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Evidence that the 116 kDa component of kinesin binds and hydrolyzes ATP

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Kinesin was prepared from bovine brain as described previously for studies of translocation. A major component of kinesin, (116 kDa) was shown to undergo specific photocrosslinking with [α-^{32}P]ATP, indicating it was an ATP-binding polypeptide. A low ATPase activity associated with kinesin was stimulated up to 5-fold by microtubules to a specific activity of 14 nmol·min^{-1}·mg^{-1}. *N*-Ethylmaleimide inhibited both [α-^{32}P]ATP binding to the 116 kDa polypeptide and microtubule-stimulated ATPase activity, suggesting that the 116 kDa polypeptide was the catalytic subunit of kinesin. Though the ATPase activity associated with kinesin is low, it may be sufficient to support motility assuming it is coupled to the velocity of translocation.

Kinesin; ATP binding; [α-^{32}P]ATP; Direct photocrosslinking; ATP hydrolysis; Mechanochemical coupling

1. INTRODUCTION

Recently, a novel factor, called kinesin, has been partially purified from squid optic lobe, bovine brain and sea urchin eggs and shown to induce both microtubule gliding and bead and organelle translocation along microtubules in vitro [1–4]. Initial characterizations have suggested that kinesin, which has an apparent molecular mass of 110–134 kDa, depending on the species, represents a new class of microtubule-based mechanoenzyme, distinct from the dynein-microtubule system of cilia and flagella. While it is generally thought that kinesin is an ATPase, demonstration and characterization of its enzymatic activity have proven difficult, as the ATPase activities associated with translocation-competent kinesin preparations have been reported to be quite low [1–3]. Recently, it has been shown for sea urchin egg kinesin that low microtubule-dependent ATPase activity and translocating activity respond similarly, although not identically, to inhibitors [4].

We report that bovine brain kinesin, prepared as described previously for translocation studies, is enriched in a 116 kDa polypeptide which binds ATP. We show that a low rate of ATP hydrolysis associated with this ATP-binding polypeptide is activated 5-fold by microtubules to a specific activity of 14 nmol·min^{-1}·mg^{-1}. Both ATP binding and ATP hydrolysis are inhibited by *N*-ethylmaleimide (NEM), suggesting that the 116 kDa polypeptide is the catalytic subunit of kinesin. We speculate that the low level of ATPase activity associated with kinesin could satisfy the energy requirements of translocation if ATP hydrolysis by kinesin were coupled to the velocity of motility, as in the dynein-microtubule system of cilia and flagella. A preliminary description of this work was reported earlier [5].

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2. MATERIALS AND METHODS

2.1. *Biochemicals*

ATP (vanadate-free), GTP, NEM, NaN_3, phospho*enol*pyruvate (PEP), pyruvate kinase and dinitrophenylhydrazine (DNPH) were purchased from Sigma (St. Louis, MO), orthovanadate from Fischer (Springfield, NJ), and *erythro*-9-[3-(2-hydroxynonyl)]adenine (EHNA) from Burroughs-Wellcome (Research Triangle Park, NC). Taxol was generously provided by Dr Matthew Suffness (National Cancer Institute).

2.2. *Preparation of bovine brain kinesin*

The protocol of Vale et al. [1] was followed without modification. Briefly, kinesin was isolated from crude soluble extracts of bovine brain by binding to microtubules in the presence of AMP-PNP followed by dissociation from microtubules by ATP. The crude kinesin (0.75 ml) was fractionated by gel-permeation chromatography on a 1 × 47 cm agarose A5m column (BioRad) equilibrated with kinesin buffer (KB) (100 mM KCl, 50 mM Tris-HCl, 5 mM $MgSO_4$, 0.5 mM EDTA, pH 7.6). In some experiments, 3 ml crude kinesin was fractionated on a 2.1 × 100 cm agarose A5m column.

2.3. *Direct photocrosslinking with [α-^{32}P]ATP*

Kinesin (0.04 mg/ml) in KB was mixed with ~5 × 10^{-8} M [α-^{32}P]ATP (3000 Ci/mmol, New England Nuclear) (total sample volume, 0.044 ml) in 96-well microtiter plates on ice and irradiated for 10 min at a distance of 4 cm with 254 nm light (Mineralight, Ultraviolet Products, San Gabriel, CA) [6–8]. Samples were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 3.2–12% gradient of acrylamide. Gels were stained with silver [9], dried and autoradiographed for 7–12 days at −70°C using RX Medical X-ray film (Fuji PhotoFilm, Japan) and an intensifying screen.

2.4. *Assays for ATP and GTP hydrolysis*

ATPase and GTPase activities were assayed principally by incubating samples with 0.1 mM [γ-^{32}P]ATP or 0.1 mM [γ-^{32}P]GTP (3 Ci/mmol, Amersham) in KB for 60–90 min at 37°C in a total volume of 0.2 ml, then determining the liberation of $^{32}P_i$ [10]. In a few experiments, ATPase and GTPase activities were determined by a coupled enzyme assay based on the colorimetric determination of pyruvate [11]. Briefly, samples were incubated with 0.5 mM PEP, 10 U/ml pyruvate kinase, and 0.1 mM ATP or 0.1 mM GTP in KB for 60 min at 37°C in 96-well microtiter plates in a total reaction volume of 0.11 ml. The reaction was quenched by adding 8 μl of a solution of 2.5 mM DNPH in 3 N HCl followed by incubation at 37°C for 10 min. Color was developed by adding 40 μl of a solution of 0.1 M EDTA in 2.5 N NaOH, and absorbance was determined at 450 nm on a microtiter plate reader (Bio-Tek model EL-307). Appropriate controls were performed to rule out effects of inhibitors on pyruvate kinase. No significant difference was noted between results obtained using the radioactive and the colorimetric assays for ATPase and GTPase activities.

2.5. *Microtubules*

Microtubules were prepared from the high-speed supernatant of bovine brain by repolymerization in the presence of 20 μM taxol and 0.5 mM GTP followed by treatment with microtubule assembly buffer (0.1 M Pipes, pH 6.7, 1 mM $MgSO_4$, 1 mM EGTA containing 0.35 M NaCl, 20 μM taxol, 0.5 mM GTP, to remove microtubule-associated proteins (MAPs) [1]. The MAP-depleted microtubules (final concentration ~10 mg/ml) were stored in liquid nitrogen.

3. RESULTS

Kinesin was identified on the basis of its method of isolation from crude extracts; its large Stokes radius as indicated by its position of elution from an agarose A5m column ($K_{av} \cong 0.7$) (identical to the elution position of 12 S cytoplasmic dynein from sea urchin eggs, which had an estimated molecular mass of 460 kDa [12]) and the presence of two major polypeptides (116 and 63 kDa; fig.1, gel) [1,2].

Irradiation of kinesin with 254 nm light in the presence of [α-^{32}P]ATP resulted in the incorporation of radioactive label into the 116 kDa polypeptide (fig.1, center lane). Labeling was shown to be specific for [α-^{32}P]ATP by controls demonstrating that label incorporation depended on irradiation and was quenched by non-radioactive ATP (fig.1,

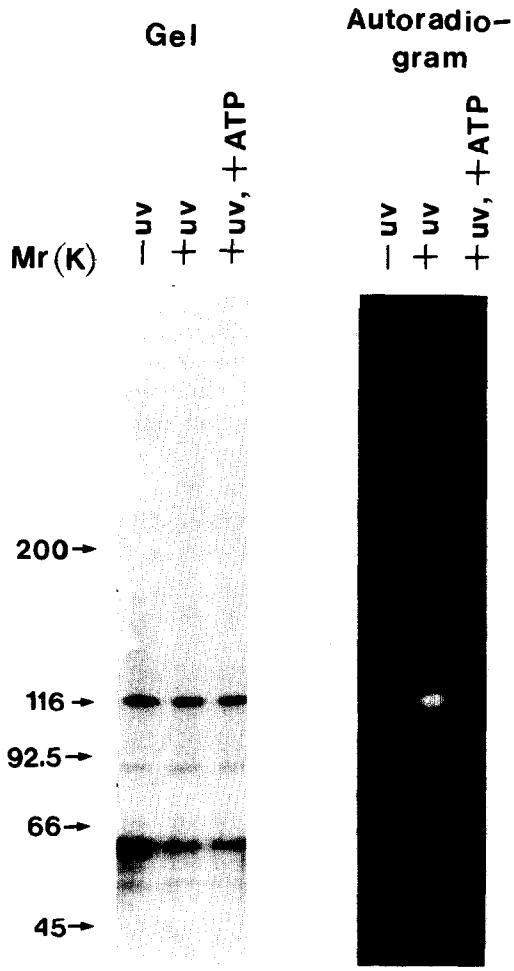


Fig.1. Direct photocrosslinking of the 116 kDa component of kinesin with [α-^{32}P]ATP. (Left-hand panel) SDS gel electropherogram of kinesin; (right-hand panel) autoradiogram of the gel on the left. (First lane) Kinesin + [α-^{32}P]ATP, not irradiated with UV light; (center lane) kinesin + [α-^{32}P]ATP, irradiated with UV light; (third lane) kinesin + [α-^{32}P]ATP + 5×10^{-5} M non-radioactive ATP (1000-fold stoichiometric excess), irradiated with UV light.

first and third lanes, respectively). Similar results were obtained in six experiments with three separate preparations of kinesin. These results indicated that one of the two major kinesin components (116 kDa) was an ATP-binding polypeptide.

When fractions from the agarose gel filtration column were assayed for ATPase activity, a peak was found at $K_{av} \cong 0.56$ (fractions 27–31) (fig.2).

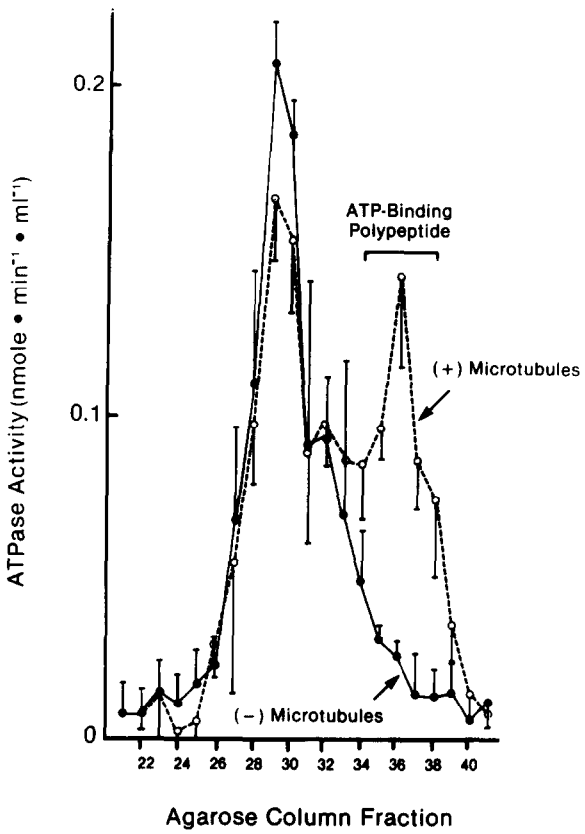


Fig.2. Stimulation by microtubules of ATPase activity which coelutes with the ATP-binding polypeptide of kinesin from an agarose A5m column. (●—●) Column fractions alone; (○---○) column fractions plus microtubules (0.4 mg/ml) stabilized with 0.1 mM GTP and 10 μM taxol (background microtubule ATPase activity has been subtracted). Bars indicate SE. Details of statistical analysis are reported in table 1.

This peak was associated with a high molecular mass polypeptide of apparent molecular mass >300 kDa (not shown). A low level of ATPase activity was associated with the ATP-binding polypeptide of kinesin (fractions 34–38, fig.2). When column fractions were assayed in the presence of microtubules, GTP and taxol, a new peak of ATPase activity was identified which co-eluted with the 116 kDa ATP-binding polypeptide of kinesin. Activation of kinesin-associated ATPase activity was 5-fold in the peak fraction and averaged 3.7-fold over the entire peak (fig.2, table 1). Activation was dependent on the presence of microtubules; controls indicated that GTP and

Table 1

Activation by microtubules of ATPase activity associated with the 116 kDa ATP-binding polypeptide of kinesin

Sample	Mean ATPase activity ($nmol \cdot min^{-1} \cdot ml^{-1}$)	Change (%)[a]	Statistical significance[a]
High molecular mass polypeptide (fractions 27–31)[b]			
Without microtubules	0.13[c]		
In the presence of microtubules	0.11[c]	−15	0.46 (not significant)
Kinesin (fractions 34–38)[b]			
Without microtubules	0.026[c]		
In the presence of microtubules	0.096[d]	+370	0.02

[a] Based on a 2-way analysis of variance (ANOVA) in which the ATPase activities of the indicated agarose column fractions in the absence and presence of microtubules were treated as repeated measures of ATPase activity [13]
[b] Refers to agarose column profile presented in fig.2
[c] Mean of indicated agarose column fractions [n = 15 (3 experiments × 5 fractions)]
[d] Mean of indicated agarose column fractions after subtraction of microtubule ATPase activity [n = 20 (4 experiments × 5 fractions)]

taxol (which were included in the assay mixture to stabilize the microtubules) failed to stimulate ATPase activity.

The activation of ATPase activity in the presence of microtubules was found not to depend on microtubule concentration (fig.3). This result indicated that the ATPase which was activated was a component of the kinesin fraction and not of the microtubule fraction.

Investigation of the enzymatic and pharmacological properties of the microtubule-activated, kinesin-associated ATPase activity revealed high GTPase activity, sensitivity to inhibition by millimolar concentrations of AMP-PNP and NEM, and relative insensitivity to inhibition by vanadate (table 2). These properties were consistent with characteristics of ATP-dependent translocation reported previously [1–4,14]. Insensitivity to inhibition by EHNA and NaN_3 ruled out contamination of the preparation by, respectively, dynein-like [10] or mitochondrial-like [15] ATPase activities.

To investigate the relationship between ATP hydrolysis and the 116 kDa ATP-binding polypeptide, we examined the effects on ATP binding of NEM and GTP. These agents acted, respectively, as an inhibitor and a substrate of the microtubule-stimulated ATPase (table 2); therefore, each could be expected to inhibit ATP binding to the ATPase active-site subunit of kinesin. The results presented in fig.4 indicate that NEM and GTP each inhibited photocrosslinking of [α-^{32}P]ATP to the 116 kDa polypeptide. Similar inhibition was observed in

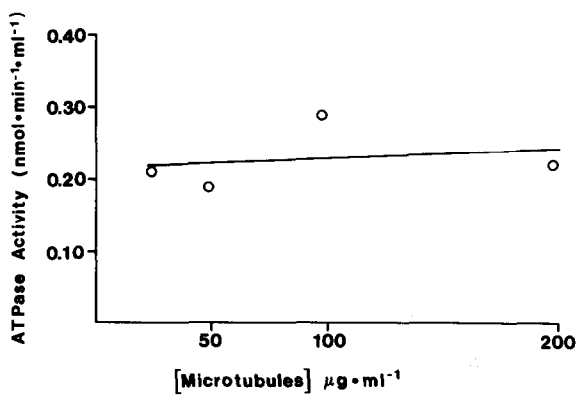


Fig.3. Stimulation of kinesin-associated ATPase activity by microtubules is not correlated with microtubule concentration. ATPase activity associated with the ATP-binding polypeptide of kinesin was assayed in the presence of increasing concentrations of taxol-, GTP-stabilized microtubules. Line was fitted by the method of least squares.

Table 2

Enzymatic and pharmacological characteristics of the ATPase activity associated with the 116 kDa ATP-binding polypeptide of kinesin

	Spec. act. (nmol·min^{-1}·mg^{-1})	GTPase/ ATPase ratio (%)[b]	% control ATPase activity[a]				
			AMP-PNP (5 mM)	Na_3VO_4 (0.05 mM)	NEM[c] (2 mM)	EHNA (0.5 mM)	NaN_3 (0.1 mM)
Kinesin-associated ATPase activity[d]	14[e]	45	52 ± 20	99 ± 40	0	78 ± 37	86 ± 23

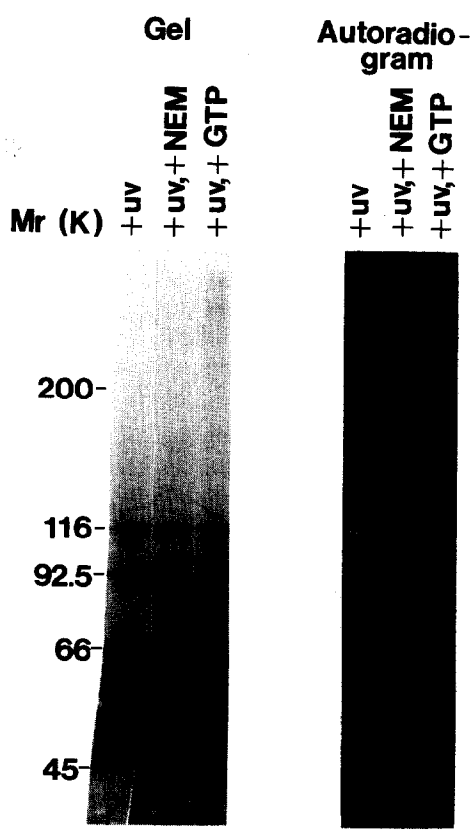
[a] Values are reported ± SD

[b] Average of two determinations

[c] Kinesin was incubated with 2 mM NEM for 30 min at room temperature, followed by addition of 10 mM dithiothreitol (DTT) and incubation for 20 min at 4°C prior to assay. Value is the result of four determinations with three separate preparations of kinesin

[d] In the presence of microtubules and after subtraction of microtubule ATPase activity

[e] Based on peak ATPase activity and a fractional concentration of the 116 kDa polypeptide, the ATP-binding polypeptide, equal to 25% of total protein (estimated from densitometric analyses of SDS gels), or 0.01 mg/ml



four experiments with two separate preparations of kinesin. These results suggested that the 116 kDa polypeptide represented the catalytic subunit of the microtubule-activated, kinesin-associated ATPase.

The microtubule preparation hydrolyzed ATP with a specific ATPase activity of 0.3 nmol·min^{-1}·mg^{-1}, about two orders of magnitude lower than preparations of microtubules described previously [2,16] and an order of magnitude lower than kinesin (table 2).

Fig.4. Direct photocrosslinking of the 116 kDa component of kinesin with [α-^{32}P]ATP is inhibited by NEM and GTP. (Left-hand panel) SDS gel electropherogram of kinesin; (right-hand panel) autoradiogram of the gel on the left. All samples were irradiated with UV light. (First lane) Kinesin + [α-^{32}P]ATP; (second lane) kinesin treated with NEM (incubated for 30 min at room temperature with 2 mM NEM followed by quenching with 10 mM DTT for 20 min at 4°C) + [α-^{32}P]ATP; (third lane) kinesin + 5 × 10^{-5} M non-radioactive GTP + [α-^{32}P]ATP. The dot in lane 2 of the autoradiogram is due to non-specific contamination with unbound radioisotope.

4. DISCUSSION

Here, we investigated the catalytic polypeptide of bovine brain kinesin. We found that the major component of kinesin (116 kDa) bound [α-^{32}P]ATP in a specific fashion. This ATP-binding polypeptide was shown to be associated with a microtubule-activated ATPase activity similar in magnitude to that of sea urchin egg kinesin [4]. Further, the microtubule-stimulated ATPase activity was pharmacologically analogous to kinesin-dependent translocation [1–3]. We found that a substrate (GTP) and an inhibitor (NEM) of the microtubule-activated ATPase activity each blocked ATP binding to the 116 kDa polypeptide. These results suggest that the 116 kDa polypeptide, the major component of bovine brain kinesin, represents the catalytic subunit of the kinesin mechanoenzyme.

It is interesting to compare the magnitude of the ATPase activity associated with kinesin to that of the dynein-microtubule system of cilia and flagella. We estimated the specific ATPase activity of the putative catalytic subunit of kinesin (116 kDa) to be 14 $nmol \cdot min^{-1} \cdot mg^{-1}$. This is approx. 50-fold lower than activities of purified outer dynein arm preparations assayed under roughly comparable conditions of ATP concentration, pH and ionic strength [10,17]. A remarkable property of dynein ATPase activity in flagella is that it is correlated with the velocity of microtubule sliding rather than with the load on the flagellum, in contrast to the load dependence of actomyosin ATPase activity in skeletal muscle [18]. Assuming that the ATPase activity of the kinesin-microtubule system is also correlated with velocity rather than load, it should be about an order of magnitude lower than dynein ATPase activity, analogous to the difference in the velocities of bead and organelle translocation in vitro ($0.5\ \mu m \cdot s^{-1}$) [1,3,14] and microtubule sliding in flagella ($5\ \mu m \cdot s^{-1}$) [19]. If it is assumed further that the efficiency of energy coupling is similar for kinesin- and dynein-powered motility, the results of this study are consistent with the concept that the motility-specific ATPase activities of kinesin and dynein are comparable (within an order of magnitude).

Kuznetsov and Gelfand [20] have reported that kinesin isolated by a novel procedure substituting tripolyphosphate for AMP-PNP is associated with an ATPase activity which is inhibited significantly by 10 μM vanadate and is stimulated up to 76-fold by microtubules to a specific activity of 4600 $nmol \cdot min^{-1} \cdot mg^{-1}$. These properties differ significantly from those of the kinesin-associated ATPase activity reported here and by Cohn et al. [4]. Possible reasons for these discrepancies include significant differences in the isolation procedures used by Kuznetsov and Gelfand [20] as well as in the conditions and methods of assay in their study.

The results of the present study provide direct evidence that the major, immunologically conserved [2,3] kinesin polypeptide is an ATPase. The next step is to elucidate the mechanisms by which the binding and hydrolysis of ATP by kinesin are coupled to translocation.

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