Adenosine 5′-O-(3-Thiotriphosphate) Hydrolysis by Dynein†

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ABSTRACT: The interaction of dynein with ATPγS, a phosphorothioate analogue of ATP, has been investigated in depth. The hydrolyses of ATPγS and of ATP were shown to be mutually competitive. ATPγS induced complete dissociation of the microtubule–dynein complex such that the time course of dissociation monitored by stopped-flow light-scattering methods followed a single exponential. The ATPγS concentration dependence of the rate of dissociation was hyperbolic, indicating that the dissociation is at least a two-step process: M·D + ATPγS ⇄ M·D·ATPγS → M + D·ATPγS. The fit to the hyperbola gives an apparent K_d = 0.5 mM for the binding of ATPγS to the microtubule–dynein complex, and the maximal rate of 45 s^{-1} defines the rate of dissociation of the ternary M·D·ATPγS complex. Rapid quench-flow experiments demonstrated that the hydrolysis of ATPγS by dynein exhibited an initial burst of product formation. The size of the burst was 1.2 mol/10^6 g of dynein, comparable to that in the case of ATP hydrolysis. The steady-state rate of ATPγS turnover by dynein was activated by MAP-free microtubules. Because the rate of ATPγS turnover is severalfold (4–8) slower than ATP turnover, the rate-limiting step must be release of thiophosphate, not ADP. Thus, microtubules can activate the rate of thiophosphate release. The stereochemical course of phosphoric residue transfer was determined by using ATPγS stereospecifically labeled in the γ position with ^{18}O. Hydrolysis of ATPγS by dynein in ^{17}O-enriched water produced inorganic [^{16}O,^{17}O,^{18}O]thiophosphate which was chiral. The configuration of this product showed that the hydrolysis proceeded with inversion at the transferred phosphoric residue. This result suggests a direct, in-line hydrolysis mechanism for the dynein ATPase and that there are no phosphorylated enzyme intermediates.

Dynein is the adenosine-5′-triphosphatase (ATPase)[1] that drives microtubule sliding in eukaryotic cilia and flagella [for reviews, see Gibbons (1981) and Johnson (1985)]. Dynein is a large molecule with a molecular weight of 1.3–1.9 million depending upon the source of isolation (Gibbons & Fronk, 1979; Johnson & Wall, 1983). The polypeptide composition of dynein is complex with two to three heavy chains, two to three intermediate chains, and four to six light chains (Tang et al., 1982; Johnson, 1985). The molecule also has complex morphology as revealed by electron microscopy, having two or three globular heads (one per heavy chain), each connected to the base by an independent strand (Johnson & Wall, 1983; Witman et al., 1982; Sale et al., 1987). Each globular head is postulated to bear an ATPase site and a microtubule binding site. The "base" is considered to be the site to anchor the dynein molecule to the A subfiber of outer doublet microtubules.

Dynein is responsible for force generation by converting chemical energy of ATP hydrolysis into mechanical force by interacting with the adjacent microtubules in reactions similar to the myosin ATPase in muscle (Johnson, 1985).

To understand the mechanism of force generation in cilia or flagella, it is of great importance to investigate the reaction mechanism of the dynein ATPase. The mechanochemical cycle of microtubule–dynein (M·D) interactions can be correlated directly to the steps in the ATPase pathway. According to previous results by Johnson and his collaborators (Johnson, 1985; Holzbaur & Johnson, 1986; Omoto & Johnson, 1986), the ATPase pathway is similar to that of the myosin ATPase in that nucleotide binding energy is used to establish the kinetically and thermodynamically preferred pathway, with the dissociation of the M·D complex preceding ATP hydrolysis. Although the rate constants and the rate-limiting steps differ, the two ATPases have similar pathways, and microtubules activate the dynein ATPase in a reaction analogous to the actin-activated myosin ATPase (Omoto & Johnson, 1986).

The use of substrate analogues is often fruitful in investigating enzymatic characteristics. It is known, however, that dynein exhibits high substrate specificity and that most analogues modified at the adenine ring are very poor substrates (Gibbons, 1966; Takahashi & Tonomura, 1978; Shimizu, 1987). We examined the effectiveness of phosphorothioate analogues of ATP as substrates of dynein in a previous study (Shimizu & Furusawa, 1986), and we reported that ATPγS was hydrolyzed slowly by dynein although we did not investigate the characteristics of ATPγS in detail partly because the ATPγS was contaminated by a small amount of ATP.

In earlier work on myosin, the identity of the reaction intermediate was controversial; some claimed that the phosphorylated myosin was an important intermediate formed during the hydrolytic cycle (Kinoshita et al., 1969), analogous to the membrane ATPases (Yamamoto & Tonomura, 1968; Makinose, 1969; Glynn & Karlish, 1975), while others

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ATPγS, adenosine 5′-O-(3-thiotriphosphate); ATPase, adenosine-5′-triphosphatase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DAPP, P^1,P^5-di(adenosine-5′) pentaphosphate; HEPES, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; MAP(s), microtubule-associated protein(s); PIPES, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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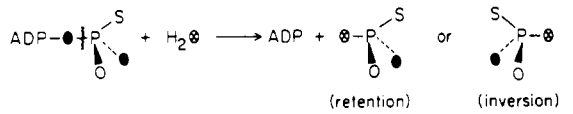
Scheme I: Hydrolysis of [γ-^{32}P]ATP Stereospecifically Labeled with ^{18}O in ^{17}O-Enriched Water[a]



[a] (○), (⊗), and (●) represent ^{16}O, ^{17}O, and ^{18}O, respectively.

questioned the existence of a phosphorylated intermediate of myosin (Wolcott & Boyer, 1973). This important issue was addressed in an extremely elegant manner. In 1978, Richard and Frey (1978) reported that they synthesized stereospecific ATPγS; although the phosphorus atoms in ATPγS are tetrahedral, the γ-thiophosphate is not stereospecific because it has two indistinguishable oxygen atoms. Their success is based upon replacement of one oxygen by oxygen-18 in a stereospecific manner, thanks to the high stereospecificity of adenylate kinase. Webb and Trentham (1980a,b) utilized this stereospecific ATPγS in elucidating the stereochemical nature of the myosin ATPase reaction. The ATPase reaction is based upon the nucleophilic attack on the γ-phosphorus, and with each nucleophilic event, the configuration around the phosphorus is inverted. If the reaction proceeds with a simple displacement reaction by water, the product phosphate has a stereochemical configuration opposite to that of the substrate. However, if the reaction proceeds via a phosphorylated enzyme intermediate, there are two consecutive nucleophilic attacks, first by an enzyme nucleophile and the second by water. As a consequence of the two inversions, the overall configuration of the phosphate is retained (Scheme I). Webb and Trentham (1980b) successfully determined that the configuration around the phosphorus of released thiophosphate was indeed inverted. This strongly argues against the existence of a phosphorylated intermediate in the myosin pathway.

This method has been applied to a considerable number of ATP-splitting enzymes [for reviews, see Frey (1982) and Eckstein (1983, 1985)]. In accordance with the prediction, the sarcoplasmic reticulum Ca-ATPase, which proceeds by a phosphorylated intermediate (Yamamoto & Tonomura, 1986; Makinose, 1969), catalyzed the hydrolysis of ATPγS with retention of the configuration (Webb & Trentham, 1981).

In this paper, thanks to our success in removing this ATP from ATPγS, we now report some enzymatic characteristics of dynein toward ATPγS as a substrate. In addition, we report that the hydrolysis of ATPγS by dynein proceeds with inversion of the configuration, which strongly suggests that the hydrolysis is a one-step process and no phosphorylated intermediates of the enzyme are formed.

Materials and Methods

Purification of ATPγS. ATPγS was purchased from Boehringer and was purified on a DEAE-Sephadex A-25 column with a triethylammonium bicarbonate buffer gradient. The ATP contaminant appeared on the leading edge of the ATPγS peak as revealed by high-performance liquid chromatography with a C_{18} reverse-phase column, and the fractions containing ATPγS only but no ATP-like materials were combined and evaporated after the addition of 3 molar equiv of Tris base. [γ-^{35}S]ATPγS was from New England Nuclear. A portion of this radioactive nucleotide diluted by a proper amount of cold ATPγS was purified as above, or through a MonoQ column (Pharmacia) with an isocratic solution of 0.3 M NH_4HCO_3. In the latter case, the radioactive peak fractions were lyophilized to obtain purified ATPγS. Other chemicals were the same as reported previously (Shimizu & Johnson, 1983a).

Protein Purification. Dynein was obtained from the cilia of *Tetrahymena thermophilia* strain SB-255 as described (Porter & Johnson, 1983a,b); all experiments were performed with 22S dynein, and the term dynein will be used to refer to this species. To concentrate the dynein solution, Aquacide III (Calbiochem) was used. Microtubule proteins were prepared from bovine brains (Porter & Johnson, 1983a). The MAP-free microtubules were prepared and stabilized by using taxol as described by Omoto and Johnson (1986). Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard.

Enzyme Assays. ATPase assay was carried out at 28 °C in an assay solution consisting of 50 mM HEPES–NaOH (pH 7.0), 4 mM $MgCl_2$, 1 mM ATP, and 20 μg/mL dynein unless otherwise described. After an appropriate length of incubation, the reaction was stopped by adding trichloroacetic acid to a final concentration of 0.3 M, and the phosphate liberated was determined (Fiske & SubbaRow, 1925). In some cases, [γ-^{32}P]ATP was used instead of cold ATP. In this case, the reaction was stopped by 0.5 M perchloric acid, and the radioactivity in the supernatant due to inorganic phosphate after charcoal treatment was determined by Cerenkov counting.

The hydrolysis of ATPγS by dynein was assayed in the same solution as above with radioactive ATPγS instead of ATP. After the reaction was stopped with perchloric acid, the radioactivity in the supernatant after charcoal treatment was determined with ScintiVerse II (Fischer) as scintillation cocktail. It should be noted that ATPγS is not stable in an acidic solution so that care was taken to treat all the samples in the same manner, and to minimize the duration of exposure of ATPγS to acid to less than 15 s. The method to determine the overall hydrolysis rate employed previously (Shimizu & Furusawa, 1986) using pyruvate kinase and lactate dehydrogenase after stopping the dynein reaction by vanadate gave similar results.

Stopped-flow and quench-flow experiments were done as described previously (Porter & Johnson, 1983a,b; Johnson, 1983, 1987).

Electron microscopic observations were also made as described by Porter and Johnson (1983a).

Stereochemistry of ATPγS Hydrolysis. Stereospecific ATPγS was synthesized as described (Richard & Frey, 1978; Webb & Trentham, 1980b). Oxygen-17- or oxygen-18-enriched water was purchased from MSD Isotopes. The contents of ^{16}O, ^{17}O, and ^{18}O were 14.9, 56.4, and 28.7%, respectively, for oxygen-17-enriched water, and were 2.8, 1.8, and 95.4%, respectively, for oxygen-18 enriched water.

A 1-mL sample of the stereospecific ATPγS at 25 mM in 70 mM Tris-HCl (pH 8.0), 25 mM $MgCl_2$, 0.01 mM DAPP, and 1 mM DTT was lyophilized. After sucrose density gradient centrifugation, 22S dynein peak fractions were pooled, sucrose was removed by dialysis, and the solution was lyophilized (Shimizu & Katsura, 1988). Lyophilized dynein was dissolved in 1 mL of oxygen-17-enriched water at 0.88 mg/mL, and the dynein solution was added to the lyophilized ATPγS to start the reaction. The course of the hydrolysis was monitored as described (Webb & Trentham, 1980b), and when about 80% of the substrate was hydrolyzed, the entire solution was frozen in liquid nitrogen. The oxygen-17-enriched H_2O was removed by lyophilization, and the residue was dissolved in 5 mL of 10 mM EDTA (pH 7) in H_2O.

The product, thiophosphate, was isolated by DEAE-Sephadex A-25 chromatography with DTNB as the coloring reagent for detection. It was incorporated into the β position of ATPβS by the method of Webb and Trentham (1980b).

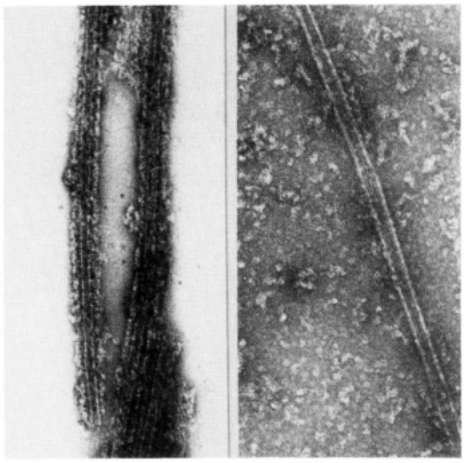


FIGURE 1: Electron micrograph of the microtubule–dynein complex. The microtubule–dynein complex was made by mixing polymerized microtubules (0.32 mg/mL) and dynein (0.5 mg/mL) in 50 mM PIPES–NaOH (pH 7.0) and 4 mM $MgCl_2$ at 28 °C for 20 min. The suspension was diluted 2-fold with fresh, warm buffer solution and then examined by negative-stain electron microscopy before (left) and after (right) the addition of 0.5 mM ATPγS. The bar in the figure indicates 200 nm (75200×).

The ^{31}P NMR spectrum of the β-phosphorus of ATPβS was resolved on a Nicolet NT-360 NMR spectrometer operating at 145.79 MHz; 20 000 scans covering ±1000 Hz were accumulated for resolving the thiophosphate species. The data was analyzed by using a computer program to address the inversion retention problem, as described (Domanico et al., 1985).

Results

Dissociation of the Microtubule–Dynein Complex by ATPγS. ATPγS induced the complete dissociation of the microtubule–dynein complex as shown by electron microscopic observations (Figure 1). The dissociation time course was monitored as a decrease in light scattering (Figure 2A). The amplitude of the light-scattering change induced by ATPγS was the same as that by ATP, thereby providing quantitative data to demonstrate that the dissociation of the dynein from the microtubule was complete. The light-scattering traces could be fit by a single exponential (Figure 2A) as was previously shown in the case of the ATP-induced dissociation. The ATPγS concentration dependence of the rate is shown in Figure 2B. The rate increased with increasing concentration but approached a maximum at high concentration. The data could be fit to a hyperbola with the maximal rate of 45 s^{-1} and with the concentration giving a half-maximal effect being 0.5 mM. This hyperbolic relationship implies that the dissociation in a two-step process according to the equation:

$$M{\cdot}D + ATP\gamma S \rightleftarrows M{\cdot}D{\cdot}ATP\gamma S \rightarrow M + D{\cdot}ATP\gamma S$$

Moreover, the data demonstrated that ATPγS was not contaminated by ATP because the rate of dissociation observed with ATP would continue to increase linearly (Porter & Johnson, 1983b; Shimizu & Johnson, 1983b) with increasing concentration.

Initial Burst of ATPγS Hydrolysis by Dynein. It was previously established that the dynein ATPase exhibits an initial burst of phosphate production, the rate of which is 60 s^{-1} or higher at 28 °C and pH 7 (Johnson, 1983). The burst phenomenon directly indicates that the substrate binding and hydrolysis are fast but that the product release is slow and rate limiting. The early phase of ATPγS hydrolysis by dynein was analyzed in this study to demonstrate a similar burst (Figure 3). The precision of the data is limited by our difficulty in reducing the background value of radioactive ATPγS in spite of our efforts to purify the ATPγS immediately prior to the experiment. Moreover, concentrations of dynein greater than 2 mg/mL could not be achieved because of precipitation of the dynein. The rate of the burst was ~5 s^{-1}, although the scatter in the data precluded a precise estimate of the rate of the burst.

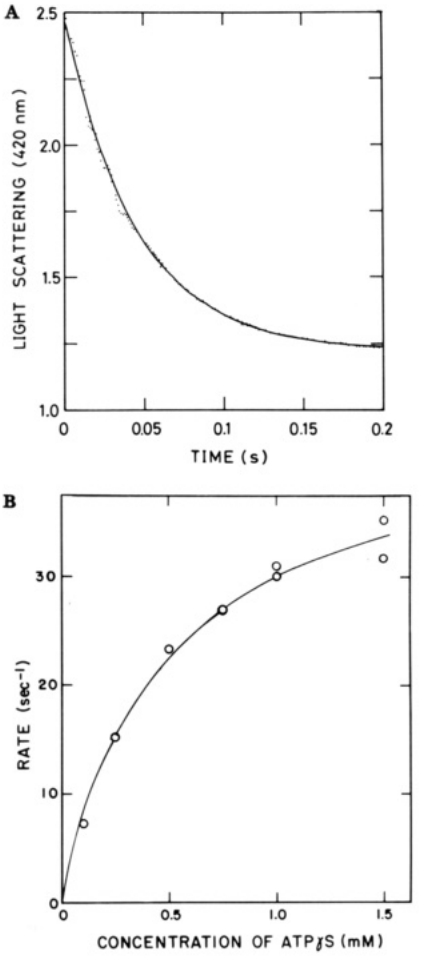


FIGURE 2: Kinetics of dissociation of the microtubule–dynein complex. The microtubule–dynein complex was made as described in the legend to Figure 1 using 0.25 mg/mL dynein and 0.14 mg/mL microtubules with 2.5 μM taxol. This suspension was mixed in the stopped-flow with an equal volume of various concentrations of ATPγS in the same buffer, 50 mM PIPES–NaOH (pH 7.0) and 4 mM $MgCl_2$. (A) Time dependence of the change in light scattering observed at 0.5 mM ATPγS. The smooth line represents the best fit to the single exponential. (B) Concentration dependence of the rate of dissociation. For each concentration of the nucleotide, the traces of the light-scattering decay were fitted to a single exponential to estimate the dissociation rate. The rates thus obtained are shown as a function of ATPγS concentration. The solid curve is the best fit to a hyperbola with a maximum rate of 45 s^{-1} and with a half-maximal rate being given at 0.5 mM.

The amplitude of the burst was ~1.2 mol of thiophosphate per 10^6 g of dynein, similar to that observed with ATP as a substrate. In the presence of vanadate, the burst amplitude was slightly larger. This is expected because the rate of ATP turnover in the absence of vanadate reduces the steady-state concentration of enzyme-bound products. At a low temperature (0 °C), the size of the burst was smaller (data not shown), suggesting a large temperature dependence of the hydrolysis step.

Steady-State Hydrolysis of ATPγS by Dynein. The Lineweaver–Burk plot of the steady-state rate of ATPγS hydrolysis by dynein was biphasic (Figure 4). Above 5 μM, the

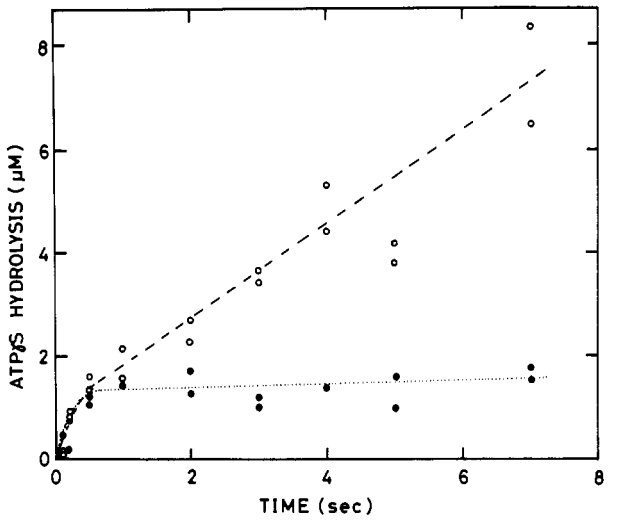


FIGURE 3: Early phase of ATPγS hydrolysis of dynein. The early phase of ATPγS hydrolysis by dynein was investigated by using a quench-flow apparatus at 28 °C as described by Johnson (1983). The final concentrations were 0.82 mg/mL dynein and 60 μM ATPγS. The experiment was performed in the absence (○) or in the presence of 0.1 mM vanadate (●). The lines represent a fit to the data with a burst rate of 5 s^{-1}.

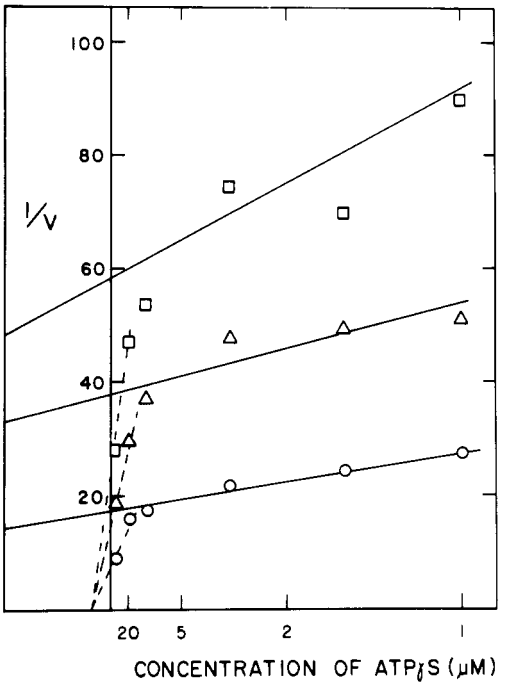


FIGURE 4: Lineweaver–Burk plot of ATPγS hydrolysis by dynein. Hydrolysis of ATPγS by dynein was assayed as described under Materials and Methods at 28 °C using radioactive ATPγS. The enzyme activity is shown in micromoles per minute per milligram of protein (V). The abscissa is linear in the reciprocal of the substrate concentration but is expressed as the concentration. Results are shown in the absence of vanadate (○), in the presence of 0.1 μM vanadate (△), and in the presence of 0.5 μM vanadate (□).

plot was linear, giving an apparent $K_m \simeq 20$ μM. Below 5 μM, the apparent K_m was less than 1 μM. This relationship was also observed in the presence of vanadate, which was a noncompetitive inhibitor for ATPγS hydrolysis as in the case of ATP hydrolysis (Shimizu, 1981).

ATP was a competitive inhibitor of ATPγS hydrolysis by dynein and vice versa (data not shown). This result indicates that ATPγS binds to the ATP catalytic sites.

Recently, Omoto and Johnson (1986) demonstrated that the repolymerized MAP-free microtubules at high concentration enhanced the ATPase activity of dynein severalfold.

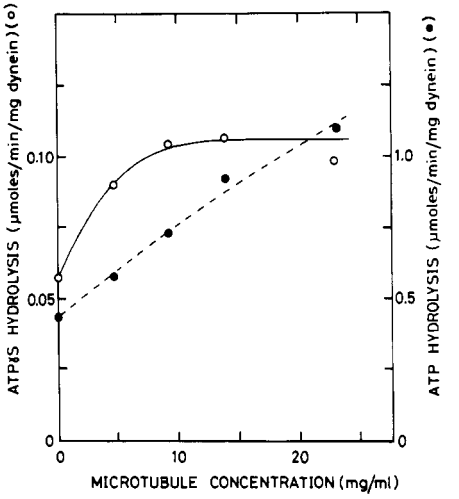


FIGURE 5: Microtubule activation of the hydrolysis of ATP and ATPγS. Hydrolysis of 0.5 mM ATPγS (○) or 0.1 mM ATP (●) was assayed by the use of radioactive substrates as described under Materials and Methods in the presence of various concentrations of the repolymerized MAP-free microtubule protein at 28 °C.

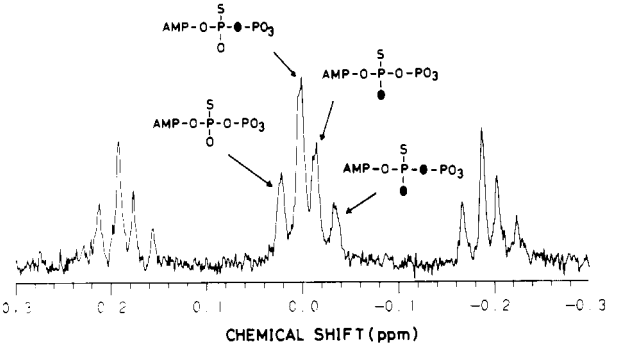


FIGURE 6: NMR spectral analysis of the reaction products. The hydrolysis product of stereospecifically labeled [γ-^{32}P]ATP by the enzyme action of 22S dynein, thiophosphate, was incorporated into the β position of ATPβS by the method of Webb and Trentham (1980b). The NMR spectrum of the β-phosphorus was recorded as described under Materials and Methods. The assignment of the peaks is made according to Webb and Trentham (1980b).

This was confirmed in the present study as shown in Figure 5. The ATPase activity increased almost linearly with the microtubule concentration up to 20 mg/mL. The magnitude of enhancement observed was almost the same as that reported previously (Omoto & Johnson, 1986).

Microtubules also enhanced the hydrolysis rate of ATPγS by dynein (Figure 5). However, the pattern of enhancement is different from that with ATP as a substrate. The enhancement had an obvious plateau, its maximum being about 2-fold.

Stereochemistry of Hydrolysis. Stereospecific ATPγS was hydrolyzed by dynein at a rate of ~0.1 μmol min^{-1} mg^{-1} at 28 °C. The incubation was continued for 3.5 h to get hydrolysis of 80% of the 25 mM substrate at the dynein concentration of 0.88 mg/mL. The product thiophosphate was recovered and converted into ATPβS through a series of enzyme reactions as described by Webb and Trentham (1980b). The final yield of ATPβS from thiophosphate was about 20%.

The NMR spectrum of the β-phosphorus of the resultant ATPβS is shown in Figure 6. Because the oxygen-17-enriched water was only 56% pure with 29% being oxygen-18, the spectrum had four peaks. The assignment of the peaks was done according to Webb and Trentham (1980b) as shown in Figure 6. At a glance, the spectrum resembles that reported previously with myosin, which has been shown to hydrolyze

Table I: Relative Peak Intensities[a]

	unlabeled	$^{18}O_1$(bridge)	$^{18}O_1$(nonbridge)	$^{18}O_2$
observed	19.3	42.1	24.7	13.9
predicted	19.3	44.4	24.8	11.5

[a] Predicted and observed peak distributions for the ^{31}P NMR spectrum of the β-phosphorus of ATPβS derived from the thiophosphate product of the 22S dynein-catalyzed hydrolysis. The predicted distribution was calculated by assuming 95% ^{18}O enrichment in the substrate ATPγS, and no racemization according to the computer program described by Domanico et al. (1985). Similar predictions were obtained with 85–95% ^{18}O enrichment in the substrate ATPγS, and up to 10% racemization of the product.

Table II: Rate Constants for Hydrolysis of ATP and ATPγS by Dynein[a]

reaction	step	ATP[b]	ATPγS[c]
substrate binding (M^{-1} s^{-1})	1	5×10^6	10^5
M·D dissociation (s^{-1})	2	≥1000	45
ATP hydrolysis (s^{-1})	3	~100	≥5
product release (s^{-1})	4	4	1–2
ATP release (s^{-1})	−1	0.1	
ATP synthesis (s^{-1})	−3	30	

[a] Constants were determined at 28 °C and pH 7 in the presence of 4 mM $MgCl_2$. Rate constants not listed here have not been determined. The step numbers are those described in the Scheme II. [b] Porter and Johnson (1983b), Johnson (1983), and Holzbaur and Johnson (1986, 1989). [c] This study.

ATPγS with inversion of configuration.

By quantitative analysis with the computer program, it was determined explicitly that the configuration around the phosphorus of released thiophosphate was inverted (Table I). Thus, the hydrolysis took place with an odd number of nucleophilic attacks of the γ-phosphorus of the original ATPγS. Of course, the most likely case is the single attack of phosphorus by oxygen of a water molecule.

Discussion

The kinetic analysis presented in this paper showed that ATPγS was hydrolyzed at the dynein ATPase sites and that the mechanism of the hydrolysis is the same as that of ATP hydrolysis although the rate constants differ greatly.

For ATP hydrolysis by dynein, Johnson (1983, 1985) proposed Scheme II where D and M denote dynein and microtubules, respectively. The rate constants determined so far are given in Table II.

Scheme II

$$\mathrm{M{\cdot}D + ATP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{M{\cdot}D{\cdot}ATP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{M + D{\cdot}ATP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{D{\cdot}ADP{\cdot}P_i} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{D + ADP + P_i}$$

The rate of ATPγS binding to dynein was at least 10-fold slower than that of ATP as evident from the kinetics of dissociation of the microtubule–dynein complex. This supports the importance of the γ position of ATP in enzyme–substrate binding in dynein (Shimizu & Furusawa, 1986). However, a more important feature of the dissociation kinetics was that the ATPγS concentration dependence was hyperbolic, indicating a two-step dissociation. Previously (Porter & Johnson, 1983b), a two-step dissociation was presumed to occur, but the ATP-induced dissociation was too fast to allow observation of the second step. From the result shown in Figure 3, the dissociation by ATPγS was shown to have a rate constant of about 45 s^{-1}, defining the rate constant for dissociation of the M·D·ATPγS complex.

The ATPγS hydrolysis rate was much lower than that of ATP. However, we have to note that the apparent rate of ATPγS binding to dynein in the quench-flow experiment may have been only 6 s^{-1} [$(10^5$ M^{-1} $s^{-1}) \times (6 \times 10^{-5}$ M ATPγS)], such that the observed rate of hydrolysis might have been limited by the rate of substrate binding. Accordingly, the rate of ATPγS hydrolysis at the active site could be higher than we observed.

The observation of the initial burst indicates that the rate-limiting step follows ATPγS hydrolysis, as in the case of ATP turnover. Actually, the hydrolysis rate observed, ~5 s^{-1}, was faster than the overall turnover rate of 1–2 s^{-1}. In the case of myosin, the initial burst of product formation was not observed with ATPγS as the substrate (Bagshaw et al., 1972), although it bound to the myosin site quite tightly (Bagshaw et al., 1974).

As in the case of myosin ATPase (Kodama et al., 1986), the burst size was smaller at low temperature, suggesting a large temperature dependence of the equilibrium constant of the hydrolysis step.

With ATP as substrate, a biphasic concentration dependence of steady-state turnover was observed (Shimizu, 1981). In the present study, a more pronounced downward bent in the double-reciprocal plot was observed with ATPγS. This seems to confirm our previous conclusion that dynein has some complex feature as an enzyme; heterogeneity, or negative cooperativity. This important question is still to be resolved.

ATP hydrolysis and ATPγS hydrolysis by dynein were inhibited by vanadate in a similar manner (Shimizu & Furusawa, 1986). Because vanadate is thought to bind to the D·ADP intermediate at the γ-phosphate site, and was shown to inhibit dynein after a single turnover (Figure 3), the order of product release must be thiophosphate first and ADP second. If ADP release were rate limiting, the overall rate should be the same for ATP and ATPγS hydrolysis. Obviously, this is not the case. In the case of ATPγS turnover, the thiophosphate release must be at least partially rate limiting while in the ATP turnover, ADP release has been shown to be rate limiting (Holzbaur & Johnson, 1989).

Microtubules activated the rate of ATPγS steady-state turnover, and so the rate of thiophosphate release was accelerated by microtubules. We observed a maximum of about 2-fold activation of ATPγS hydrolysis. This could be explained in two ways. (1) This type of dependence on the microtubule concentration is one of the properties of the thiophosphate releasing step. (2) Another step such as ATPγS hydrolysis has a rate constant about 2-fold higher than thiophosphate release and becomes rate limiting at higher microtubule concentrations.

The results presented here demonstrate that microtubules can activate the release of thiophosphate, suggesting that a similar reaction may occur in vivo to activate phosphate release. Although solution studies indicate that phosphate release is faster than the rates of microtubule binding, this need not be the case in vivo. At the high microtubule concentrations in the axoneme, the microtubule may bind to either the D·ADP·P_i or the D·ADP intermediate to complete the ATPase cycle.

Analysis of the stereochemical course of the ATPase reaction demonstrated that the reaction proceeded with inversion of configuration, suggesting a direct hydrolysis mechanism with no phosphorylated intermediates. Although the mitochondrial F_1-ATPase was shown to hydrolyze ATPγS with inversion of configuration (Webb et al., 1980), some membrane ATPases such as (Na–K)-ATPase and sarcoplasmic reticulum Ca-ATPase are known to yield phosphate through phosphorylated intermediates (Webb & Trentham, 1981; Glynn & Karlish, 1975; Inesi, 1985).

In conclusion, the present data strongly suggest that the hydrolytic activity of the dynein ATPase is a single-step process and that no phosphorylated intermediates are formed during the catalysis.

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