Phosphorothioate Analogues of Adenosine 5′-Triphosphate as Substrates of Dynein from *Tetrahymena* Cilia

Takashi Shimizu* and Kiyotaka Furusawa

Research Institute for Polymers and Textiles, Yatabe-Higashi, Tsukuba, Ibaraki 305, Japan

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ABSTRACT: The substrate specificity of dynein from *Tetrahymena* cilia was investigated with phosphorothioate analogues of ATP. All analogues including ATPγS were hydrolyzed by dynein. At 0.5 mM nucleotide and 4 mM $MgCl_2$ or 0.4 mM $CdCl_2$, the activity of dynein toward these analogues was as follows (the first and second values after each nucleotide indicate the relative activity with Mg^{2+} and Cd^{2+}, respectively): ATP, 100, 13.4; ATPαS(S_P), 254, 32.1; ATPαS(R_P), 151, 18.8; ATPβS(S_P), 9.8, 1.2; ATPβS(R_P), 0.7, 32.7; ATPγS, 11.6, 1.2. These values suggest that dynein exhibits stereospecificity at the β-phosphate but not at the α-phosphate. This was confirmed by the determination of the apparent K_m and V_{max} for each analogue with Mg^{2+} or Cd^{2+} as the supporting cation. In the presence of Mg^{2+}, the susceptibility of the enzyme activity of dynein to vanadate-induced inhibition was in the following order: ATP > ATPγS ≥ ATPαS(S_P) > ATPαS(R_P) ≫ ATPβS(S_P). The complex of dynein with brain microtubules was dissociated completely by ATP or by ATPαS(S_P). ATPαS(R_P) at 0.2 mM induced a partial dissociation by itself and complete dissociation in the presence of vanadate. ATPβS isomers did not induce the dissociation even in the presence of vanadate.

The ciliary or flagellar movement of eukaryotic cells is induced by a cyclic interaction of dynein ATPase[1] with microtubules coupled to ATP hydrolysis in such a way that the chemical energy of ATP hydrolysis is converted into the mechanical force needed to produce sliding between adjacent microtubules (Gibbons, 1981; Johnson et al., 1984; Johnson, 1985). These sliding movements are coordinated by mechanisms, which are not well understood, to produce the waveforms typical of cilia or flagella (Sugino & Naitoh, 1982). The recent investigations by Johnson and co-workers on the kinetics of dynein ATPase (Johnson, 1985; Holzbaur & Johnson, 1986; Omoto & Johnson, 1986) have revealed that the basis of the interaction between dynein and microtubules has considerable similarity to that of the interaction between myosin and actin (Taylor, 1979; Adelstein & Eisenberg, 1980), although there are some significant differences in the rate constants.

Dynein is known to be very specific for ATP as substrate (Gibbons, 1966; Takahashi & Tonomura, 1979) and has relatively little activity toward most of the ATP analogues that have been useful in studying myosin ATPase. Moreover, attempts to detect a physicochemical change of dynein when it binds its substrate has so far been unsuccessful, which is a serious limitation in investigating its ATPase mechanism.

Over the past decade, a new series of ATP analogues has been introduced and widely used in the study of kinases: phosphorothioate analogues (Eckstein & Goody, 1976; Eckstein, 1983). In these analogues, the substitution of a sulfur atom for an oxygen atom at a nonbridging position on the triphosphate moiety leads to stereoisomeric forms that one can use to probe stereospecificity of the enzyme.

We have begun using the phosphorothioate analogues of ATP to study dynein from *Tetrahymena* cilia with three aims in mind. First, we might be able to see if there is an enzymatic heterogeneity among the ATPase sites in the dynein preparation, such as might parallel the structural heterogeneity observed by scanning transmission electron microscopy (STEM) in the head portions of solubilized dynein (Johnson & Wall, 1983). Second, it is of enzymological interest to determine what kind of specificity the dynein ATPase has and to compare it with that of other ATPases, especially myosin. Third, by the use of these analogues, it might be possible to classify different forms of cell motility into several categories according to specificity with respect to the analogues. We hope the present paper will contribute to the first step in the broader applicability of the analogues in cell biology.

Materials and Methods

Preparation of Nucleotide Analogues. Phosphorothioate analogues of ATP and ADP were prepared basically according to Eckstein and Goody (1976), which is described briefly as follows.

ADPβS was chemically synthesized from AMP and *S*-carbamoylthiophosphate (Cook, 1970) by the method described by Eckstein and Goody (1976). For the purification of the product (and also of the other nucleotides described below), the DEAE-Sephadex A-25 (Pharmacia) column chromatography was carried out with a triethylammonium bicarbonate buffer gradient. As will be described later, the ADPβS thus obtained contained some impurities but was a suitable starting material for syntheses of the ATPβS analogues.

ATPβS(S_P) was synthesized with pyruvate kinase and phosphoenolpyruvate and was treated with hexokinase to remove ATPβS(R_P) contamination as far as possible (Eckstein & Goody, 1976; Yee et al., 1979). ATPβS(R_P) was synthesized by using acetyl kinase and acetylphosphate and was

* Correspondence should be addressed to this author.

[1] Abbreviations: AMPS, adenosine 5′-phosphorothioate; ADPαS, adenosine 5′-*O*-(1-thiodiphosphate); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); MOPS, 3-(*N*-morpholino)propanesulfonic acid; ATPase, adenosine-5′-triphosphatase; Ap_5A, P^1,P^5-bis(5′-adenosine) pentaphosphate; M-ATPase, M^{2+}-dependent adenosine-5′-triphosphatase; P_i, inorganic phosphate.

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treated with myosin Ca-ATPase to remove ATPβS(S_P) contamination (Eckstein & Goody, 1976; Yee et al., 1979). In order to obtain pure ADPβS, the ADPβS preparation described above was converted into ATPβS(S_P), which was purified by the DEAE-Sephadex method: the resultant ATPβS(S_P), now free from the contaminants whose elution profiles were similar to that of ADPβS, was hydrolyzed to yield pure ADPβS with Ca^{2+}-dependent enzyme activity of myosin.

AMPS and the mixture of ADPαS diastereomers were synthesized by the method described by Eckstein and Goody (1976). The ATPαS(S_P) and ADPαS(R_P) isomers were obtained with pyruvate kinase and phosphoenolpyruvate, while the ATPαS(R_P) and ADPαS(S_P) isomers were made with creatine kinase and creatine phosphate. The ATPαS(R_P) obtained in this way was treated with hexokinase to eliminate possible ATPαS(S_P) contamination (Midelfort & Sarton-Miller, 1978).

ATPγS was purchased from Boehringer Mannheim and was purified on a DEAE-Sephadex column. However, this preparation still seemed to contain 1–2% of ATP-like material. This ATPγS was used only to test the ability of dynein to hydrolyze this analogue with an assumption that 1–2% ATP contamination would not affect the bulk rate of ATPγS hydrolysis.

Analysis by high-performance liquid chromatography with a C_{18} reverse-phase column revealed that the ATPαS isomers and ATPβS(R_P) were not appreciably contaminated with the other analogues or with ATP. The ATPβS(S_P) contained about 10% of the (R_P) isomer, which will be discussed later. The ADPβS purified by the method above appeared free from contaminants. The ADPαS isomers were contaminated by each other, but the purity of each was better than 90%.

Proteins. Pyruvate kinase and lactate dehydrogenase were obtained from Boehringer Mannheim. Creatine kinase, acetate kinase, and hexokinase were purchased from Sigma. Myosin was prepared from rabbit hind leg and back muscles by the method described by Perry (1955).

The preparation method of the dynein has already been described (Shimizu & Kimura, 1974; Porter & Johnson, 1983a) and will only be outlined. Cilia of *Tetrahymena pyriformis* str. W were detached by the ethanol–calcium method and demembranated with 0.25% Triton X-100. After several washes, the axonemes were extracted with a high salt solution, and the resultant crude dynein solution was applied to a DEAE-Sephacel column (Porter & Johnson, 1983a). The dynein was eluted with 0.2 M NaCl and fractionated into the 22S and 14S forms by sucrose density gradient centrifugation. In this paper, the term "dynein" is used for simplicity to represent the "22S dynein" except where otherwise stated. The specific Mg-ATPase activity of dynein thus obtained varied from 0.5 to 0.85 μmol of P_i min^{-1} mg^{-1} of protein, depending upon the preparation.

Porcine brain microtubule protein, containing microtubule associated proteins, was prepared as described previously (Porter & Johnson, 1983a).

Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Assays of the Hydrolysis of Nucleotide Analogues. Assays were carried out at 25 °C in 0.1–0.3 mL of solution containing 50 mM MOPS–NaOH (pH 7.0), 4 mM $MgCl_2$, 5–50 μg of dynein/mL, and 0.5–1.0 mM ATP or a nucleotide analogue, unless otherwise stated. The enzyme reaction was usually allowed to proceed to 5–30% conversion of the substrate into products. After such a duration of incubation, the reaction was terminated by adding trichloroacetic acid to a final concentration of 4%, and the phosphate liberated was determined by the method of Fiske and SubbaRow (1925). The linearity of the enzyme reaction was checked to be, at least, satisfactory.

In the case of ATPγS hydrolysis, the products are ADP and thiophosphate, and the latter cannot be determined by the usual phosphate assay. Therefore, the amount of ADP liberated was measured as follows. After the reaction had proceeded for 20–40 min as above, an equal volume of a solution containing 50 mM MOPS–NaOH (pH 7.0), 4 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 20 μg/mL pyruvate kinase, 50 mM KCl, and 0.2 mM metavanadate was added. The resultant mixture was incubated for 2 min to allow the pyruvate kinase reaction to go to completion, while the dynein activity was more than 95% inhibited by the metavanadate present. The pyruvate released was determined by the method of Reynard et al. (1961). Five-micromolar Ap_5A was incorporated into the initial assay solution to inhibit possible adenylate kinase contamination. The presence of 0.1 mM metavanadate did not inhibit the pyruvate kinase activity (Kobayashi, personal communication). There were two possible sources of error in this assay of ATPγS hydrolysis: the contamination by 1–2% ATP-like material and the possibility of ATPγS hydrolysis during the second incubation in the presence of vanadate. The first source of error would not be serious for assays of hydrolysis. The second might seem more serious, since during the pyruvate kinase action ATP rather than ATPγS was regenerated by this enzyme, and ATP hydrolysis by dynein was about 8 times faster than ATPγS hydrolysis. However, ATP hydrolysis is more sensitive to vanadate, so that at least 99% of the ATPase activity is inhibited by 0.1 mM vanadate (Shimizu & Johnson, 1983a). For these reasons, the above errors are considered to be small and comparable to random experimental errors.

Determination of Apparent K_m and V_{max}. In order to determine the apparent K_m and V_{max} for each of ATPαS and ATPβS analogues, the modified malachite green assay for phosphate was employed (Kodama et al., 1986). The assay solution was the same as described above except for the concentration of divalent metal cations: for $MgCl_2$, the concentration was the sum of 4 mM and that of the nucleotide to maintain the free Mg^{2+} concentration to be 4 mM; for $CdCl_2$, the concentration was chosen to be the same as that of the nucleotide. After an appropriate duration of incubation, perchloric acid was added to give a final concentration of 0.3 M, and a malachite green solution of the same volume as the acid-quenched solution was added. The malachite green solution contained 2 g of sodium molybdate, 0.3 g of malachite green oxalate, and 0.5 g of Triton X-100 in 1 L of 0.7 M HCl. After a 30–40 min incubation at 25 °C, the absorbance at 650 nm was recorded.

In practice, the time course of the phosphate liberation was measured at a nucleotide concentration between 5 and 500 μM, and a rough estimation of the Michaelis constants was made. The concentration range was then selected for carrying out the Lineweaver–Burk plot. Care was also taken that less than 30% of the nucleotide was hydrolyzed by dynein: in this range, the time course was practically linear.

For the apparent K_m and V_{max} of Cd-ATPase of dynein, the same method as above was used, whereas those of Mg-ATPase were determined by the coupled assay method using pyruvate kinase and phosphoenolpyruvate as described previously (Shimizu, 1981).

Turbidimetric Assay. The microtubule–dynein complex was made by mixing porcine brain microtubules reconstituted from 3-times cycled microtubule protein and dynein purified by

Table I: Hydrolysis Rates of ATP and Analogues by 22S Dynein[a]

nucleotide	hydrolysis rate (μmol of substrate consumed min^{-1} mg^{-1} of protein) with $MgCl_2$	with $CdCl_2$	Mg/Cd
ATP	0.722 (100)	0.097 (13.4)	7.4
ATPαS(S_P)	1.833 (254)	0.232 (32.1)	7.9
ATPαS(R_P)	1.091 (151)	0.136 (18.8)	8.0
ATPβS(S_P)	0.071 (9.8)	0.0084 (1.2)	8.5
ATPβS(R_P)	0.005 (0.7)	0.236 (32.7)	0.021
ATPγS	0.084 (11.6)	0.0089 (1.2)	9.4

[a] The method of determining the hydrolysis rates is described under Materials and Methods. The reaction mixture contained 50 mM MOPS–NaOH (pH 7.0, 4 mM $MgCl_2$ or 0.4 mM $CdCl_2$, 0.1 mM EDTA, 0.5 mM of one of the nucleotides, and 5–50 μg of 22S dynein/mL. The reaction was allowed to proceed at 25 °C for 10–100 min depending on the nucleotide species and protein concentration. The figures in parentheses indicate the relative values of the hydrolysis rate by taking the Mg-ATP hydrolysis as 100.

DEAE-Sephacel chromatography (Porter & Johnson, 1983a) in a medium containing 50 mM MOPS–NaOH (pH 7.0) and 4 mM $MgCl_2$. A sample (0.4 mL) of the suspension was transferred into a cuvette, and the turbidity was measured at 420 nm before and after addition of ATP or ATP analogue as described below.

Results

Hydrolysis Rate of Phosphorothioate Analogues of ATP by 22S Dynein. Both ATPαS isomers were good substrates for 22S dynein in the presence of Mg^{2+} (Table I). It is notable that these analogues were catalyzed faster than ATP itself, which is not seen with kinases but with myosin (Goody & Hofmann, 1980; Connolly & Eckstein, 1981). ATPβS(S_P) was hydrolyzed only at about 10% of the rate of ATP hydrolysis, and ATPβS(R_P) was a very poor substrate. Although the ATPβS(S_P) was contaminated by the (R_P) isomer, the contribution of the (R_P) isomer hydrolysis to the observed rate of the (S_P) isomer hydrolysis should be negligible because of the very slow hydrolysis of the (R_P) isomer. ATPγS was hydrolyzed by dynein, but only at a low rate.

It has been established that Mg^{2+} coordinates to phosphorus through an oxygen atom, while Cd^{2+} has a strong tendency to form a chelate complex through a sulfur atom (Pecoraro et al., 1984). The chelate complexes of diastereomers of phosphorothioate analogues of ATP have stereochemically different configurations depending upon these divalent metal cations (Eckstein, 1983). In practice, kinetic constants of an enzyme reaction on these analogues are determined in the presence of Mg^{2+} and Cd^{2+} separately and are compared to see if there is a switchover of preferred isomer upon changing the divalent metal cation from Mg^{2+} to Cd^{2+}. Such a switchover allows one to conclude that the enzyme has a stereospecificity (Eckstein, 1983).

Now, in order to check the stereospecificity of dynein at the α- and β-phosphates, the hydrolysis rate at a fixed concentration of nucleotide (0.5 mM) was measured in the presence of Cd^{2+}. However, since Cd^{2+} is a well-known SH poison and dynein is sensitive to such agents (Ogawa & Mohri, 1972; Shimizu & Kimura, 1974), the dependence of the hydrolysis rate on Cd^{2+} concentration was studied first (Figure 1). Because of the presence of 0.1 mM ethylenediaminetetraacetic acid (EDTA), almost no activity was observed up to 0.1 mM $CdCl_2$. The activity increased as the $CdCl_2$ concentration was raised from 0.1 to 0.4 mM with the analogues, whereas the hydrolysis of ATP showed a maximum at 0.3 mM. A $CdCl_2$ concentration of 0.4 mM was chosen for the experiments below except for those for determining the Michaelis constants.

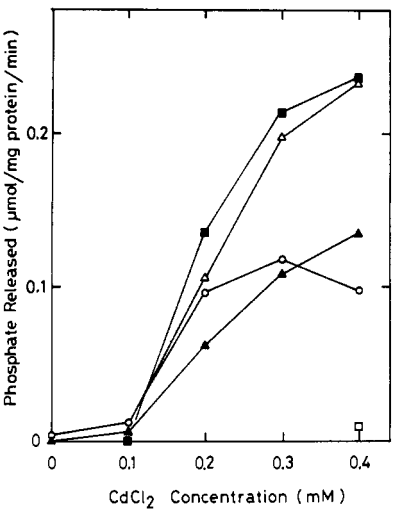


FIGURE 1: Hydrolysis of ATP and analogues by 22S dynein in the presence of $CdCl_2$. The reaction mixture contained 50 mM MOPS–NaOH (pH 7.0), 0.1 mM EDTA, 0.5 mM nucleotide, 20–40 μg/mL 22S dynein and various concentrations of $CdCl_2$ as indicated. After a 10–30-min incubation at 25 °C, the reaction was stopped with 4% trichloroacetic acid, and the phosphate liberated was determined: (○) ATP; (△) ATPαS(S_P); (▲) ATPαS(R_P); (□) ATPβS(S_P); (■) ATPβS(R_P).

With Cd^{2+} as the divalent metal cation, both the ATPαS isomers were fairly good substrates (Figure 1 and Table I). While ATPβS(S_P) was hydrolyzed very slowly, ATPβS(R_P) was a good substrate. ATPγS was hydrolyzed slowly.

It should be mentioned that the hydrolysis of ATPβS(S_P) in the presence of $CdCl_2$ had a kind of burst in the first 30 min and a steady state after that. We interpret this as the preferential hydrolysis of the contaminant ATPβS(R_P) (see Materials and Methods) in the initial phase, and we take the subsequent steady-state phase to represent the true hydrolysis rate of ATPβS(S_P). On the other hand, ATPβS(R_P) hydrolysis with $MgCl_2$ proceeded linearly with time, which agreed well with the lack of (S_P) isomer contamination in our (R_P) isomer preparation.

These results are listed in Table I. The ratio of the hydrolysis rate in the presence of $MgCl_2$ to that with $CdCl_2$ was in the range of 7–10 for ATP and for all the analogues, except ATPβS(R_P) which gave a ratio of only 0.02. From these data, we suggest that the metal chelation to β-phosphate is important during the course of the enzymatic activity of dynein.

Michaelis Constants for Each Analogue. Next, in order to reach an unambiguous conclusion about stereospecificity, we determined apparent K_m and V_{max} for each analogue. In the presence of Mg^{2+}, the Lineweaver–Burk plots with all the αS and βS analogues were practically straight and simple Michaelis–Menten relationships were observed in the nucleotide concentration range of 5 μM to 2 mM. This indicates no obvious diversity of the dynein sites with these analogues as substrates. The apparent K_m for ATPαS(S_P) was fairly low (8.2 μM), but the others gave larger values (67–105 μM).

As the Cd^{2+} concentration dependence of the ATPase shown in Figure 1 might have suggested the complicated situation in the use of $CdCl_2$ as a supporting cation for dynein, we had difficulty in determining Michaelis constants in the presence of $CdCl_2$: at a higher nucleotide (i.e., also $CdCl_2$) concentration such as 0.5 mM, the activity was lower, probably due to the ability of free Cd^{2+} to poison the enzyme activity even though the concentration of $CdCl_2$ was the same as that of nucleotide; at lower concentrations, such as less than 10 μM, the concentration of the Cd^{2+}–nucleotide chelate complex would be significantly lower than the total concentration [the

Table II: Apparent K_m and V_{max} Values for ATP and Analogues in the Presence of Mg^{2+} or Cd^{2+} [a]

nucleotide	with $MgCl_2$			with $CdCl_2$		
	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	V_{max}/K_m	K_m (μM)	V_{max} (nmol min^{-1} mg^{-1})	V_{max}/K_m
ATP	1.1, 2.8	404, 530	367, 189	5.3	180	22
ATPαS(S_P)	8.2	1340	163	11	290	26
ATPαS(R_P)	83	820	9.9	65	140	2.2
ATPβS(S_P)	67	87	1.3	70	13	0.19
ATPβS(R_P)	105	7.1	0.068	10	550	55

[a] The method of determining the apparent K_m and V_{max} values is described under Materials and Methods. The cation levels were 4 mM plus the concentration of the nucleotide ($MgCl_2$) and the same concentration as that of the nucleotide ($CdCl_2$). It should be noted that this preparation of 22S dynein had a little lower activity than that used in the experiment shown in Table I and that the V_{max} value for ATPβS(R_P) with $CdCl_2$ as a supporting cation is considerably different from the value listed in Table I, probably due to the effect of variation of the $CdCl_2$ concentration as described in the text. As reported previously (Shimizu, 1981), the Lineweaver–Burk plot of the Mg-ATPase had a downward bent. The two apparent K_m's and V_{max}'s are listed here.

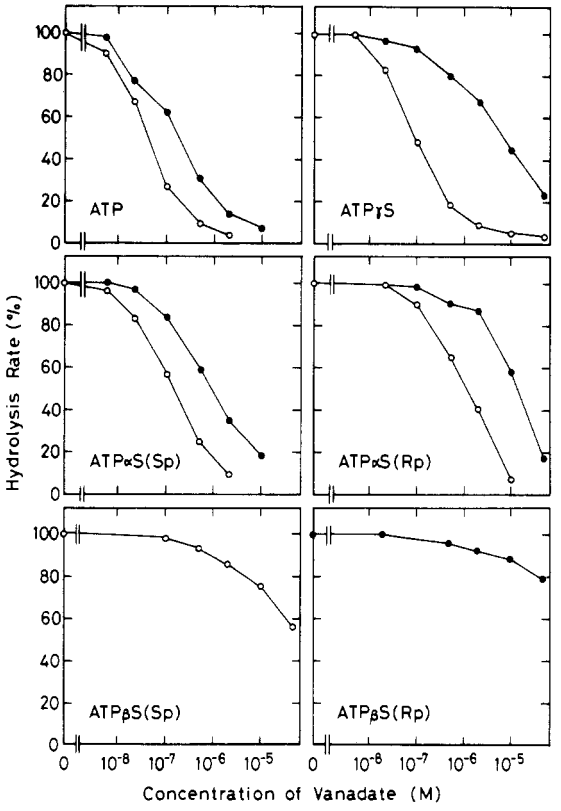


FIGURE 2: Vanadate inhibition of the hydrolysis of ATP and analogues by 22S dynein. The enzyme assay was carried out as described under Materials and Methods with various concentrations of metavanadate as indicated. Open and closed circles indicate that 4 mM $MgCl_2$ and 0.4 mM $CdCl_2$ were used as a supporting cation, respectively.

dissociation constants of the chelate complexes are around 10 μM (Pecoraro et al., 1984)]. Therefore, the Lineweaver–Burk plots were made in relatively narrow ranges of nucleotide concentration, so the values listed in Table II may not be very accurate but should not be far from the true values.

Again, we see a switchover of the preferred isomer of the βS analogues by changing the divalent metal cation, while there were no such switchovers with the αS isomers.

Vanadate Inhibition of Analogue Hydrolysis. When $CdCl_2$ was substituted for $MgCl_2$ with ATP as the substrate, the sensitivity to vanadate inhibition decreased about 5-fold (Figure 2), as has been described previously for $CaCl_2$ (Kobayashi et al., 1978; Shimizu, 1981). A similar decrease was observed with the analogues, although its magnitude varied from one analogue to another (Figure 2). In the presence of $MgCl_2$, the order of sensitivity to vanadate was ATP > ATPγS $\geq$ ATPαS(S_P) > ATPαS(R_P). The hydrolysis of ATPβS(S_P) with $MgCl_2$ or of ATPβS(R_P) with $CdCl_2$ was much less sensitive to vanadate. Because the hydrolysis rates of

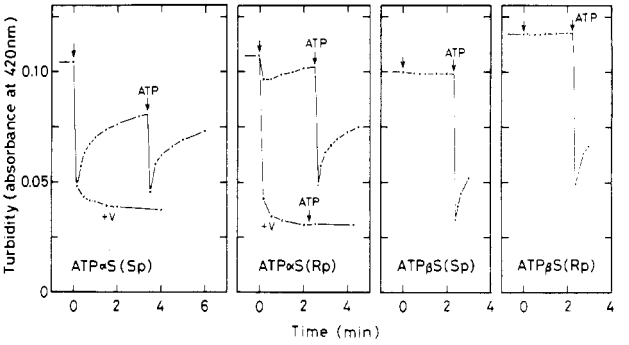


FIGURE 3: Turbidimetric analyses of the microtubule–dynein complex. Porcine brain microtubules containing microtubule-associated proteins were mixed with DEAE-Sephacel purified dynein at 28 °C to form the complex in the presence of 4 mM $MgCl_2$ as described under Materials and Methods. The final concentrations of microtubules and dynein were 0.27 and 0.40 mg/mL, respectively. At time zero, 0.2 mM ATP analogue was added, and the reading due to turbidity at 420 nm on a spectrophotometer was recorded. +V in the figure means that 0.05 mM vanadate was added at time zero together with the nucleotide. Arrows with "ATP" in the figure indicate that final 0.05 mM ATP was added at the time indicated.

ATPβS(R_P) with Mg^{2+} and of ATPβS(S_P) with Cd^{2+} were so low, we did not investigate their sensitivity to vanadate.

It should be mentioned that we did not find out what would indicate unambiguous diversity of the dynein sites in relation with vanadate inhibition of the analogue hydrolysis.

Turbidity Decrease of the Microtubule–Dynein Complex Suspension by Analogues. Mixing of reconstituted brain microtubules with DEAE-Sephacel purified dynein leads to microtubule–dynein complex formation, and the complex is dissociated by addition of ATP (Haimo et al., 1979; Porter & Johnson, 1983a). Previous kinetic analysis by stopped flow has demonstrated that the dissociation is due to the binding of ATP to dynein (Porter & Johnson, 1983b). This type of experiment can, therefore, provide additional information about the substrate specificity of dynein.

In this study, turbidimetric experiments were performed with a conventional spectrophotometer to examine the ability of the analogues to dissociate the microtubule–dynein complex.

At a concentration of 0.2 mM, neither ADPβS nor either of the ADPαS isomers induced a significant decrease in turbidity, irrespective of the absence or presence of 0.5 mM vanadate. This may be a result of very weak binding of these analogues or may indicate that they do not induce a conformational change required for dissociation of the dynein from the microtubules. The ATPβS analogues did not induce a turbidity decrease, even in the presence of vanadate.

On the other hand, ATPαS(S_P) induced a rapid turbidity decrease of the same magnitude as occurred upon addition of ATP and vanadate. After this initial decrease, the turbidity recovered (Figure 3). The turbidity recovery was prevented

if vanadate was added together with the analogue.

Addition of 0.2–1.0 mM ATPαS(R_P) induced an incomplete turbidity decrease in the absence of vanadate, whereas in the presence of vanadate the turbidity decrease was of the same magnitude as occurred with ATP.

Discussion

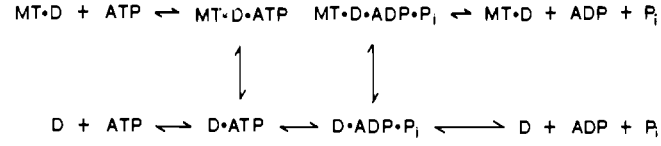
Under the experimental condition described here, dynein ATPase appears to exhibit a stereospecificity at the β-phosphate of ATP. In the presence of the physiological cation, Mg^{2+}, dynein showed significant activity toward ATPβS(S_P), whereas in the presence of Cd^{2+} ATPβS(R_P) was the preferred substrate. On the other hand, the αS diastereomers were not distinguished by the enzyme in such a way as the βS isomers were. However, it was shown that the ATPαS(S_P) isomer was the better substrate for dynein with both Mg^{2+} and Cd^{2+}. Although we have little information about the significance of cation chelation to the γ-phosphate, a β–γ bidentate form of Mg-ATP would be the actual substrate for dynein. Some exchange inert chelate complexes of ATP with certain trivalent metal ions, such as Cr(III)$(NH_3)_4$ATP (Cleland & Mildvan, 1979; Dunaway-Mariano & Cleland, 1980a,b), may be useful in further probing of the true structure of the substrate for dynein, although with myosin these chelate complexes were shown to have little affinity for the active site (Eccleston & Trentham, 1978).

The stereospecificity determined for dynein ATPase is the same as that for myosin ATPase (Goody & Hofmann, 1980; Connolly & Eckstein, 1981). However, there are subtle differences. For example, Mg-ATPβS(S_P) hydrolysis by myosin was faster than Mg-ATP hydrolysis, whereas Mg-ATPβS(S_P) hydrolysis by dynein occurred at about 10% of the rate for Mg-ATP. Thus, though dynein and myosin appear to have the same basic stereospecificity, there still is a possibility that these analogues might be useful in determining whether dynein or myosin or some other ATP-utilizing enzyme is involved in a certain cellular process.

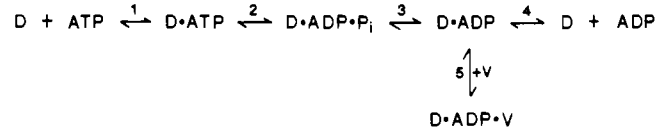
In this study, we also investigated the substrate specificity for dissociation of the microtubule–dynein complex. The kinetics of the process by which ATP binding to dynein causes dissociation of the microtubule–dynein complex have been analyzed by stopped-flow light scattering measurements (Porter & Johnson, 1983a,b). In this work, we used turbidimetric assay rather than light scattering, but both methods are considered to give the same information. The completeness of dissociation induced by ATP or ADP plus vanadate (Porter & Johnson, 1983a; Shimizu & Johnson, 1983a), determined optically, correlates well with that observed by electron microscopy. Therefore, in the discussion below, the ligands that induce a complete turbidity decrease are regarded as competent to fully dissociate the microtubule–dynein complex. On the other hand, a partial turbidity decrease is interpreted as nondissociation, and ligands that induce no or a partial turbidity decrease are regarded as incompetent. Preliminary observations using electron microscopy support these interpretations for the ATP analogues (Shimizu, Marchese-Ragona, and Johnson, unpublished results).

Among the analogues, only ATPαS(S_P) appeared competent to dissociate the microtubule–dynein complex (Figure 3). It was rather surprising that ATPαS(R_P) induced only a small turbidity decrease, indicating that it was not competent in complete dissociation of the complex, although in the presence of vanadate it did give complete dissociation. On the other hand, both ATPβS isomers were totally ineffective even in the presence of vanadate, which was less surprising since they were hydrolyzed slowly.

Scheme I



Scheme II



There are several possible explanations of why ATPαS(R_P) that was hydrolyzed at an even faster rate than ATP did not induce complete dissociation: (1) Binding of the dynein to the microtubules may limit the accessibility of the analogue to the dynein catalytic sites. This is not likely since ATP and ATPαS(S_P), which are similar to ATPαS(R_P) in structure, have good access to the sites. (2) Heterogeneity of dynein sites in a single molecule. Since 22S dynein from *Tetrahymena* cilia is known to consist of a common base and three globular heads that interact with the microtubules in an ATP-sensitive manner (Johnson & Wall, 1983; Shimizu & Johnson, 1983b), there is a possibility that only one or two of the three heads might be able to bind and hydrolyze ATPαS(R_P), whereas the other(s) might have little access to this analogue. While the former would be detached and actively hydrolyzing the analogue, the latter would remain associated with the microtubule. Thus, the dynein molecule would not come off the microtubule. The presence of vanadate will somehow freeze all the heads in a state favorable for dissociation. However, this interpretation suggests that all three sites have affinity for ATPαS(R_P) with different binding constants. Since we did not detect possible diversities of the sites in vanadate inhibition or in the Lineweaver–Burk plot with this analogue, this explanation also seems unlikely. (3) A kinetic explanation. According to the ATPase pathway of dynein (Scheme I; MT and D represent the microtubule and the dynein catalytic site, respectively) (Johnson, 1983, 1985), in the presence of sufficient Mg-ATP, most dynein sites are found in the D•ADP•P_i state, and these intermediates have little affinity for the microtubule binding. When ATPαS(R_P) is the substrate, the corresponding intermediates might have a short lifetime relative to the duration of the overall hydrolysis cycle. Thus, the dissociation would not be favored even if the dynein sites were actively catalyzing this analogue. This situation would be reinforced by the fact that dynein has multiple heads as discussed above: in order for a dynein molecule to dissociate from the microtubule, all three heads need to have bound ATPαS(R_P) at the same time.

Possibility 1 is very unlikely as we have discussed above. Possibility 2 also seems unlikely, although we could not rule it out. Further detailed kinetic studies on the elementary steps of the pathway scheme of ATPαS(R_P) hydrolysis may be able to address this question.

Our previous study on the pre-steady-state kinetics of vanadate inhibition (Shimizu & Johnson, 1983a) indicated that vanadate would bind to the γ-phosphate site of the D•ADP intermediate to form a D•ADP•V dead-end complex (Scheme II). It is natural to assume that hydrolysis of the analogues is inhibited by the same mechanism. Therefore, the sensitivity of the hydrolysis of the analogues to inhibition by vanadate would be considered to be governed by the lifetime of the intermediate corresponding to D•ADP and by its conformation. It is not surprising that ATPγS hydrolysis was only a little less sensitive to vanadate than that of ATP since the products

of hydrolysis are thiophosphate and ADP, and D·ADP is the intermediate of the ATPγS hydrolysis to bind vanadate. The present results also indicate that the substitution of the sulfur atom for oxygen in ATPα$S(R_P)$ had a pronounced effect, while that in ATPα$S(S_P)$ did not. The sulfur at the β-phosphate position had a greater effect, which seems consistent with the inability of the ATPβS isomers plus vanadate to dissociate the microtubule–dynein complex (Figure 3). Better understanding of the difference in sensitivity would only be obtained by direct measurements of rate constants of the steps, although we do not know some of them even with ATP as a substrate.

We are currently working to establish which analogues are able to induce dissociation in the presence of Cd^{2+} instead of Mg^{2+}. There is a possibility that we may be able to determine whether α-coordination is of any significance for dynein. It will also be of interest to learn which analogues are capable of inducing motility in detergent-treated models of cilia and flagella. Since dissociation of the microtubule–dynein complex is presumably prerequisite for the mechanochemical coupling of hydrolysis to motility (Taylor, 1978; Adelstein & Eisenberg, 1980; Johnson, 1985), ATPα$S(S_P)$ appears to be the most probable candidate for inducing motility.

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Registry No. ATP, 56-65-5; ATPα$S(S_P)$, 58976-48-0; ATPα$S(R_P)$, 58976-49-1; ATPβ$S(S_P)$, 59261-36-8; ATPβ$S(R_P)$, 59261-35-7; ATPγS, 35094-46-3; ATPase, 9000-83-3; Mg, 7439-95-4; Cd, 7440-43-9; VO_4^{3-}, 14333-18-7.

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