urchin embryo (6, 10). Egg microtubules purified using taxol have been reported (11) to contain cytoplasmic dynein, but this represented only a small fraction of the total dynein in the egg.

We report here on the existence of a prominent ATPase activity in sea urchin egg cytosol that is fully dependent on assembled microtubules. Because of this characteristic, the enzyme has been undetected until now. It is distinct from cytoplasmic dynein and represents a new candidate for a role in mitotic motility.

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MATERIALS AND METHODS

Preparation of Cytosolic Extract. Unfertilized eggs were collected from the sea urchin *Strongylocentrotus purpuratus* and prepared for use as described (1). An egg homogenate was prepared in PEM buffer [100 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)/1 mM EGTA/1 mM $MgSO_4$, pH 6.6] containing 0.5 M mannitol, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 0.1 mg of soybean trypsin inhibitor per ml, and an additional 4 mM EGTA. The homogenate was centrifuged at 30,000 × *g* for 30 min at 2°C. The supernatant was recovered and centrifuged at 180,000 × *g* for 90 min at 4°C to obtain a second supernatant, referred to as the cytosolic extract. This was further fractionated by sucrose density gradient centrifugation or treated with taxol to assemble microtubules.

Preparation of Microtubules. Egg microtubules were assembled from the cytosolic extract by addition of 20 μM taxol (10 mM stock solution in dimethyl sulfoxide, obtained from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute). After warming at 37°C for 5 min, the microtubules were collected by centrifugation of the extract through a 10% sucrose cushion at 30,000 × *g* for 30 min at 2°C. The pellets were resuspended in PEM buffer.

Pure calf brain tubulin (3 mg/ml) was prepared by DEAE-Sephadex chromatography (12) and induced to assemble with equimolar taxol (30 μM). The microtubules were washed twice by centrifugation and resuspension in PEM buffer to remove unbound taxol. *S. purpuratus* sperm flagellar outer-doublet microtubules were prepared as in ref. 13.

Sucrose Density Gradient Centrifugation. Density gradient centrifugation of egg cytosolic extracts was carried out on 11-ml linear gradients of 5–20% sucrose in PEM buffer. Samples (1.25 ml) were applied to the gradients, which were centrifuged in a Beckman SW 41 rotor for 16 hr at 31,500 rpm. Gradient fractions were collected by puncturing a hole in the bottom of the centrifuge tube. The following sedimentation markers were centrifuged in duplicate gradients: flagellar latent-activity dynein (21 S), prepared from *S. purpuratus* sperm (14), and thyroglobulin (19 S), catalase (11.3 S), and rabbit IgG (7 S), obtained from Sigma.

Analytical Methods. ATPase assays were performed by mixing samples with an equal volume of 100 mM Tris Cl (pH 8.0) containing 2 mM [γ-^{32}P]ATP (0.5 μCi/μmol, New England Nuclear; 1 Ci = 37 GBq) and 4 mM $MgSO_4$. After incubation at 30°C for 30 min, the reaction was stopped by adding cold perchloric acid to 5%. Charcoal was added to

each sample to adsorb residual nucleotide, and the samples were centrifuged in an Eppendorf microcentrifuge for 2 min. Cerenkov radiation due to the ^{32}P in the supernatant was assayed by scintillation counting to determine the amount of P_i released during the reaction. GTPase assays were conducted in the same manner, using [γ-^{32}P]GTP (New England Nuclear) as substrate. Protein concentration was determined (15) using bovine serum albumin as a standard.

RESULTS

Microtubule Activation of ATPase Activity in Unfractionated Cytosol. In the course of characterizing the proteins associated with taxol-purified sea urchin egg microtubules, we determined the fraction of cytosolic ATPase activity that cosedimented with the microtubules. To our surprise, the total activity in microtubule pellet and post-microtubule supernatant fractions always exceeded the cytosolic level. One explanation for this effect was that ATPase activity was stimulated by the formation of the microtubules.

To evaluate this possibility, we examined more fully the effect of microtubule assembly on cytosolic ATPase activity (Fig. 1). Since self-assembly of microtubules does not occur appreciably in this system, taxol was added to promote assembly. In the absence of taxol, with no assembled microtubules present, a significant amount of ATPase activity was detected, representing the combined activity of a number of cytosolic enzymes (see below). With added taxol, ATPase activity was observed to increase. The extent of the increase was dependent on taxol concentration, up to a level (between 2 and 5 μM taxol) at which maximal assembly of the endogenous tubulin was determined to occur. At the highest taxol concentration, ATPase activity reached a value almost 50% greater than that of the control sample.

Similar stimulation of activity was also observed with addition of exogenous microtubules. Fig. 2 shows the change in activity in the cytosolic extract upon addition of flagellar

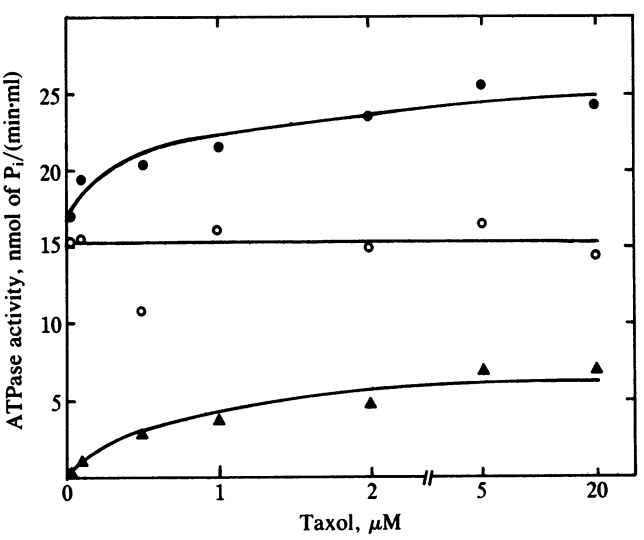

FIG. 1. Stimulation of sea urchin egg cytosolic ATPase activity by microtubule assembly. Taxol was added to a cytosolic extract to the final concentrations indicated, and the samples were incubated at 37°C for 5 min to ensure efficient microtubule polymerization. A portion of each sample was set aside on ice for later ATPase assay. The remainder was centrifuged at 30,000 × g for 30 min at 2°C to sediment microtubules. The pellets were resuspended to volume in PEM buffer containing 20 μM taxol, and both supernatant and pellet were stored on ice. ●, Cytosolic extract; ○, post-microtubule supernatant; ▲, microtubule pellet. Dimethyl sulfoxide (solvent for taxol stock solution) alone had no effect on cytosolic ATPase activity.

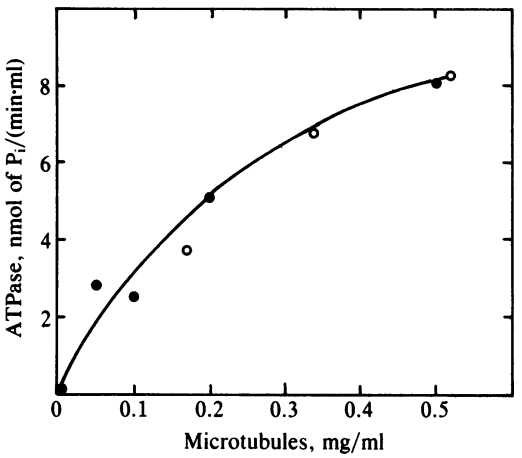

FIG. 2. Stimulation of egg cytosolic-extract ATPase activity by exogenous microtubules. Taxol-stabilized microtubules prepared from pure brain tubulin (●) or flagellar outer-doublet microtubules (○) at the final concentrations indicated were preincubated with egg cytosolic extract for 10 min prior to ATPase assay. Results are expressed as the increase in extract ATPase activity over the level obtained in the absence of microtubules. Data for the outer-doublet microtubules have, in addition, been corrected for the residual dynein activity in the preparation (2 nmol of ATP hydrolyzed per min per mg of protein).

outer-doublet microtubules or microtubules formed from purified brain tubulin. Activity is expressed as the increase over the basal cytosolic level in the absence of microtubules. In both cases, large increases in cytosolic ATPase activity were observed, indicating that the effect was independent of the source of microtubules and was not due to taxol alone.

To analyze the behavior of the increased cytosolic ATPase activity during microtubule purification, the taxol-treated cytosol samples in Fig. 1 were centrifuged to sediment microtubules. The activity of supernatant and microtubule pellet fractions was determined. Sedimentable activity was observed to increase with taxol concentration in a manner closely paralleling the increase in activity in the unfractionated cytosol. The post-microtubule supernatant, however, showed activity equivalent to that observed in the cytosol in the absence of assembled microtubules. This behavior suggested that all of the microtubule-stimulated activity had bound to the assembled microtubules and had then been removed from the cytosolic extract by centrifugation.

In a separate experiment, ATP was added to the cytosolic extract before taxol-induced microtubule assembly to determine whether the apparent association of the ATPase activity with microtubules would be affected (Table 1). The stimulation of cytosolic activity and the level of activity in the microtubule pellets were found to be unchanged by the added nucleotide.

Sedimentation Analysis of the Microtubule-Activated ATPase. To determine whether the stimulated activity corresponded to a known cytosolic enzyme, density gradient centrifugation was employed to fractionate the cytosolic ATPases (Fig. 3). In the absence of added microtubules, ATPase activity resolved into two major peaks corresponding to sedimentation coefficients of 20 S and 6 S (Fig. 3A, filled circles). This pattern of ATPase activity has also been observed in egg cytosolic extracts by Asai and Wilson (16), though under different buffer conditions other investigators have observed major peaks at 12 S and 6 S (8, 9, 17, 18). The 20S and 12S peaks have both been identified as forms of cytoplasmic dynein (8–10, 16–18). We find that the relative abundance of the two forms is dependent on solvent conditions (unpublished data), and a shoulder or minor 12S peak is

Table 1. Cosedimentation of ATPase activity with microtubules prepared in the presence or absence of ATP

Fraction	Total ATPase activity, nmol of P_i/min	
	− ATP	+ ATP
Cytosolic extract	20.0	20.0
Extract plus taxol	29.3 ± 1.6	31.6 ± 1.5
(Increase)	9.3 ± 1.7	11.6 ± 1.5
Post-microtubule supernatant	17.8 ± 1.0	19.3 ± 1.2
Microtubule pellet	10.0 ± 1.6	9.6 ± 1.5

Cytosolic extract, post-microtubule supernatant, and microtubule pellet fractions were prepared as for Fig. 1 or with the addition of 2 mM ATP just prior to microtubule assembly. Taxol (20 μM) was used to promote microtubule assembly. Total activity was normalized from four separate experiments, in which extractable activity ranged from 10.2 to 23.8 nmol per min per ml. The specific activity of the extract ATPase in these preparations was 2.0 ± 0.2 nmol of P_i per min per mg of protein. Total activity in − and + ATP samples showed no significant difference.

sometimes seen under the gradient conditions employed in the present study (see below).

To determine the gradient position of the microtubule-activated ATPase activity, taxol-stabilized microtubules composed of purified brain tubulin were added to each of the gradient fractions during the ATPase assay. This revealed a new peak of ATPase activity at 10 S (Fig. 3*A*, open circles). No stimulation of the 20S and 6S activities was observed. The addition of taxol alone or of unassembled brain tubulin had no effect on activity (Table 2), indicating that, as determined in the unfractionated cytosolic extracts (Figs. 1 and 2), assembled microtubules were specifically required for the appearance of the new activity.

Pharmacological Characterization and Substrate Specificity. To distinguish further between the several cytosolic ATPases, we compared their enzymatic properties under a variety of conditions (Fig. 3 *B* and *C*; Table 3). Data for the 12S cytosolic dynein activity were obtained from those gradients in which enough of this species was present to be assayed accurately (see, for example, Fig. 3*C*).

A peak of GTPase activity was observed at 10 S in the presence of added brain tubulin microtubules, corresponding to the position of the microtubule-activated ATPase (Fig. 3*b*; see Table 3). This suggested that the activated ATPase could also hydrolyze GTP. In contrast, the cytoplasmic dynein fractions at 12 S and 20 S showed relatively little GTP hydrolysis. The 10S GTPase activity was considerably higher than that contributed by the added tubulin, which is itself a well-characterized GTPase (19).

Na_3VO_4 (100 μM) had little effect on the microtubule-dependent 10S ATPase activity, but strongly inhibited the 20S and 12S dynein species (Fig. 3*C*, Table 3). *N*-ethylmaleimide had an opposite effect, inhibiting the dyneins only slightly and the 10S species almost completely. Addition of NaCl to 0.25 M resulted in a 2- to 3-fold stimulation of ATPase activity in the 20S and 12S peaks. In contrast, the microtubule-activated 10S activity was lost. Finally, stimulation of cytoplasmic dynein by nonionic detergent (16, 20), a property shared with latent-activity dynein obtained from sea urchin sperm flagella (14), was observed for both the 20S and 12S activities but was not seen with the microtubule-activated 10S species. Thus, the 10S enzyme has a number of properties strikingly different from those of cytoplasmic dynein.

Characterization of the enzymatic properties of microtubules assembled and sedimented out of egg cytosolic extracts is also presented in Table 3. The results are complicated by the presence of a small amount of dynein (11) in these preparations, as well as a low level of GTPase activity due to the tubulin. Nonetheless, the pharmacological characteristics of this preparation are similar to those of the cytosolic,

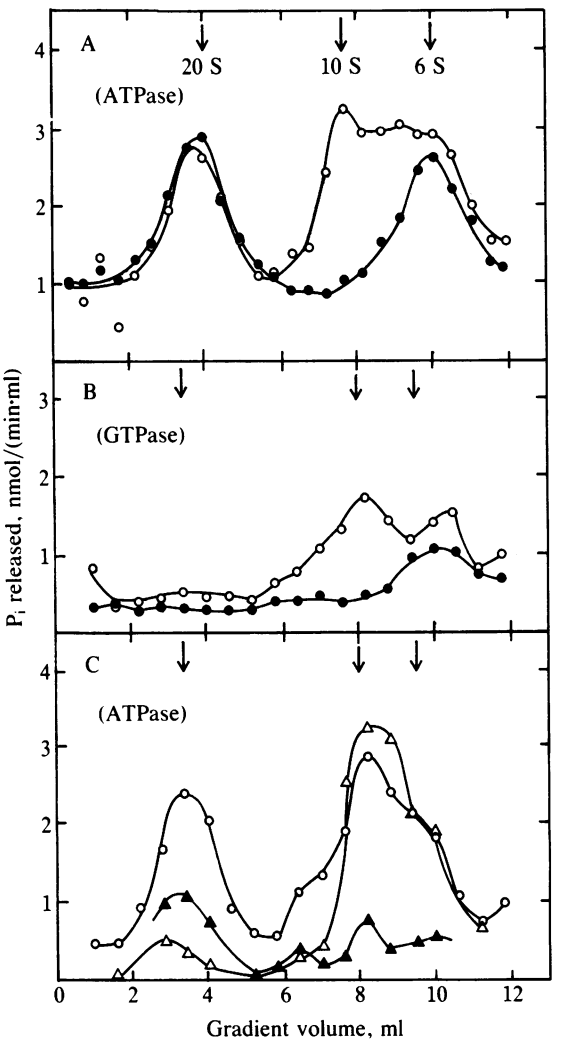

FIG. 3. Sucrose density gradient analysis of egg cytosolic extract. The effect of microtubules on enzymatic activity was determined by addition of purified calf brain tubulin (0.5 mg/ml) that had been assembled into microtubules with the aid of taxol and then washed free of excess drug. Gradient fractions were preincubated with microtubules and, where indicated, pharmacological agents for 5 min at 30°C prior to assay. Arrows show calculated 20S, 10S, and 6S positions. (*A*) ATPase activity in the presence (○) or absence (●) of microtubules. (*B*) GTPase activity in the presence (○) or absence (●) of microtubules. (*C*) ATPase activity in the presence of microtubules, either without enzymatic inhibitors (○) or with 100 μM Na_3VO_4 (△) or 2 mM *N*-ethylmaleimide (▲). Data in *B* and *C* were from a different experiment than that in *A*. GTPase activity due to added microtubules was less than 0.4 nmol per min per mg of tubulin.

Table 2. Requirement for polymeric tubulin in activating 10S ATPase

Addition	ATPase activity, nmol of P_i/(min·ml)	
	Value	Increase over control
None (control)	1.1	—
Taxol	0.9	−0.2
Tubulin	1.2	0.1
Microtubules	3.2	2.1

The 10S sucrose gradient peak fraction from an experiment similar to that shown in Fig. 3 was used. Taxol (10 μM), purified brain tubulin (0.5 mg/ml), or taxol-stabilized brain tubulin microtubules (0.5 mg/ml) were added to the enzyme sample for a preincubation period of 5 min at 30°C prior to assay.

Table 3. Characterization of sucrose gradient fractions and purified microtubule protein

Sample	GTPase/ ATPase ratio	ATPase activity				
		Control, nmol per min per ml	% of control			
			Na_3VO_4 (100 μM)	MalNEt* (2 mM)	NaCl (0.25 M)	Triton X-100 (0.25%, vol/vol)
20 S	0.15	2.02	23.2	62.4	202	256
12 S	ND	0.46	11.8	79.0	285	167
10 S	1.42	2.07	107	12.0	11.1	73.9
6 S	0.52	1.89	112	55.6	96.3	102
Microtubules	0.96	21.0†	75.8	38.4	32.8	77.0

Data for the sedimentation species were from the sucrose density gradient depicted in Fig. 3 *B* and *C*. Values for peak fractions are shown. Data for the 10S peak are presented as the difference between the activity in the presence and in the absence of taxol-stabilized brain tubulin microtubules. Data for the microtubule preparation are from an independent experiment in which sea urchin egg microtubules were sedimented in the presence of 5 mM ATP (*cf.* Table 1) and then washed once in PEM buffer. ND, not determined.

*N-ethylmaleimide.

†Specific activity, 35.0 nmol of P_i per min per mg of protein.

microtubule-activated 10S ATPase. In particular, the microtubule-associated ATPase activity showed very little inhibition by Na_3VO_4 and no apparent stimulation by Triton X-100. The microtubule-associated activity was largely inhibited by 0.25 M NaCl. These characteristics are all consistent with the conclusion that most of the microtubule-associated activity was due to binding of the cytosolic microtubule-activated enzyme, with relatively little due to cytoplasmic dynein. We have, so far, been unable to reextract soluble microtubule-stimulated ATPase activity from microtubules for further purification.

DISCUSSION

We have detected a previously undescribed ATPase activity in the cytosol of the sea urchin egg. The activity has the following characteristics.

Activation by Microtubules. Cytosolic ATPase activity was stimulated by assembly of the pool of unpolymerized tubulin present in the sea urchin egg (Fig. 1) and by addition of preformed microtubules (Figs. 2 and 3). The levels of microtubule polymer that were effective were low and fully consistent with cellular levels. Microtubules composed of egg tubulin, mammalian brain tubulin, or sea urchin flagellar outer doublets were all effective in stimulating activity. The brain microtubules contained only tubulin and, thus, non-tubulin-associated factors were not required. Activity was unaffected by unassembled tubulin subunits (Fig. 1, Table 2) or by taxol alone (Fig. 2, Table 2). From these observations, we conclude that the effective agent in activating the cytosolic ATPase was polymeric tubulin. The data also suggest that the ability of microtubules to activate the enzyme is phylogenetically conserved.

An ATPase in brain tissue has been described (21) that depends on unpolymerized tubulin in the presence of 10 mM Ca^{2+} for its activity. A subsequent report (22) indicated that what was apparently the same enzyme was stimulated ≈2-fold by taxol, though not in a manner entirely correlated with microtubule assembly. We believe that the previously described species is distinct from that described here because of the differences in properties. In addition, we have not detected any microtubule stimulation of ATPase activity in brain cytosolic extracts under the conditions used for our sea urchin egg experiments. We point out that the activity described by us is more likely to be of biological interest because activation occurs under conditions much more nearly approaching those experienced in the cell.

Other ATPases present in microtubule preparations have been described (23–29). None has pharmacological properties comparable to our activity, and the activity of these species was unaffected by microtubules.

Binding of the ATPase to Microtubules. The microtubule-activated ATPase activity appeared to cosediment with microtubules. This conclusion was based on two types of observation. First, all microtubule-stimulated activity was removed from the cytosolic extract by centrifugation, and an equivalent amount of activity appeared in the microtubule pellet (Fig. 1). Second, the pelletable activity showed pharmacological characteristics comparable to those observed for the microtubule-activated 10S ATPase characterized in sucrose density gradients. Since the activity described here appears to be totally dependent on microtubules, we cannot be certain that all of the available enzyme in the cytosolic extract associates with the microtubules. However, this is strongly implied by the observation that the level of ATPase activity in the cytosolic extract and the amount of sedimentable activity appeared to saturate at sufficiently high concentrations of polymerized microtubules (Fig. 1).

Binding of the ATPase activity to microtubules occurred under normal microtubule-assembly conditions (Fig. 1, Table 1). Even with added ATP, full recovery of activity in the microtubule pellet was obtained (Table 1). Thus, the microtubule-activated ATPase behaves as expected for a classical microtubule-associated protein (1, 2). Presumably, therefore, it corresponds to one of the polypeptides already identified in sea urchin egg microtubule preparations (1), though we do not know which one.

Distinctions from Cytoplasmic Dynein and Kinesin. The biochemical and pharmacological properties of the microtubule-activated ATPase, as well as its behavior during microtubule purification, reveal numerous distinctions from cytoplasmic dynein. In addition, they indicate that the activity is not associated with kinesin, a protein of 110–135 kDa that has been implicated in organelle motility in neuronal tissue and in the sea urchin egg (30, 31).

The microtubule-activated 10S enzyme differed from cytoplasmic dynein in sedimentation coefficient, microtubule binding characteristics, and several pharmacological properties. Most notable among these was the insensitivity of the microtubule-activated ATPase to vanadate, which is a potent inhibitor of dyneins (32).

Cytoplasmic dynein was unaffected by added microtubules, in contrast to the microtubule-activated enzyme. Evidence has been presented (33) that the ATPase activity of *Tetrahymena* ciliary dynein can be stimulated by micro-

tubules. This phenomenon is observed only at very high polymer concentrations and seems to be unrelated to that observed by us in the sea urchin egg.

The properties of the microtubule-activated ATPase differ from those determined or expected for kinesin. Our activity appeared to associate with microtubules both in the presence and in the absence of added ATP, in marked contrast to kinesin (30, 31). We have monitored the fate of kinesin in our preparations with the use of a polyclonal antibody (31) and have confirmed that only a small amount of that protein cosediments with microtubules under our microtubule-purification conditions (data not shown).

An additional important distinction with our enzyme is that kinesin has been found not to have detectable ATPase activity (30). Brady (34) noted an increase in ATPase activity in microtubule pellets prepared in the presence of adenosine 5'-[β,γ-imino]triphosphate, which promotes the binding of kinesin (30). However, it was not determined whether the activity was associated with kinesin itself or with other proteins and organelles induced to bind to the microtubules by the ATP analog.

The pharmacological characteristics of our activity cannot be compared directly to those of kinesin, because of its uncertain enzymatic character. However, the pharmacological properties determined for *in vitro* motility associated with kinesin were distinct from those we determined for the sea urchin egg ATPase. Most notably, 2 mM *N*-ethylmaleimide totally inactivated the ATPase but had no effect on kinesin-associated motility. In addition, 100 μM vanadate had no effect on the ATPase but inhibited kinesin effectively.

Functional Implications. We do not know what function the ATPase plays in the cell. However, its activation by microtubules provides a biochemical indication of its likely physiological significance. Activation of the myosin ATPase by polymeric actin was described many years ago, but a comparable effect for a microtubule-related enzyme has not been found until now. Whether this ATPase represents a comparable mechanochemical enzyme, in the present case with a role in mitotic motility, remains to be determined.

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