Bovine brain kinesin is a microtubule-activated ATPase

(axonal flow/organelle transport/intracellular motility/mechanochemical protein)

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ABSTRACT Recently, a protein called kinesin was described, which is capable of inducing movement of inert particles along microtubules. To purify this protein from bovine brain, we used the ability of kinesin to bind to taxol-stabilized microtubules in the presence of inorganic tripolyphosphate. The brain kinesin preparation contained one major polypeptide of 135 kDa and four minor polypeptides of 45–70 kDa. The minor polypeptides were eluted from a gel-permeation chromatography column at the same position as the major component. All the polypeptides of the preparation were capable of binding to the microtubules under identical conditions. The kinesin molecule is most probably a complex of these polypeptides. Brain kinesin had a very low ATPase activity (0.06–0.08 μmol·min^{-1}·mg^{-1} in 3 mM Mg^{2+} at pH 6.7). ATPase activity was strongly stimulated by microtubules (V_{max} = 4.6 μmol per min per mg of kinesin). Microtubule-activated kinesin ATPase had a K_m for ATP between 10 and 12 $\times$ 10^{-6} M and a K_{app} for microtubules (i.e., polymerized tubulin concentration required for a half-maximal activation) of 12–14 $\times$ 10^{-6} M. Kinesin had a significant ATPase activity even without microtubules if 2 mM Ca^{2+} was substituted for Mg^{2+} (V_{max} = 1.6 μmol·min^{-1}·mg^{-1}; K_m = 800 $\times$ 10^{-6} M). Kinesin is therefore a mechanochemical ATPase that is activated by microtubules.

Rapid organelle transport is a typical feature of most eukaryotic cells (1). The movement of small vesicles and inclusions at velocities of >10 μm/min has been especially well-described for nerve cells, where it is called fast axonal transport (2).

Selective depolymerization of microtubules inhibits many forms of organelle motility, including fast axonal transport (reviewed in ref. 1). Furthermore, video-enhanced differential interference contrast microscopy (3) has demonstrated that organelles are able to move along single microtubules (4, 5) and that organelles are capable of moving in cytoplasm extruded from axons in an ATP-containing buffer (6).

Experiments with extruded axoplasm showed that the nonhydrolyzable ATP analog adenyl-5'-yl imidodiphosphate (p[NH]ppA) induced the formation of a stable microtubule–organelle complex (7). Microtubules pelleted from nerve-cell extract after incubation with p[NH]ppA contained a component with a molecular mass of 110 kDa (squid axons) or 120–130 kDa (chicken or bovine brain) (8, 9). This protein was not pelleted with microtubules if the extract was supplemented with ATP.

The protein that was capable of binding to the microtubules in the presence of p[NH]ppA has since been purified and called kinesin (8). Purified kinesin can be adsorbed on carboxymethylated latex beads and induce the movement of latex beads along microtubules. Kinesin-induced latex movement is ATP dependent.

Brady (9) showed that an ATPase activity cosedimented with microtubules mixed with crude brain extract in the presence of p[NH]ppA. However, Vale *et al.* (8) showed that purified kinesin has a very low ATPase activity ($\approx$0.01 μmol·min^{-1}·mg^{-1}). To resolve the apparent discrepancy between the low ATPase activity of kinesin and its ability to induce the ATP-dependent movement of beads, we developed a purification method in which the ATPase activity of kinesin is retained, and we examined the conditions under which purified kinesin could hydrolyze ATP.

MATERIALS AND METHODS

Microtubules and tubulin were purified from bovine brain in buffer A (50 mM imidazole HCl, pH 6.7/0.5 mM MgCl$_2$/0.1 mM EDTA/1 mM 2-mercaptoethanol) supplemented with 50 mM KCl. Microtubules were obtained by one cycle of temperature-dependent polymerization (10) as modified in ref. 11. Tubulin was prepared by phosphocellulose purification of microtubule proteins (12) in buffer A supplemented with 50 mM KCl. Purified tubulin (2.0–2.5 mg/ml) was polymerized in the presence of 20 μM taxol (kindly provided by M. Suffness, Natural Products Branch, National Cancer Institute, Bethesda, MD) for 30 min at 37°C. Polymerization was performed in buffer A with 50 mM KCl/1 mM EGTA.

ATPase activity was determined in buffer B (50 mM imidazole HCl, pH 6.7/50 mM KCl/3 mM MgCl$_2$/0.1 mM EDTA/1 mM EGTA/1 mM 2-mercaptoethanol) unless otherwise indicated. The incubation mixture also contained 2 mM ATP (or different ATP concentrations for the determination of K_m). When we measured the K_m for ATP in the presence of microtubules, concentrations of ATP were too low to measure the cleaved inorganic phosphate directly by colorimetric procedures.

In this case, we used an ATP-regenerating system containing 2 mM phospho*enol*pyruvate and pyruvate kinase at 0.5 unit/ml. Control experiments showed that under these conditions ATP regeneration by pyruvate kinase never limited the rate of ATP hydrolysis. Incubation was carried out at 37°C and was stopped by 3% perchloric acid. The amount of inorganic phosphate released during incubation was determined as described (13).

NaDodSO$_4$/PAGE was carried out using a discontinuous buffer system (14) on 9% gels containing acrylamide/*N,N'*-methylene(bisacrylamide) in a 100:1 (wt/wt) ratio. Gels were stained with Coomassie blue R-250, and the content of individual polypeptides was determined by densitometric scanning.

Protein concentration was determined with Folin phenol reagent (15), using bovine serum albumin as a standard.

Kinesin purification was carried out at 4°C unless otherwise stated. Bovine brain (2.0–2.5 kg) was homogenized in

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Abbreviations: p[NH]ppA, adenyl-5'-yl imidodiphosphate; PPP$_i$, inorganic tripolyphosphate.
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buffer A (pH 7.2) at a ratio of 1 liter per kg of brain. The homogenate was centrifuged at 10,000 × *g* for 30 min and phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM. After this step, all the buffers for kinesin isolation were supplemented with 0.1 mM phenylmethylsulfonyl fluoride.

The supernatant fraction (1 liter, 20–30 mg of protein per ml) was centrifuged again at 20,000 × *g* for 60 min and then 700–800 ml of DEAE-cellulose (Whatman DE-52) in buffer A (pH 6.7) was added. After 1 hr of stirring, the resin was washed with buffer A and proteins were batch-eluted with 2 liters of 150 mM KCl in buffer A. Phosphocellulose (150–175 ml) (Whatman P-11) in buffer A with 150 mM KCl was added to the DEAE-cellulose eluate, and after washing with the same buffer, phosphocellulose was poured into the column. Proteins were eluted from the column with buffer A containing 0.5 M KCl. The eluate (100–200 ml) with a protein concentration of 1.5–1.7 mg/ml was dialyzed against buffer A, and when the KCl concentration in the eluate reached 150 mM, the dialysate was applied to a 20-ml column of DEAE-cellulose equilibrated with buffer A containing 150 mM KCl.

The flow-through fraction (120–130 ml; protein concentration, 1.0–1.2 mg/ml) was dialyzed against buffer A with 1 mM EGTA, clarified by centrifugation at 100,000 × *g* for 30 min, and used for affinity purification. The supernatant (100 ml) was mixed with 30 ml of buffer A supplemented with 12 M glycerol, after which 50 ml of a microtubule preparation was added. Microtubules were assembled by polymerizing phosphocellulose-purified tubulin (2.0–2.5 mg/ml) in buffer A containing 50 mM KCl, 1 mM EGTA, 0.5 mM GTP, and 20 μM taxol. The microtubule/supernatant mixture was supplemented with 2.5 mM inorganic tripolyphosphate (PPP$_i$), and after 20 min of incubation at 20°C, the microtubules were sedimented for 1 hr at 100,000 × *g* and 20°C through a layer of 4 M glycerol in buffer A supplemented with 50 mM KCl/5 μM taxol/1 mM EGTA/2.5 mM PPP$_i$. The pellets were resuspended in 10–12 ml of buffer A containing 2 M glycerol/150 mM KCl/5 μM taxol/5 mM ATP. Dissociated proteins were separated from microtubules by centrifugation at 150,000 × *g* for 20 min at 20°C and further purified by gel-permeation chromatography.

The supernatant was applied to a Sephacryl S-300 column (2.6 × 90 cm) and eluted with buffer A. The column was run at 25 ml/hr; 6-ml fractions were collected and analyzed by NaDodSO$_4$/PAGE. Fractions containing purified kinesin were concentrated on a 2-ml column of DEAE-cellulose. Kinesin was eluted with 150 mM KCl in buffer A.

RESULTS

Brain Kinesin Purification. The original procedure of kinesin purification (6) was based on the affinity binding of kinesin to the microtubules in the presence of 5 mM p[NH]ppA. However, this affinity purification seemed to yield fractions that were inhibited with respect to ATPase activity. We therefore decided to (*i*) explore alternative compounds that could induce microtubule–kinesin binding, and (*ii*) enrich the material used for the affinity purification of kinesin.

Among substances tested, we found that PPP$_i$ could also induce the binding of a 130- to 135-kDa protein to the microtubules. Fig. 1 shows the polypeptide composition of microtubules polymerized in the crude brain extract in the presence of 20 μM taxol and pelleted through a layer of 4 M glycerol in buffer A with 50 mM KCl. The major nontubulin components contained in the pellets were high molecular weight microtubule-associated proteins 1 and 2. The microtubules pelleted from a p[NH]ppA-containing aliquot also contained a 135-kDa polypeptide, most likely, brain kinesin (lane a). A polypeptide with the same electrophoretic
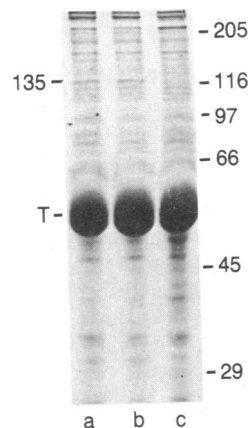

FIG. 1. Cosedimentation of soluble bovine brain proteins with microtubules in the presence of 5 mM p[NH]ppA (lane a), 2.5 mM PPP$_i$ (lane b), and in the absence of exogenous nucleotides or PPP$_i$ (lane c). Microtubules were polymerized in 100,000 × *g* brain supernatant by adding taxol and EGTA (final concentrations, 20 μM and 1 mM, respectively); after 30 min incubation at 37°C, the microtubules were pelleted through a glycerol cushion. The pellets were solubilized in a NaDodSO$_4$ sample buffer and analyzed by electrophoresis. Positions of tubulin (T) and 135-kDa component (135) are shown on the left; positions of molecular size standards are indicated on the right.

mobility was found in the pellet obtained from the PPP$_i$-containing sample, but it was absent from the control (sample without added p[NH]ppA or PPP$_i$) (lanes b and c). It was therefore very likely that PPP$_i$ as well as p[NH]ppA induced kinesin binding to microtubules, and so we decided to use the ability of the putative kinesin to bind to microtubules in the presence of PPP$_i$ for the affinity isolation of this protein.

Before the affinity purification stage, it seemed essential to enrich and concentrate kinesin, because as seen from Fig. 1 (lanes a and b) kinesin was a minor component among other microtubule-associated proteins. Batch chromatography on DEAE-cellulose (with 150 mM KCl elution) resulted in the removal of ≈80% of total protein (Fig. 2, lane b). The DEAE-cellulose eluate was directly applied to phosphocellulose and eluted with 500 mM KCl (lane c). As a result, significant concentration and some additional purification were achieved. After dialysis, which reduced the KCl con-
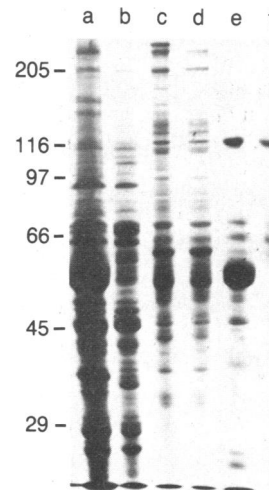

FIG. 2. Electrophoretic analysis of brain kinesin purification. Lanes: a, crude brain supernatant; b, DEAE-cellulose eluate; c, phosphocellulose eluate; d, second DEAE-cellulose eluate; e, microtubule eluate; f, purified kinesin after concentration on DEAE-cellulose.

centration to 150 mM, the phosphocellulose eluate was again applied to a DEAE-cellulose column. At this KCl concentration, kinesin did not bind to the resin, whereas many other extraneous proteins partially removed during the first DEAE-cellulose chromatography (including high molecular weight microtubule-associated proteins) were now completely removed from the preparation (lane d).

The flow-through fraction after the second DEAE-cellulose chromatography was clarified by centrifugation and then used for binding kinesin to microtubules. As described above, 2.5 mM PPP$_i$ was used to induce binding and, after pelleting through a glycerol cushion, kinesin was dissociated with 5 mM ATP and 150 mM KCl from the microtubules stabilized with 5 μM taxol and 2 M glycerol. The preparation was significantly enriched at this stage, with tubulin being the major contaminant (Fig. 2, lane e). (In our latest experiments, we found that the tubulin content of the eluate could be significantly reduced by using buffer containing 20 μM taxol and no glycerol.)

The final purification stage was gel filtration on Sephacryl S-300 in buffer A. Most of the minor components and tubulin aggregates were found in the void volume of the column; the 135-kDa component and four minor polypeptides (see below) were eluted immediately after the void volume, and depolymerized tubulin was eluted several fractions later (Fig. 3).

The fractions containing the 135-kDa polypeptide were

pooled, concentrated on DEAE-cellulose, and analyzed by NaDodSO$_4$/PAGE. In addition to the major 135-kDa component, the preparation also contained minor polypeptides of 70, 66, 58, and 45 kDa (Fig. 2, lane f). The relative proportions of these polypeptides in the purified kinesin fraction were determined to be 54.5% ± 3.9%, 10.5% ± 1.9%, 21.0% ± 2.0%, 9.4% ± 1.5%, and 4.6% ± 1.8%, respectively (10 analyses in three independent isolations).

Incubation of this preparation with the microtubules in the presence of PPP$_i$ resulted in the binding of the 135-kDa polypeptide. The 45- to 70-kDa components also associated with the microtubules in PPP$_i$-containing buffer (Fig. 4, lane b). All of these polypeptides were also found in the microtubule pellet after incubation with p[NH]ppA (lane a). However, none of them cosedimented with the microtubules after incubation with ATP (lane c).

p[NH]ppA-dependent microtubule-binding activity is a characteristic feature of kinesin (8). Therefore, the results presented above show that the 135-kDa component purified from bovine brain is in fact a kinesin polypeptide. The capacity of the minor components for p[NH]ppA-dependent microtubule binding and their coelution together with the 135-kDa polypeptide on a Sephacryl S-300 column suggest that these components are also kinesin polypeptides. These results also show that (as suggested above) not only p[NH]ppA but also PPP$_i$ can induce kinesin binding to microtubules.

ATPase Activity of Kinesin. Under our standard assay conditions, purified bovine brain kinesin had a low ATPase activity (0.06–0.08 μmol·min^{-1}·mg^{-1}) in Mg^{2+}-containing buffers, as originally described (8). However, as shown by Vale et al. (8), purified kinesin can induce the ATP-dependent movement of latex beads on microtubules and of microtubules on glass. Probably the main difference between the conditions of ATPase activity and motility assays was the presence of the microtubules in the latter but not in the former case. Therefore, we added taxol-stabilized microtubules, made of pure tubulin in buffer B with 30 μM taxol, to purified kinesin (the final concentrations of microtubules, kinesin, and taxol being 1.0 mg/ml, 9.4 μg/ml, and 15 μM, respectively). The addition of microtubules dramatically increased the ATPase activity of the kinesin preparation to as high as 2.1 μmol per min per mg of kinesin in this particular experiment. The ATPase activity of pure taxol-stabilized microtubules was undetectable by our colorimetric assay for inorganic phosphate, while the ATPase activity of kinesin alone was <0.08 μmol·min^{-1}·mg^{-1} as mentioned above. Thus, microtubules containing phosphocellulose-purified tubulin were capable of stimulating the ATPase activity of the kinesin preparation.
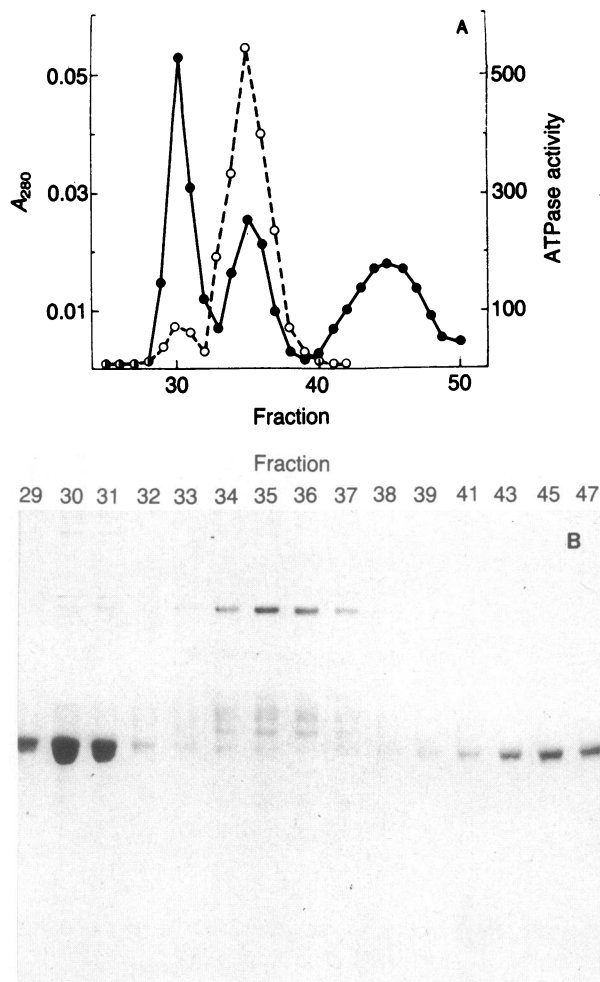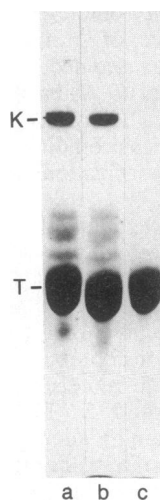

FIG. 3. Gel filtration chromatography of brain kinesin on Sephacryl S-300. Bovine brain proteins obtained by microtubule affinity purification were applied to Sephacryl S-300 column. (A) Elution profile (●) and ATPase activity (○; expressed as nmol of P$_i$ per min per fraction) of column fractions. (B) NaDodSO$_4$/PAGE analysis of fractions.

FIG. 4. Cosedimentation of purified kinesin with microtubules in the presence of 5 mM p[NH]ppA (lane a), 2.5 mM PPP$_i$ (lane b), and 5 mM ATP (lane c). Positions of kinesin (K) and tubulin (T) are shown on the left.

Microtubule-activated ATPase was eluted from a Sephacryl S-300 gel-filtration column as a single peak corresponding precisely to the elution peak of the kinesin polypeptides. Furthermore, the specific ATPase activity of the kinesin peak fraction was nearly constant across the peak of eluted kinesin polypeptides. These data indicate that kinesin itself, rather than contaminating proteins, possesses ATPase activity.

Table 1 shows the influence of Mg^{2+} and Ca^{2+} on ATPase activity of pure kinesin (without microtubules). In a Ca^{2+}- and Mg^{2+}-free buffer with 5 mM EDTA (added to bind any residual divalent cations), kinesin had a very low activity. No activity was detected in 5 mM EDTA in a high ionic strength buffer (0.55 M KCl), optimal for the ATPase activity of myosin. However, kinesin had a substantial activity in 2 mM Ca^{2+}.

The kinetic parameters of ATP hydrolysis by kinesin were also studied. At a low ATP concentration, the amount of inorganic phosphate released by kinesin was too small for our colorimetric assay. An ATP regenerating system including phospho*enol*pyruvate and pyruvate kinase was therefore added to our usual ATPase assay system. Control experiments showed that if the kinesin concentration was <15 μg/ml, increasing the phospho*enol*pyruvate and pyruvate kinase concentrations above the levels present in the regenerating system (2 mM and 0.5 unit/ml, respectively) did not change the amount of phosphate released by kinesin. Therefore, the rate of ATP regeneration did not limit the nucleotide hydrolysis by kinesin.

A Lineweaver–Burk plot of ATP hydrolysis by a kinesin/ microtubule mixture is shown in Fig. 5A. Kinesin ATPase fits simple Michaelis kinetics with the corresponding K_m of kinesin for ATP being 10–12 × 10^{-6} M.

The same kinetics were observed when ATP was hydrolyzed by kinesin alone in 2 mM Ca^{2+}, but the K_m for ATP was much higher than in the case of microtubule-activated ATPase ($\approx$800 × 10^{-6} M) (Fig. 5B).

The plot of kinesin activation by microtubules is shown in Fig. 6. This activation was studied at four kinesin concentrations (4.7, 9.4, 18.8, and 37.6 μg/ml). At all the kinesin concentrations explored, the K_{app} (i.e., the polymerized tubulin concentration required for half-maximal activation) was 12–14 × 10^{-6} M. The V_{max} of ATP hydrolysis at saturating concentrations of ATP and microtubules was 4.6 μmol·min^{-1}·mg^{-1} of kinesin (Fig. 6), and the V_{max} in 2 mM Ca^{2+} without microtubules was 1.6 μmol·min^{-1}·mg^{-1}.

Kinesin was not activated by depolymerized tubulin or muscle F-actin.

Microtubule-activated kinesin ATPase was not strictly specific for ATP. It could hydrolyze all the other nucleoside triphosphates (GTP, ITP, UTP, and CTP), and the rates of their hydrolysis at 2 mM initial concentrations were, respec-

Table 1. Effects of mono- and divalent cations on kinesin ATPase

Ionic condition*					ATPase activity, μmol·min^{-1}·mg^{-1}
KCl, mM	CaCl$_2$, mM	MgCl$_2$, mM	EDTA, mM	EGTA, mM	
50	—	—	—	—	0.15
50	—	—	5	—	0.03
550	—	—	5	—	0.02
50	—	2	—	—	0.08
50	—	10	—	—	0.02
50	—	3	—	1	0.06
50	2	—	—	—	1.15
50	10	—	—	—	0.57

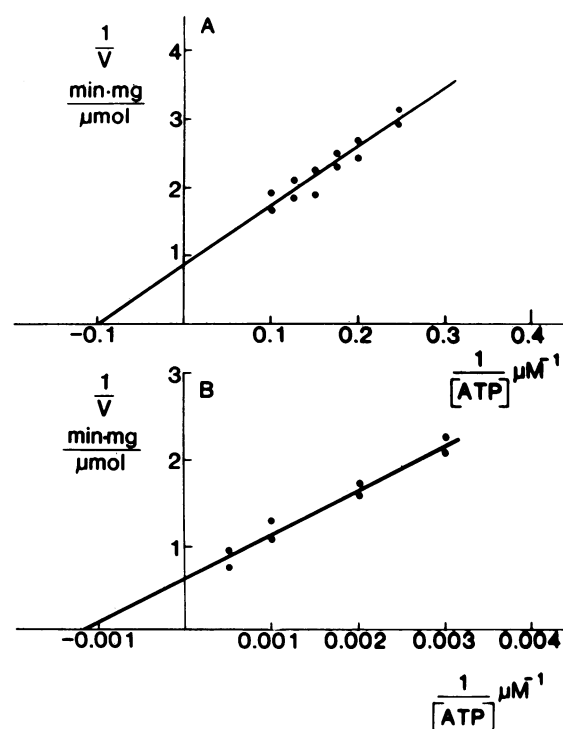*The buffer also contained 50 mM imidazole hydrochloride (pH 6.7), 1 mM 2-mercaptoethanol, 2 mM ATP.

FIG. 5. Determination of the K_m of kinesin for ATP in the presence of microtubules (A) (kinesin concentration, 10.5 μg/ml; microtubule concentration, 0.8 mg/ml) and in the absence of microtubules (B) (kinesin concentration, 20 μg/ml; Ca^{2+} concentration, 2 mM).

tively, 81%, 68%, 41%, and 85% of the ATP hydrolysis rate. ADP was not hydrolyzed by kinesin.

As shown above, PPP_i and p[NH]ppA induced the forma-
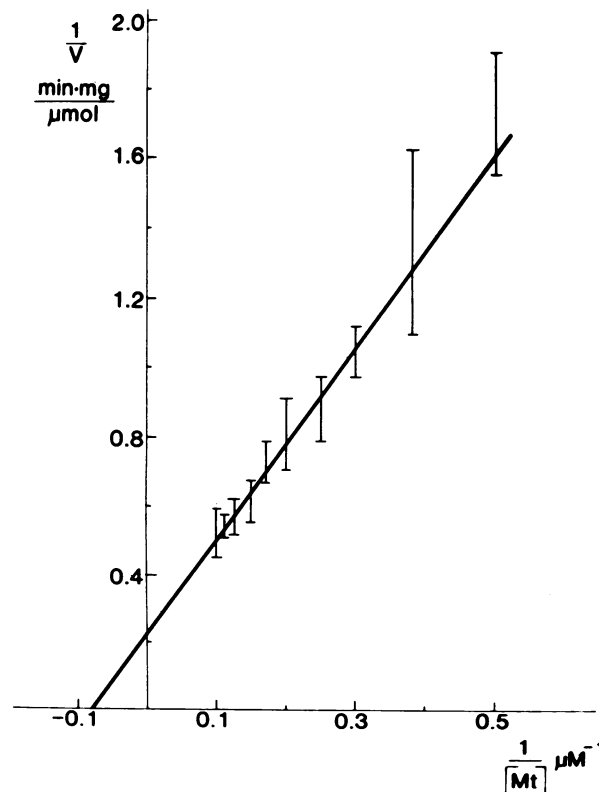

FIG. 6. Kinetics of kinesin activation by microtubules (Mt). Kinesin concentrations 4.7, 9.4, 18.8, and 37.6 μg/ml.

tion of a stable kinesin–microtubule complex. One may suggest that both analogs inhibit ATP hydrolysis. In fact, they were competitive inhibitors of microtubule-activated kinesin ATPase with $K_i = 60 \times 10^{-6}$ M for p[NH]ppA and $K_i = 250 \times 10^{-6}$ M for PPP$_i$.

When kinesin was treated with 4 mM *N*-ethylmaleimide followed by 40 mM 2-mercaptoethanol inactivation, its ATPase activity fell only to 80% of the control value. Vanadate (2 μM) did not inhibit kinesin ATPase, while vanadate (10 μM) inhibited it, but only slightly (27%).

DISCUSSION

The results presented above demonstrate that the protein binding to microtubules in the presence of PPP$_i$ possesses ATPase activity and that this activity is strongly stimulated by the presence of microtubules. It is highly probable that the protein we isolated from bovine brain is identical to the kinesin described by Vale *et al.* (8). Several data are consistent with this suggestion. First, the polypeptide composition of our preparation is similar (although not identical) to that of kinesin (8). Second, the protein described in this work can bind to microtubules not only in the presence of PPP$_i$, but also in the presence of p[NH]ppA, which is a typical property of kinesin. Third (probably most important) is the existence of ATPase activity, the very property expected for kinesin, because it can perform ATP-dependent mechanical work (8).

Our kinesin preparation contained not only a major 135-kDa protein, but also four minor 45- to 70-kDa proteins. We found that these polypeptides could not be resolved from the major kinesin chain by chromatography or microtubule-binding, which is in good agreement with the results reported by Vale *et al.* (8). They also found that the low molecular mass components could also bind to a column with an immobilized monoclonal antibody to the major polypeptide (16). Taken together, these results strongly suggest that the minor components form a complex with the 135-kDa subunit, although the exact quaternary structure of a kinesin molecule has yet to be determined.

Our results also clearly indicate that kinesin itself, rather than some contaminating protein, exhibits ATPase activity. First, the amount of the contaminating proteins in our preparation could not exceed 5%. If the contaminating protein is an ATPase, its specific activity would have to be at least 80 μmol·min^{-1}·mg^{-1}, which is too high to be reasonable. Second, the elution position of ATPase activity during Sephacryl S-300 chromatography exactly coincided with the elution position of kinesin polypeptides.

Any mechanochemical system that works cyclically should periodically associate with the structure along which it moves and then dissociate from this structure. For the two known mechanochemical ATPases (myosin, dynein) this association–dissociation cycle is coupled to ATP hydrolysis. It is likely that the same relationship pertains for kinesin. The ATPase activity of kinesin is stimulated by microtubules in much the same way as dynein and myosin ATPases are stimulated by microtubules and F-actin, respectively (17, 18).

However, the mechanism of kinesin stimulation differs from that of myosin and dynein. The rate-limiting step in the cycle of ATP hydrolysis by myosin and dynein is the dissociation of the products of hydrolysis. This step is accelerated by the binding of cytoskeletal structures, which promote dissociation. ATP, on the other hand, dissociates the cytoskeletal structures from the active center, preparing

the ATPase for the next cycle (17, 18). The addition of p[NH]ppA stops the cycle at this stage, inducing dissociation of an ATPase (myosin or dynein) from the second protein component.

On the other hand, it may be reasonable to assume that under physiological conditions the rate-limiting step of the mechanochemical cycle of kinesin is the hydrolysis of ATP itself and that the active site of kinesin ATPase may be formed only after the binding of the kinesin–ATP complex to microtubules. After ATP hydrolysis, the kinesin and hydrolysis products may dissociate from the microtubules, thus allowing kinesin to again become available for another round of ATP and microtubule binding. Under these conditions, the addition of p[NH]ppA could "freeze" the cycle at the state of ternary complex formation (kinesin–p[NH]ppA–microtubule). Although this mechanism remains to be proved for kinesin, it has been clearly demonstrated in several other cases—e.g., during GTP hydrolysis by complexes of elongation factor Tu (or G) with a ribosome (see ref. 19 for a review). Two questions therefore remain to be elucidated concerning kinesin ATPase: the molecular mechanism of the kinesin ATPase working cycle, and, most important, the mechanism of coupling of ATP hydrolysis to mechanochemical work.

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