Nucleotide Specificity of the Enzymatic and Motile Activities of Dynein, Kinesin, and Heavy Meromyosin

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Abstract. The substrate specificities of dynein, kinesin, and myosin substrate turnover activity and cytoskeletal filament-driven translocation were examined using 15 ATP analogues. The dyneins were more selective in their substrate utilization than bovine brain kinesin or muscle heavy meromyosin, and even different types of dyneins, such as 14S and 22S dynein from *Tetrahymena* cilia and the β-heavy chain–containing particle from the outer-arm dynein of sea urchin flagella, could be distinguished by their substrate specificities. Although bovine brain kinesin and muscle heavy meromyosin both exhibited broad substrate specificities, kinesin-induced microtubule translocation varied over a 50-fold range in speed among the various substrates, whereas heavy meromyosin-induced actin translocation varied only by fourfold. With both kinesin and heavy meromyosin, the relative velocities of filament translocation did not correlate well with the relative filament-activated substrate turnover rates. Furthermore, some ATP analogues that did not support the filament translocation exhibited filament-activated substrate turnover rates. Filament-activated substrate turnover and power production, therefore, appear to become uncoupled with certain substrates. In conclusion, the substrate specificities and coupling to motility are distinct for different types of molecular motor proteins. Such nucleotide "fingerprints" of enzymatic activities of motor proteins may prove useful as a tool for identifying what type of motor is involved in powering a motility-related event that can be reconstituted in vitro.

EUKARYOTIC cells exhibit many types of motility, including muscular contraction, ciliary or flagellar movement, chromosome movement, and organelle transport. Two general classes of motility mechanisms have been defined according to the "track" on which the "molecular motor" works. Movements along actin filaments are powered by various myosins, while dynein (Gibbons and Rowe, 1965; Gibbons, 1988) and kinesin (Vale et al., 1985) motors power movements along microtubules. All known myosin move unidirectionally toward the actin barbed end, and until recently it was thought that dyneins move towards the microtubule minus end and kinesins move towards the microtubule plus end. However, a kinesin-like motor (the product of the *ncd* gene) from *Drosophila* induces minus end–directed microtubule movement (Walker et al., 1990). Other motors may have the capacity to move bidirectionally along microtubules (Schliwa et al., 1991).

Muscle myosin, ciliary or flagellar dynein and kinesin are structurally quite different from one another. Muscle myosin has two globular beads connected to an α-helical coiled-coil tail (Lowey et al., 1969). Kinesin has somewhat similar structure to myosin, but is considerably smaller, also having a tail with fan-shaped tip (Hirokawa et al., 1989). Dynein, in contrast, has no tails but has two or three heads connected by stems to a common base (Johnson and Wall, 1983; Johnson, 1985; Toyoshima, 1987). 14S dynein from *Tetrahymena* cilia, whose localization in cilium is still controversial, is unlike other other dyneins in having only one head with or without a short tail (Marchese-Ragona et al., 1988).

Myosin, dynein, and kinesin all turn over the substrate, ATP, by a rather similar mechanism (Johnson, 1985; Hibberd and Trentham, 1986; Hackney, 1988). The ATP turnover cycle consists of binding to the active site, hydrolysis, and product release. For those molecular motor enzymes, the ATP binding and hydrolysis are rapid but the release of products (ADP and phosphate) is slow and rate limiting: it is this release of products that is linked to the power production.

Although the kinetic mechanisms of ATP turnover are similar for all three mechanochemical enzymes, other characteristics of the ATPase, including the substrate specificity, are somewhat different. Unlike myosin, dynein has been shown to have a highly preferred substrate specificity (Gibbons, 1966; Ogawa and Mohri, 1972). Recently we con-

firmed this in a quantitative manner, determining the apparent K_ms and V_{max}s of turnover for naturally occurring NTPs with 22S dynein from *Tetrahymena* cilia (Shimizu, 1987). For ATP, this dynein has an apparent K_m in the micromolar range, while the apparent K_m is two orders or more higher for other NTPs. Kinesin is shown to have broad substrate specificity with naturally occurring NTPs (Kuznetsov and Gelfand, 1986; Porter et al., 1987; Cohn et al., 1989).

In the last few years, a cytoplasmic form of dynein (Paschal and Vallee, 1987; Schroer et al., 1989), numerous myosin-like motors (Kiehart, 1990) and several kinesin-like motors (Vale and Goldstein, 1990) have been identified. One of the challenges of the next few years is to identify the biological functions of these various motors. In some instances, the phenotype of a cell with a mutation in the gene encoding a motor protein provides some clue as to the motors. In some instances, the phenotype of a cell with a mutation in the gene encoding a motor protein provides some clue as to the motor's physiological role. Another approach, which is also amenable in cells which do not have well-developed genetics, is to reconstitute a motility event in vitro and probe the system biochemically. Previously, the ATPase inhibitors vanadate and EHNA and an alkylating reagent NEM have been used as the probes to identify what type of motor may be powering a motile event. Dyneins, for example, are generally thought to be more sensitive to vanadate, EHNA and NEM than kinesins. However, such generalizations using a very limited number of perturbing agents could lead to erroneous interpretations (see Walker et al., 1990).

In the present study, we examined a different strategy for distinguishing among different motor proteins. When different ATP analogues were tested for their ability to be turned over or support in vitro motility by dynein, kinesin and heavy meromyosin motors, we found that the substrate utilization by each motor tested was unique. Nucleotide "fingerprints" may therefore be a useful diagnostic means of identifying which type of motor is involved in a form of cellular motility that can be reconstituted in vitro. One such application is described in the accompanying paper (Schliwa et al., 1991).

Glossary

ATPαS	adenosine 5′-*O*-(1-thiotriphosphate)
ATPβS	adenosine 5′-*O*-(2-thiotriphosphate)
ATPγS	adenosine 5′-*O*-(3-thiotriphosphate)
dideoxy ATP	2′,3′-dideoxy-adenosine 5′-triphosphate
dimethyl ATP	N^6,N^6-dimethyl-adenosine 5′-triphosphate
8-azido ATP	8-azido-adenosine 5′-triphosphate
8-bromo ATP	8-bromo-adenosine 5′-triphosphate
EHNA	*erythro*-9-[3-(2-hydroxynonyl)]adenine
etheno ATP	1,N^6-etheno-adenosine 5′ triphosphate
FTP	formycin 5′-triphosphate
HMM	heavy meromyosin
MAP	microtubule-associated protein
monomethyl ATP	N^6-methyl-adenosine 5′-triphosphate
NTP	nucleoside 5′-triphosphate
PRTP	purine riboside triphosphate
2′dATP	2′-deoxy-adenosine 5′-triphosphate
3′dATP	3′-deoxy-adenoside 5′-triphosphate

Materials and Methods

Proteins

22S and 14S dyneins were obtained from cilia of *Tetrahymena thermophila* SB-255 by the method of Porter and Johnson (1983). The β-heavy chain–containing particle (β-particle) was prepared from the 21S outer arm dynein of sperm flagella from Japanese sea urchin, *Pseudocentrotus depressus*, according to the method of Tang et al. (1982).

Kinesin was purified according to a method developed by F. Malik and R. Vale (manuscript in preparation). Briefly, a bovine brain high speed supernatant was warmed in the presence of 33% glycerol and 1 mM GTP to polymerize microtubules. Then apyrase, to deplete nucleoside triphosphates, and 5′-adenylylimidodiphosphate (AMPPNP) (20 μM) were added to the suspension to induce binding of kinesin to microtubules. Microtubules pelleted by centrifugation were incubated with 5 mM Mg-ATP to release kinesin. Kinesin was purified by phosphocellulose (Whatman, Kent, England) and Biogel A5M (Bio-Rad Laboratories, Richmond, CA) gel filtration chromatography. As a last step, the enzyme was adsorbed onto a 2 ml S-Sepharose (Pharmacia, Uppsala, Sweden) column and then eluted with 80 mM KCl. Fractions containing kinesin were pooled and then frozen in liquid nitrogen in the presence of 10% sucrose; storage was at −80°C.

Microtubules were obtained from bovine or porcine brains by cycles of polymerization and depolymerization, and MAP-free tubulin was prepared by phosphocellulose (for translocation assay) or DEAE-Sephacel (for substrate turnover assay) column chromatography. Polymerization of the MAP-free microtubules was induced by 7% dimethylsulfoxide (Vale and Toyoshima, 1989*a*). In some cases, especially where the tubulin concentration was low, an equimolar concentration of taxol was added to further stabilize the microtubules. No detectable differences were observed with those microtubules in both assays irrespective of the starting materials or of the preparation methods.

Heavy meromyosin and actin were prepared from rabbit back muscles by the methods of Okamoto and Sekine (1985) and of Spudich and Watts (1971), respectively.

The protein concentrations of dyneins, actin, heavy meromyosin, and tubulin were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. The concentration of kinesin was estimated by the method of Hackney (1988), where the densitometry of the kinesin heavy chain band on an SDS-polyacrylamide gel was performed using bovine serum albumin as a standard.

ATP Analogues

For the chemical structure of ATP analogues used herein, see Fig. 1. ATP, 2′dATP, dideoxy ATP, UTP, CTP, GTP, 8-bromo ATP, and etheno ATP were purchased from Sigma Chemical Co. (St. Louis, MO), Boehringer Mannheim GmbH (Mannheim, Germany), or Pharmacia (Uppsala, Sweden) and purified by a DEAE-Sephadex A-25 column chromatography with triethylammonium bicarbonate buffer (pH 7.6) gradient (from 0.1 to 0.6 M). 3′dATP and FTP were made from the corresponding monophosphate forms (Sigma Chemical Co.) with a trace amount of triphosphate form by the adenylate kinase and pyruvate kinase (both enzymes from Boehringer Mannheim GmbH) reaction and purified as above. To get rid of contaminating ATP (ca. 0.4%) from 8-azido ATP (Sigma Chemical Co.), it was treated with 22S dynein until about half of 8-azido ATP was converted to the diphosphate form, when most of contaminating ATP must have been turned over because of highly preferred specificity of this enzyme to ATP. Then the mixture was chromatographed as above and the triphosphate form was obtained. PRTP, monomethyl ATP, and dimethyl ATP were synthesized from the corresponding nucleosides (Sigma Chemical Co.) by two-step chemical phosphorylation to the diphosphate forms (Shimizu and Furusawa, 1986) and then by enzymatic phosphorylation to the triphosphate forms by pyruvate kinase with phosphoenolpyruvate. The final products were also purified as above. The preparation method of the phosphorothioate analogues of ATP was described previously (Eckstein and Goody, 1976; Shimizu et al., 1990). The purification of ITP to separate contaminating ATP was carried out with a Dowex 1 column as described (Shimizu, 1987).

The purity of the nucleotides was checked by high performance liquid chromatography with a C_{18} reverse phase column. All nucleotides were substantially free from contamination, except for 8-azido ATP and ATPβS(Sp). 8-azido ATP purified as above still contained ATP-like material, but not more than 0.1%. ATPβS(Sp) contained about 10% of the (Rp) isomer.

Substrate Turnover Assay

The turnover rate of ATP or an analogue was determined from the time course of the enzyme reaction. First, a rough estimate of the turnover rate was obtained and then the assay was repeated under a suitable condition to get a better value for the rate. The assay mixture contained: 50 mM MOPS-NaOH (pH 7.0), 4 mM $MgCl_2$, 1 mM ATP or analogue for dynein; 80 mM imidazole-HCl (pH 6.8), 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM ATP or

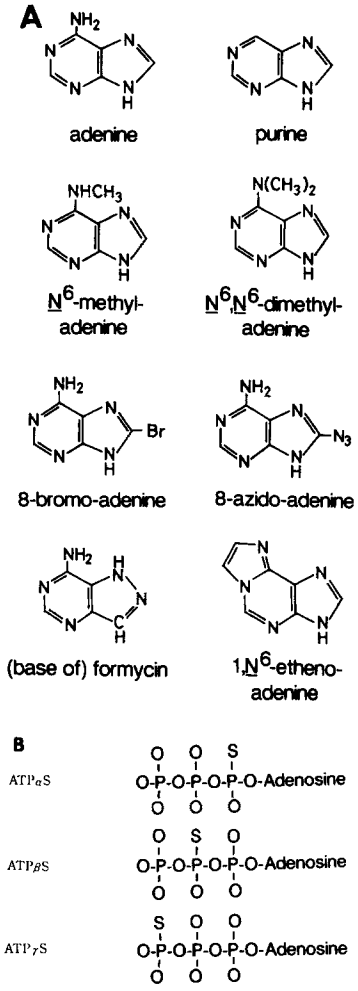

Figure 1. Chemical structures of ATP analogues. (*A*) Structure of modified adenines used in this study. (*B*) Structure of the phosphorothioate analogues of ATP. Because of the tetrahedral nature of phosphorus atom, two stereoisomers exist for ATPαS and for ATPβS, i.e., (Sp) and (Rp) isomers. They are referred to as ATPαS(Sp) and so on. Negative charge symbols and double bonds are omitted to make the formula simpler and because of equivalence of all four bonds of a phosphorus atom.

an analogue for kinesin; 25 mM Hepes-NaOH (pH 7.4), 25 mM KCl, 4 mM $MgCl_2$, 1 mM ATP or an analogue for HMM. The enzyme concentration in the assay solution was 5–25 μg/ml. After incubation at 25°C, the reaction was terminated by the addition of final 0.3 M perchloric acid. Precipitates, if formed, were removed by centrifugation and the supernatant was assayed for phosphate by the modified malachite green method (Shimizu and Furusawa, 1986).

When measuring the microtubule-activation of kinesin or actin-activation of HMM, the polymerized microtubules or actin filaments were diluted with a nucleotide-free buffer solution, pelleted by centrifugation, and resuspended in the same nucleotide-free solution containing trace amounts of taxol or phalloidin, respectively, in order to reduce the background of GTP or ATP contained in the protein solution. By repeating this treatment once more, the contribution of GTP or ATP from the protein solution to the assay solution was significantly <1 μM.

For determining the Michaelis constant of 22S dynein for ATP, 2'dATP, or 3'dATP, the coupled assay method using pyruvate kinase and phosphoenolpyruvate was used, and the production of corresponding diphosphate was measured to determine the substrate turnover rate of dynein (Shimizu, 1981).

In Vitro Translocation Assay

The in vitro microtubule translocation assay was carried out as described (Vale and Toyoshima, 1989*b*) using a flow chamber method. A dynein or kinesin (50 μg/ml) solution was introduced into a flow chamber consisting of a slide glass and a coverslip separated by spacers; after washing with a buffer solution, a mixture of taxol-stabilized microtubules (20 μg/ml) and ATP or an analogue (1 mM) was then perfused. The buffer solution ingredients were: 10 mM Tris-acetate (pH 7.5), 50 mM potassium acetate, 4 mM $MgSO_4$, 1 mM EGTA, and 1 mM DTT for 14S and 22S dynein; 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT for β-particle; 80 mM Pipes-NaOH (pH 6.8), 2 mM $MgCl_2$, and 1 mM EGTA for kinesin. The translocation of microtubules was observed with a darkfield microscope and recorded on a video tape. The speed of translocation was analyzed by replaying the videotapes afterwards. 20 microtubules were analyzed for one substrate to get the average and standard deviation.

In the case of actin translocation assay, an HMM (50 μg/ml) solution was applied to a flow chamber covered with a nitrocellulose film and a solution of actin (0.5 μg/ml) labeled with rhodamine-phalloidin was perfused as described by Toyoshima et al. (1987). The movement of actin upon addition of ATP or an analogue (1 mM) was observed with a fluorescence microscope and analyzed as above. The buffer solution consisted of 25 mM imidazole-HCl (pH 7.4), 25 mM KCl, 4 mM $MgCl_2$, and 1 mM DTT.

Turbidimetric Assay for the Dissociation of the Microtubule–22S Dynein Complex

A suspension of microtubule (0.1 mg/ml)–22S dynein (0.18 mg/ml) complexes in 50 mM MOPS-NaOH (pH 7.0) and 4 mM $MgCl_2$ maintained at 28°C was transferred into a cuvette and a small amount of a nucleotide solution was added. The time course of the decrease in absorbance (turbidity) at 420 nm due to nucleotide-induced dissociation was recorded for up to 4 min. The first time point available with this method was ~5 s.

Detergent Models of Tetrahymena

The *Tetrahymena* models were made according to the method of Goodenough (1983) using NP-40 as a detergent with a slight modification (Shimizu et al., 1990). The motion of reactivated cilia was recorded on videotapes and the beat frequency was measured with slow speed playing. For 1 determination, 20 cilia were analyzed.

Results

Substrate Specificities of Enzymatic and Motility Activities of Dynein

Substrate Turnover Activities of 22S and 14S Dyneins from Tetrahymena Cilia. The enzymatic activity of 22S dynein toward the ATP analogues (Fig. 1 for the structures) was investigated using Lineweaver-Burk plots (Table I). The V_{max} and apparent K_m of turnover of ATP, 2'dATP, or 3'dATP were comparable, whereas removal of both 2' and 3' oxygens (dideoxy ATP) increased the apparent K_m from 2 to 40 μM. All three of these deoxy derivatives gave simple Michaelis-Menten type relationships.

Monomethyl ATP was the only ATP analogue giving complex kinetics amongst the substrates investigated; the Lineweaver-Burk plot gave a downward bend as in the case of ATP and ATPγS (Shimizu et al., 1989), with the apparent K_m and V_{max} being comparable to those of ATP turnover. FTP, one of the two fluorescent ATP analogues investigated herein, was also a good substrate. The V_{max} of 8-bromo ATP was even higher than that of ATP, although the apparent K_m was high (30 μM).

Dimethyl ATP, PRTP, etheno ATP (another fluorescent ATP analogue), and 8-azido ATP (often used as a probe for the adenine-binding sites of certain enzymes) were all turned over poorly by 22S dynein.

The other form of dynein from *Tetrahymena* cilia is 14S dynein. The catalytic activity of 14S dynein toward the ATP analogues was measured at 1 mM substrate as shown in Ta-

Table I. Nucleotide Specificity of Microtubule-22S Dynein System

Nucleotide	Microtubule translocation		Substrate turnover	
	− Triton	+ Triton (0.1%)	V_{max}	Apparent K_m
	μm/s	*μm/s*	*μmol/min/mg*	*μM*
ATP	4.52 ± 1.06 (100%)	11.2 ± 1.38 (248%)	0.25, 0.30	0.69, 2.0
2'dATP	1.26 ± 0.31 (28)	N.M.	0.31	3.5
3'dATP	2.00 ± 0.52 (44)	N.M.	0.51	6.3
Dideoxy ATP	0.94 ± 0.31 (21)	4.80 ± 0.85 (106)	0.56	41
Monomethyl ATP	0.28 ± 0.075 (6.2)	0.90 ± 0.19 (20)	0.063, 0.19	1.0, 7.1
Dimethyl ATP	–	–	0.016	83
PRTP	–	–	0.13	96
8-Bromo ATP	–	–	0.55	29
8-Azido ATP	–	–	0.044	170
FTP	–	0.054 ± 0.017 (1.2)	0.29	10
Etheno ATP	–	–	0.0088	210
ATPαS(Sp)	0.83 ± 0.24 (18)	1.86 ± 0.45 (41)	1.34*	8.2*
ATPαS(Rp)	–	–	0.82*	83*
ATPβS(Sp)	–	–	0.087*	67*
ATPβS(Rp)	–	–	0.007*	105*
ATPγS	0.21 ± 0.11 (4.6)	0.26 ± 0.19 (5.8)	0.052, 0.11‡	<1, 20‡

The substrate turnover activity and the microtubule translocation speed were measured at 25°C as described in Materials and Methods. The V_{max} and apparent K_m for each substrate were estimated from the Lineweaver-Burk plot. This dynein exhibits a downward bend in this plot with ATP (Shimizu, 1981), ATPγS (Shimizu et al., 1989) or with monomethyl ATP, so that two values are listed for these substrates. The values for the phosphorothioate analogues of ATP except for ATPγS are taken from the previous study with 22S dynein from *T. pyriformis* (Shimizu and Furusawa, 1986), which are marked with an asterisk. Those for ATPγS are from Shimizu et al. (1989) with 22S dynein from *T. thermophila* assayed at 28°C, marked with a dagger. V_{max} is expressed in μmol/min/mg 22S dynein. + Triton indicates that the substrate solution for the translocation assay contained 0.1% Triton X-100. The relative translocation speed is also shown in the parentheses with the speed with ATP in the absence of Triton X-100 as 100%. N.M., not measured.

ble II. The relative turnover rates to that of ATP were similar to those of 22S dynein except for 8 substituted analogues whose relative turnover rates were somewhat different between the two dyneins.

In Vitro Microtubule Translocation by 22S Dynein, 14S Dynein, and the β-Particle from Sea Urchin Sperm Flagellar Outer Arm Dynein. Each analogue (at 1 mM concentration) was examined for its ability to support in vitro microtu-

Table II. Nucleotide Specificity of Microtubule-14S Dynein and Microtubule-β-Particle System

Nucleotide	14S dynein		β-Particle microtubule translocation
	Substrate turnover rate	Microtubule translocation	
	μmol/min/mg	*μm/s*	*μm/s*
ATP	0.112	4.29 ± 0.70 (100%)	9.65 ± 1.20 (100%)
2'dATP	0.112	4.24 ± 0.51 (99)	8.85 ± 1.11 (92)
3'dATP	0.138	3.30 ± 0.36 (77)	11.9 ± 1.03 (123)
Dideoxy ATP	0.158	2.86 ± 0.48 (67)	5.28 ± 0.76 (55)
Monomethyl ATP	0.0665	0.73 ± 0.098 (17)	3.52 ± 0.38 (36)
Dimethyl ATP	0.0127	–	=
PRTP	0.0359	–	=
8-Bromo ATP	0.0908	–	=
8-Azido ATP	0.0676	1.17 ± 0.24 (27)	3.42 ± 0.53 (35)
FTP	0.0845	0.084 ± 0.019 (2.0)	4.16 ± 0.52 (43)
Etheno ATP	0.0042	–	=
ATPαS(Sp)	0.157	1.20 ± 0.13 (28)	8.43 ± 1.19 (87)
ATPαS(Rp)	0.141	–	=
ATPβS(Sp)	0.0040	–	=
ATPβS(Rp)	0.0013	–	=
ATPγS	0.0843	0.52 ± 0.24 (12)	0.51 ± 0.17 (5.3)

The substrate turnover rate of 14S dynein toward each nucleotide (1 mM) was assayed at 25°C in the same manner as that of 22S dynein as described in Materials and Methods. The turnover rate is expessed as μmol/min/mg 14S dynein. The microtubule translocation assay at 1 mM nucleotide was also performed in the same manner. = indicates that the microtubules did not associate with the β-particle-coated glass surface but were floating in the solution. The relative translocation speed is also shown in the parentheses with the speed with ATP as 100%.

Table III. Reactivation of Ciliary Motility

Nucleotide	Beat frequency (s^{-1}) at the substrate concentration of		
	1 mM	0.3 mM	0.1 mM
ATP	12.4 ± 1.0		8.5 ± 1.1
2'dATP	12.6 ± 1.4		7.2 ± 1.1
3'dATP	12.7 ± 0.8		3.5 ± 0.3
dideoxy ATP	9.0 ± 0.7	4.5 ± 0.5	–
PRTP	–		
Dimethyl-ATP	–		
Monomethyl-ATP	2.9 ± 0.2	0.62 ± 0.08	–
8-Bromo ATP	–		
8-Azido ATP	–		
FTP	–		
Etheno ATP	–		

The preparation of the NP-40 models of *T. thermophila* and the analysis of the ciliary reactivation were performed as described in Materials and Methods. Dideoxy ATP or monomethyl ATP did not support the ciliary motility at 0.1 mM but at 0.3 mM, the reactivation was observed and the beat frequency is listed. Others were not checked at 0.3 mM. –, no reactivation observed. As for the phosphorothioate analogues, only ATPαS(Sp) was shown to be able to support the motility (Shimizu et al., 1990); beat frequency was 4.6 ± 0.6 s^{-1} at 1 mM.

bule translocation on 22S dynein-coated glass surface (Table I). All the deoxy ATP derivatives supported the translocation of the microtubules, although the speed was slower. Among adenine-modified analogues, only monomethyl ATP was competent for supporting the movement. Of the phosphorothioate analogues, ATPαS(Sp), which was a good substrate for dynein catalytic activity (Shimizu and Furusawa, 1986), supported the microtubule translocation. Vale and Toyoshima (1989*a*) reported that ATPγS could induce slow but significant microtubule translocation mediated by 22S dynein, and we confirmed this with column-purified, substantially ATP-free ATPγS at concentrations between 0.3 and 2 mM.

As Vale and Toyoshima (1989*a*) demonstrated, addition of 0.1% Triton X-100 improved the 22S dynein-mediated microtubule translocation by increasing the speed, making movement more continuous and making more microtubules move (Table I). In addition, slow movement was observed with FTP in the presence of Triton X-100, but other NTPs were not competent even in its presence.

Next the microtubule translocation assay at 1 mM substrate on the 14S dynein-adsorbed glass surface was performed (Table II). The results were also similar to those of 22S dynein, although the specificity was somewhat broader. In addition to those analogues that supported the 22S dynein-mediated microtubule translocation, FTP and, surprisingly, 8-azido ATP supported the motility. We believe that 8-azido ATP itself, and not contaminating ATP-like material in 8-azido ATP, was responsible for the movement. The maximum of ATP contamination in our 8-azido ATP was 0.1%, but motility was observed at 0.1 mM 8-azido ATP (contamination of ATP was 0.1 μM at most) at about half the speed of that at 1 mM. ATPγS supported the microtubule translocation as in the case of 22S dynein as above, although more slowly.

Dialysis of the outer arm 21S dynein from sea urchin

Table IV. Nucleotide Specificity of Dissociation of the Microtubule–22S Dynein Complex

Nucleotide	Dissociation at the nucleotide concentration of	
	0.25 mM	1 mM
ATP	Rapid	
2'dATP	Rapid	
3'dATP	Rapid	
Dideoxy ATP	Rapid	
Monomethyl ATP	Rapid	
Dimethyl ATP	None	Slight
PRTP	Slow, incomplete	Slow (faster), complete
8-Bromo ATP	Slow, incomplete	Slow (faster), almost complete
8-Azido ATP	None	Slight
FTP	Rapid	
Etheno ATP	None	None
ATPαS(Sp)	Rapid	
ATPαS(Rp)	Slow, incomplete	Slow, incomplete
ATPβS(Sp)	None	
ATPβS(Rp)	None	
ATPγS	Rapid	
GTP (0.5 mM)	None	
ITP (0.5 mM)	Slight	
UTP (0.5 mM)	None	
CTP (0.5 mM)	Slow, almost complete	

The dissociation of the microtubule–22S dynein complex was measured by monitoring the turbidity of the suspension at 420 nm for up to 4 min after addition of ATP or analogue as described in Materials and Methods. Terms to describe the dissociation are defined as follows: the extent of dissociation is complete (saturating amount of ATP, e.g., 0.2 mM, would not cause further turbidity drop) or not complete (addition of ATP did cause further turbidity drop), and the latter is subdivided into: almost complete (>80% completion of turbidity change), incomplete (<80%), slight (<20%), or none (<5%). The extent of incomplete dissociation varies considerably depending on the substrate concentration. As for the rate of dissociation, it is rapid (complete within 5 s) or slow (not complete within 5 s).

Table V. Nucleotide Specificity of Microtubule-Kinesin System

Nucleotide	Microtubule translocation	Substrate turnover rate	
		− microtubules	+ microtubules
	μm/s	*μmol/min/mg kinesin*	
ATP	0.422 ± 0.062 (100%)	0.0056	0.572
2'dATP	0.382 ± 0.074 (91)	0.0046	0.532
3'dATP	0.304 ± 0.059 (72)	0.0125	0.353
Dideoxy ATP	0.290 ± 0.049 (69)	N.M.	N.M.
Monomethyl ATP	0.198 ± 0.052 (47)	0.0061	0.603
Dimethyl ATP	0.086 ± 0.018 (20)	0.0169	0.449
PRTP	0.0075 ± 0.0051 (1.8)	0.0335	0.284
8-Bromo ATP	0.013 ± 0.0067 (3.1)	0.0151	0.251
8-Azido ATP	−	0.0245	0.196
FTP	0.037 ± 0.019 (8.8)	0.0210	0.427
Etheno ATP	0.054 ± 0.028 (13)	0.0235	0.377
ATPαS(Sp)	0.077 ± 0.023 (18)	0.0094	0.520
ATPαS(Rp)	−	0.0451	0.194
ATPβS(Sp)	−	0.0113	0.303
ATPβS(Rp)	−	N.D.	N.D.
ATPγS	0.0091 ± 0.0027 (2.2)	N.M.	N.M.

The substrate turnover assay and the microtubule translocation assay were performed at 25 and 22–24°C, respectively, as described in Materials and Methods. The substrate turnover by kinesin (10 μg/ml) was measured in the absence or the presence of the MAP-free microtubules (0.95 mg/ml). The concentration of nucleotide was 1 mM for both types of assay. The relative translocation speed is shown in the parentheses with the speed with ATP as 100%. N.D. and N.M., not detectable and not measured, respectively.

sperm flagella against a low ionic strength solution dissociates the two-headed dynein into single-headed α- and β-particles (Tang et al., 1982). While the α-particle does not mediate microtubule translocation, the β-particle adsorbed on glass coverslip is able to induce motility (Sale and Fox, 1988; Vale et al., 1989). As shown in Table II, the nucleotide specificity of microtubule translocation on β-particle–coated glass surfaces was similar to that of 14S dynein-mediated movement, except that ATPαS(Sp) and FTP supported better microtubule translocation on β-particle–coated glass surface than on 14S dynein-coated surface. Previously, it was noted that microtubules did not adhere to glass surfaces coated with β-particles in the absence of ATP (Sale and Fox, 1988; Vale et al., 1989) in contrast to the results with 14S or 22S dynein. With several ATP analogues, microtubules did not adhere to the β-particle–coated surface, which may indicate a weak interaction between the β-particle and those ATP analogues.

Reactivation of Ciliary Motility of Detergent Models of Tetrahymena. NP-40 mdels of *Tetrahymena* cilia were reactivated only by ATP, deoxy ATP derivatives and monomethyl ATP (Table III). Neither FTP, 8-azido ATP, nor ATPγS (at 1 mM) supported ciliary reactivation. Each deoxy ATP derivative at 1 mM produced a beat frequency similar to that evoked by ATP, whereas the frequency was smaller at 0.1 mM than that at 0.1 mM ATP. Dideoxy ATP at 0.1 mM did not support motility. Monomethyl ATP induced slower ciliary beating and, as dideoxy ATP, it did not support the motility at 0.1 mM.

Turbidimetric Measurements of Dissociation of the Microtubule–22S Dynein Complex. We also examined the ability of the various ATP analogues to induce the dissociation of the microtubule–22S dynein complex (Porter and Johnson, 1983; Shimizu and Furusawa, 1986). As shown in Table IV, each of the deoxy ATP derivatives, monomethyl ATP, and FTP (0.25 mM) induced rapid dissociation (complete within 5 s). ATPαS or ATPγS were previously demonstrated to rapidly dissociate the complex (Shimizu and Furusawa, 1986), confirmed herein. Of the other analogues that did not induce rapid dissociation, only PRTP and 8-bromo ATP induced nearly complete, but slow dissociation at 1 mM concentration. Naturally occurring NTPs at 0.5 mM could not substitute for ATP except CTP which induced slow but complete dissociation within 2 min.

Nucleotide Specificity of the Microtubule-Kinesin System

Kinesin-induced motility of the microtubules was reported to exhibit a rather broad substrate specificity; each of naturally occurring NTPs at 10 mM could support motility of microtubules along kinesin-coated surfaces, though the translocation speed varied from one substrate to another (Porter et al., 1987; Cohn et al., 1989).

As shown in Table V, most ATP analogues supported kinesin-induced microtubule movement while only 8-azido ATP, ATPαS(Rp), or ATPβS isomers were incompetent for inducing the motility. The speed of microtubule translocation induced by the ATP analogues, however, varied to a considerable extent. ATP itself was the best substrate in terms of the speed of translocation and the deoxy derivatives were next. Those with modified adenine moieties produced slow translocation. ATPγS or PRTP (1 mM) induced movement at 2% the speed of 1 mM ATP.

The substrate turnover activity of kinesin in the absence or presence of 0.95 mg/ml microtubules also varied depending on the substrate. The turnover was accelerated by microtubules in all cases tested (Table V) including naturally occurring NTPs (data not shown). With ATP, the activation

Table VI. Nucleotide Specificity of Actin–HMM System

Nucleotide	Actin translocation	Substrate turnover rate	
		− actin	+ actin
	μm/s	*μmol/min/mg HMM*	
ATP	5.21 ± 1.04 (100%)	0.0105	0.257
2'dATP	4.51 ± 0.87 (87)	0.0141	0.343
3'dATP	4.62 ± 0.53 (89)	0.0200	0.626
Dideoxy ATP	5.43 ± 0.79 (104)	0.0300	0.352
Monomethyl ATP	3.80 ± 0.53 (73)	0.0454	0.442
Dimethyl ATP	2.96 ± 0.39 (57)	0.0528	0.677
PRTP	2.38 ± 0.32 (46)	0.0547	0.477
8-Bromo ATP	–	0.215	0.412
8-Azido ATP	–	0.188	0.420
FTP	1.32 ± 0.26 (25)	0.0144	0.422
Etheno ATP	1.18 ± 0.34 (23)	0.0930	0.584
ATPαS(Sp)	10.4 ± 1.01 (200)	0.0311	0.707
ATPαS(Rp)	0.52 ± 0.16 (10)	0.357	0.630
ATPβS(Sp)	–	0.137	0.0470
ATPβS(Rp)	–	0.00082	<0.0005
ATPγS	–	N.M.	N.M.

The substrate turnover assay and the actin translocation assay were performed at 25°C as described in Materials and Methods. The concentration of nucleotide was 1 mM for both types of assay. The substrate turnover by HMM (10 μg/ml) was measured in the absence or the presence of F-actin (0.44 mg/ml). The relative translocation speed is shown in the parentheses with the speed with ATP as 100%. N.M., not measured.

by microtubules was ~100-fold. It is of interest to note that neither the relative magnitudes of activation by the microtubules nor the absolute turnover rates in the presence of microtubules corresponded to the relative speeds of the microtubule translocation induced by the ATP analogues. Even with the nucleotides that did not support the motility, activation of the turnover rate was still observed in the presence of the microtubules.

Nucleotide Specificity of the Actin–HMM System

The catalytic activity of myosin and actin-myosin–based motility systems have long been known to exhibit broad substrate specificity (Tonomura et al., 1967; Takenaka et al., 1978). The in vitro translocation system with actin and HMM is shown to be supported by CTP or UTP (Toyoshima, Y., S. Kron, J. Spudich, and H. White, manuscript in preparation).

Our experiments also demonstrated that many of the ATP analogues supported the HMM-mediated actin translocation in vitro (Table VI). Of the analogues examined, only 8-substituted ATP analogues, ATPβS isomers and ATPγS were incompetent for supporting motility. In this regard, the actin–HMM system was similar to the microtubule-kinesin system. A notable difference, however, was that while the speed of translocation varied significantly in the microtubule-kinesin system depending on the substrate species, most ATP analogues induced translocation at 1–5 μm/s in actin–HMM system. Two exceptions were the ATPαS isomers; ATPαS (Rp) supported actin movement at 10% of the speed of that by ATP, and ATPαS(Sp) induced actin translocation at twice the rate of ATP. This latter result is the only case where an ATP analogue induced filament movement at a significantly faster rate than ATP with the three in vitro translocation systems studied herein.

The substrate turnover rate of HMM was, in most cases, higher in the presence of actin than in its absence (Table VI), but again, the magnitude of activation or the turnover rate in the presence of actin does not seem to correlate with the speed of actin translocation. It should be noted that even with 8-substituted ATP analogues, which did not support the motility, the activation of substrate turnover by actin was observed, as in the case of the microtubule-kinesin system.

Discussion

Nucleotide Specificities of Dynein, Kinesin, and HMM

Previously, we have shown in a quantitative manner that the catalytic activity of 22S dynein is highly specific for ATP (Shimizu, 1987). Here, we extended the survey to include a wider variety of ATP analogues and several other molecular motors such as 14S dynein, HMM, and bovine brain kinesin. Myosin, or its proteolytic subfragment conveying the enzymatic sites such as HMM, has long been known to have a broad substrate specificity. This was confirmed by the present results that all the adenine-modified analogues and deoxy-derivatives of ATP examined were turned over faster than ATP itself. Kinesin also exhibited broad substrate specificity; ATP again was turned over slower than many analogues. Thus, these two molecular motors are similar to each other in this context, but different from dyneins.

The specificity of 22S dynein substrate turnover was investigated in a quantitative manner. Slight modification of the ribose moiety of ATP did not seem to affect the 22S dynein-substrate interaction (also see Inaba et al., 1989). On the other hand, modification of the adenine moiety of ATP resulted in a serious reduction in its capability to act as a substrate for 22S dynein in most cases (see Omoto and

Nakamaye, 1989). 14S dynein seemed to exhibit specificity similar to 22S dynein (Table I).

The dynein, kinesin, and HMM translocation systems also exhibited distinct differences in substrate specificity. While the dynein-based systems showed quite narrow specificity, the kinesin and HMM systems showed broad specificity. For example, dimethyl ATP and etheno ATP supported fairly good filament translocation with HMM and kinesin but not with dynein. An intriguing difference between the kinesin and HMM systems is found in the speed of translocation. With the HMM system, variation in the speed of actin translocation was moderate and independent of the analogues, while with kinesin system, the speed of the microtubule translocation varied to a large extent. For instance, PRTP induced fairly fast translocation with the actin-HMM system while the speed of kinesin-mediated microtubule translocation was only 2% that of the ATP-induced one. In addition, 8-bromo ATP supported the kinesin system but not HMM system.

Among dynein-mediated microtubule-translocation systems, an interesting difference was observed with 8-azido ATP. This ATP analogue was a poor substrate for turnover and translocation activity of 22S dynein but supported the microtubule translocation mediated by 14S dynein or the β-particle. 8-azido ATP has been reported to be a good substrate for *Chlamydomonas* dyneins (Pfister et al., 1984, 1985) and for sea urchin flagellar or egg dynein (Pratt, 1986). It is reported not to be a good substrate for squid dynein (Gilbert and Sloboda, 1989). Thus, 8-azido ATP may be a useful analogue for distinguishing different types of dynein molecules.

Potential Applications of Using Nucleotides to Identify Molecular Motors

Our results demonstrate that different motors, including several forms of dynein, exhibit distinct substrate specificities in the in vitro motility assay. Useful criteria for comparing the activities of different motors are (*a*) the ability or inability of a substrate to support movement, and (*b*) for those substrates that induce translocation, the relative velocity compared to that induced by 1 mM ATP. These tests performed with a battery of ATP analogues reveal a nucleotide "fingerprint" of the enzymatic activity of a motor that may be useful in identifying what type of motor may be operating in a form of cell motility. This strategy may be more convincing than using the concentration dependence of vanadate or EHNA inhibition or NEM susceptibility.

In the accompanying paper (Schliwa et al., 1991), this approach was applied to examine the motor that drives organelle transport in *Reticulomyxa*. Since it is now possible to reconstitute mitotic anaphase B movements in vitro (Cande and McDonald, 1985; Masuda et al., 1990), these ATP analogues may be useful for gaining insight into what motors are powering these processes. Kinetochore-based movements and various forms of membrane transport that can be reconstituted in vitro may similarly benefit from this type of characterization.

To interpret substrate specificity of a form of cellular motility in terms of what motor may drive this process, one must analyze more purified motors for their substrate utilization in the in vitro motility assay. In addition to the motors described herein, cytoplasmic dynein (Collins and Vallee, 1989) and dynamin (Shpetner and Vallee, 1989), two microtubule-based motors, should be examined. Furthermore, a variety of myosin-like and kinesin-like proteins have been uncovered through genetic and molecular biological techniques. Many of these motors can be expressed in bacteria and their motility is characterized (Walker et al., 1990). It will be interesting to explore whether the utilization of ATP analogues can distinguish between members of the kinesin and myosin superfamilies.

The strategy of identifying what motor may drive a cellular process based on substrate specificity may have possible problems as well. In the case of ciliary reactivation, the specificity is more strict than microtubule translocation in vitro: ATPγS or FTP, which supported dynein-mediated translocation, did not support ciliary reactivation. This may mean that a motility system might exhibit different specificity from that of reconstituted translocation system consisting of the motor obtained thereof. On the other hand, some form of motility, such as ciliary beating, may require the actions of several motors, which may make interpreting the substrate specificity difficult. The slow speed of translocation produced by some analogues in vitro may be also insufficient to initiate ciliary beating in a reconstituted assay.

Implications for the Force-generating Cycle

The finding that ATPγS supported kinesin- and dynein-driven microtubule translocation but not HMM-driven actin movement is interesting in light of the enzymatic reaction mechanisms of these molecular motors. With all three motors, product release is rate limiting and is ordered; phosphate is released first and ADP, second. According to kinetic investigations, the phosphate release step, or the step immediately after phosphate release, is likely to be related to power production in the actin-myosin system (Hibberd and Trentham, 1986). ATPγS may not support actin-HMM motility because the power production step does not function well with thiophosphate, one of the products of ATPγS hydrolysis. On the other hand, in the kinesin and dynein systems, power production is postulated to be related to ADP release and not to phosphate (thiophosphate) release (Hackney, 1988; Holzbaur and Johnson, 1989). This could explain why kinesin and dynein can use ATPγS as an energy donor for motility.

Filament-activated NTP turnover activity is generally thought to be coupled to power production. In this study, we found that actin or microtubules accelerated the turnover of certain ATP analogues by HMM or kinesin, respectively, even though these analogues did not support filament movement under similar experimental conditions. Such uncoupling may occur as the result of some steric difficulty or if the step responsible for the accelerated substrate turnover by either actin or microtubules is different from that involved in the power production. Vallee and his group (Paschal and Vallee, 1987; Shpetner et al., 1988) reported that CTP turnover by cytoplasmic dynein was much faster than that of ATP but not accelerated by microtubules, and that the in vitro microtubule translocation was not supported by CTP. Their result is distinguished from the present result in that we observed filament-activated substrate turnover of certain ATP analogues by kinesin or HMM without filament translocation.

It is of interest to note that nucleotides that supported microtubule movement on 22S dynein-coated glass surfaces also induced rapid dissociation of the microtubule-22S

dynein complex, whereas those which did not induce rapid dissociation did not support translocation. It seems likely that the rapid dissociation of dynein heads from the microtubules is prerequisite to power production in the cross-bridge cycle. If dissociation is slower than the hydrolytic step, a considerable fraction of hydrolysis and product release would take place without dissociation, which would result in the uncoupling of substrate turnover from power production, or even a resistance to the motility. Thus, the ability of a nucleotide to dissociate the motor–filament complex may correlate with its ability to induce movement. We intend to examine whether this correlation between dissociation and movement holds true for the kinesin and HMM systems as well.

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