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A DYNEIN ATPase INHIBITOR ISOLATED FROM A COMMERCIAL ATP PREPARATION

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Summary

Preparations of ATP from equine muscle contained an inhibitor of dynein Mg^{2+}-activated ATPase. The inhibitory material was separated from the ATP by molecular sieve filtration. The several molecular species of dynein extracted from three different axonemal sources were all inhibited; myosin ATPase was not. With increasing amounts of inhibitor the inhibition did not go to completion but reached a plateau when the rate had been reduced to 1/5 the uninhibited rate. A plot of $1/[S]$ against $1/v$ at several inhibitor concentrations yielded parallel lines. There was little inhibition of dynein ATPase when Mg^{2+} was replaced by Ca^{2+}. The inhibitor appeared slightly smaller in molecular size than ATP, had anionic character, and was not adsorbed to charcoal.

Introduction

Motility in eucaryotic cilia and flagella is based on active sliding of outer doublet microtubules past each other induced by dynein, energy-transducing protein(s) in paired arms attached to the A tubules [1,2]. Dynein, as solubilized by dialysis against EDTA at low ionic strength, is a heterogeneous high molecular weight ATPase which can be activated by either Mg^{2+} or Ca^{2+}. Gibbons [3] reported some time ago that with certain batches of ATP a 30 s fraction of dynein from *Tetrahymena* cilia was activated normally by Ca^{2+}, but much less well by Mg^{2+}. We have also recently encountered some ATP preparations with which the Mg^{2+}-activated ATPase of *Chlamydomonas* flagellar dynein was reduced by 80% [4]. Evidently either these preparations contained a dynein inhibitor, or all others contained an activator [5].

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Until now no specific inhibitor of dynein has been available. Such an inhibitor would be useful in studying the function of axonemal dynein in vivo and, if it did not inhibit myosin ATPase, would be particularly valuable in investigating the possible roles of dynein in cytoplasmic and chromosomal motility. We have now separated the inhibitor from ATP. In this report we describe some characteristics of the inhibition and preliminary studies of the inhibitor structure.

Materials and Methods

Materials. The primary batch of "inhibitory ATP" was Lot 112C-7470 of Sigma highest purity (No. A3127) ATP from equine muscle. Reference ATP was from Schwarz/Mann. Other nucleotides were from P-L Biochemicals, [γ-^{32}P]ATP from Amersham/Searle, and Norit A activated charcoal powder from Matheson, Coleman and Bell. Heavy meromyosin ATPase from rabbit muscle was kindly supplied by Dr. Yeu-Zu Yang.

Preparation and assay of dynein. Unless otherwise specified all experiments were done with crude dynein extracted from *Tetrahymena* cilia. Conditions have been described [4] for the culture of *Tetrahymena pyriformis* strain W (ATCC 10542), isolation of cilia, and low ionic strength extraction of dynein. Sperm were obtained from the sea urchin *Lytechinus variegatus* (from Gulf Specimens, Panacea, Florida).

Sucrose density gradient centrifugation of cilia and flagella extracts were done as previously described [4] except that the gradient was from 5 to 30% and KCl was omitted. The gradients were run after the extracts had been stored at 0°C overnight, or for not more than 2 days.

ATPase was routinely assayed essentially as previously described [4]. The standard reaction mixtures for the assay contained, in a final volume of 1 ml: 30 mM Tris · HCl (pH 7.9)/12 mM $MgSO_4$/0.45 mM EDTA/1 mM ATP and (unless otherwise specified) 15—30 μg of extract protein. A unit of ATPase is the amount liberating 1 μmol of orthophosphate in 1 min. Protein was determined by the Lowry method [6], using bovine serum albumin as standard. ATPase was also assayed with [γ-^{32}P]ATP as substrate [7].

Isolation of inhibitor by column chromatography. A 2 × 48 cm column of Sephadex G-25 (fine) was washed with water at 2°C. A sample of the "inhibitory ATP" (1.5 ml of 0.3 M adjusted to pH 7.7) was applied, and 2-ml fractions were collected while eluting with water at 18 ml/h.

A 0.8 × 20 cm column of Dowex-50 (AG50W-X8, 200—400 mesh, H^+ form, Bio-Rad) was equilibrated with 50 mM Tris · HCl (pH 7.9) at 2°C. "Inhibitory ATP" (0.2 ml of 0.1 M adjusted to pH 7.6) was added, and 1-ml fractions were collected while eluting with the same buffer at 40 ml/h.

A 0.8 × 8 cm column of a mixture of Norit A and celite was prepared by a modification of the method of Lilly et al. [8], and eluted in 0.5-ml fractions at 2°C and 2 ml/h. Total phosphate was determined by wet ashing [9] and colorimetric assay [10].

Although, as shown below, the inhibition of dynein does not go to completion and we have not defined a unit amount of inhibitor, the recovery in the eluted fractions was estimated by using aliquots small enough to give an inhibi-

tion linearly proportional to the amount added. In Figs. 2 and 5 the amount of inhibitor is expressed on the ordinate scale as the difference in the μmol of phosphate liberated in the standard ATPase mixture with and without addition of the indicated aliquot of eluate fraction.

Results

Specificity of inhibitor for dynein Mg^{2+}-ATPase

The rate of Mg^{2+}-activated hydrolysis of ATP by *Tetrahymena* dynein was about 1/5 as rapid with Lot 112C-7470 Sigma ATP from equine muscle (hereafter called ATP(i)), as with a standard or "normal" ATP. The same apparent inhibition was observed with incubation lasting from 5 to 60 min, and with two other lot numbers of ATP(i). ATP(i) added to reaction mixtures containing 1 mM ATP inhibited the dynein, and inhibition was nearly maximal with 0.7 mM ATP(i). The different rates were thus due to an inhibitor in ATP(i), and not to an activator in standard ATP. Thin-layer chromatography did not reveal any ultraviolet-absorbing impurities or any difference in ATP content between the inhibitory ATP and the standard ATP.

Table I shows that the inhibition was specific for Mg^{2+}-activated dynein, and little inhibition was observed when Mg^{2+} was replaced by Ca^{2+}. The same result was observed when EDTA was omitted from reaction mixtures. With both metals present there was substantial inhibition even with Ca^{2+} 10 times more concentrated than Mg^{2+} (Table I). ATP(i) is a preparation widely used in many laboratories and has not been reported to inhibit other ATPases. Table I documents that myosin ATPase is not inhibited under the assay conditions used for dynein; there was also little inhibition of the uncharacterized axonemal ATPase which is not solubilized by low ionic strength dialysis.

Tetrahymena dynein was fractionated by sucrose gradient centrifugation into 22-S and 15-S species, perhaps corresponding to the 30 S and 14 S previously described [3]. Both components had reduced activity with ATP(i) (Fig. 1), the inhibition being somewhat greater for the 22-S species. Some dynein preparations also revealed a 4 S Mg^{2+}-ATPase which was not inhibited by ATP(i). Similar fractionation of sea urchin sperm flagellar extracts showed

TABLE I

SPECIFIC ACTIVITIES OF VARIOUS ATPases WITH ATP OR ATP(i), AND OF *TETRAHYMENA* DYNEIN IN THE PRESENCE OF Ca^{2+} OR Mg^{2+}

ATPase preparation	Bivalent metal added to reaction mixture		ATPase specific activity with:		Activity ratio (ATP(i)/ ATP)
	(mM) Mg^{2+}	(mM) Ca^{2+}	ATP	ATP(i)	
Heavy meromyosin *	1.2		0.0081	0.0081	1.0
Non-extractable axonemal ATPase	12		0.11	0.071	0.65
Extracted dynein	12		0.39	0.09	0.23
Extracted dynein		12	0.34	0.32	0.94
Extracted dynein	1.2	12		0.21	

* Heavy meromyosin assayed under the same conditions used for dynein.

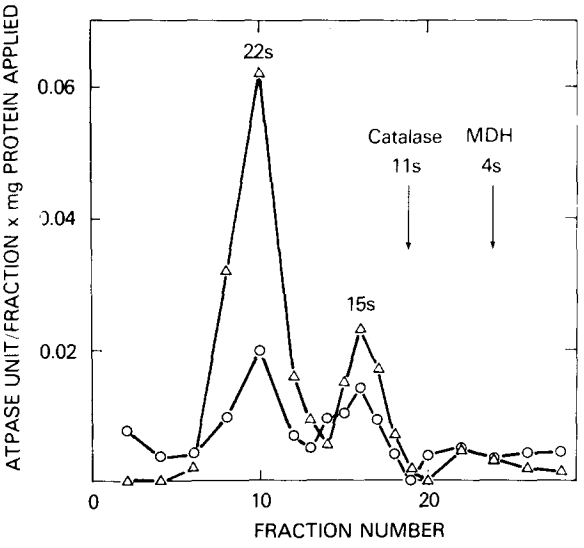

Fig. 1. Mg^{2+}-ATPase activity in sucrose density gradient fractions of *Tetrahymena* cilia extract. The fractions were assayed with 1.2 mM Mg^{2+} + ATP (△———△) or ATP(i) (○———○). So that the activities can be directly compared with those previously described [4], they are expressed as units per fraction per mg protein applied to the gradient. Catalase and malate dehydrogenase (MDH) were added as molecular size markers, and assayed as previously described [4].

15-S and 10-S species of dynein both of which were inhibited by ATP(i). Thus multiple species of dynein from *Chlamydomonas* flagella [4], *Tetrahymena* cilia and sea urchin sperm flagella all give similar results.

Isolation of the inhibitor

The inhibitor was separated from ATP with molecular sieve columns, from which it was eluted just after ATP, suggesting it may have a somewhat lower molecular weight. In the experiment of Fig. 2 the total recovery of inhibitor was 80% of that applied to the column, calculated as described in Materials and Methods. When standard ATP was applied to the column, no inhibitory material was present in the eluted fractions. The small second peak of inhibitor nearly coinciding with ATP (Fig. 2) may indicate an association between ATP and inhibitor. Some inhibition might also have been expected in the second peak fractions because they introduced a high ATP (3—4 mM) concentration into the reaction mixture (see Fig. 3). The same distribution of inhibitor was observed when the [^{32}P]ATP method was used to assay the effect of the fractions on dynein activity.

Fractions of inhibitor having no detectable absorbance at 260 nm were accumulated from several column runs, lyophilized, and redissolved in water to 1/30 the original volume. After concentration a weak absorbance at 260 nm was detectable. Under standard assay conditions dynein was not completely inhibited even by relatively enormous amounts of purified inhibitor. Since the inhibition plateaued at the same level (80%) as was observed with the original ATP(i), the latter must have contained an excess of inhibitor.

Mg^{2+}-ATPase velocity was studied as a function of ATP concentration in the

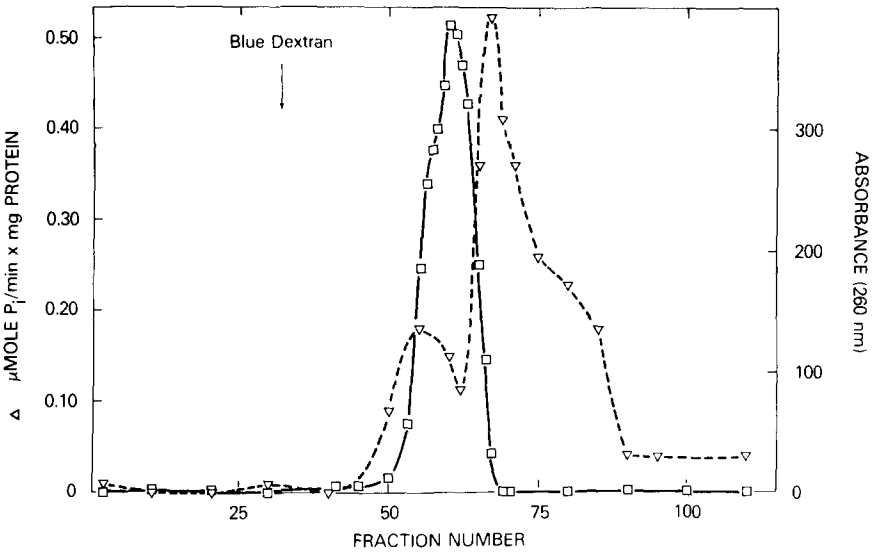

Fig. 2. Separation of the inhibitor from the ATP in ATP(i) by Sephadex G-25 gel filtration. The ATP content of fractions is indicated by absorbance at 260 nm (□———□). The inhibitor content (△———△) is expressed as the difference in the specific activity of dynein assayed in the standard reaction mixture with and without a 100 μl aliquot of the fraction. The specific activity without adding any aliquot was 0.62 μmol · min^{-1} · mg^{-1}.

presence or absence of different amounts of purified inhibitor, the Mg^{2+} concentration being maintained constant at 2.4 mM. Lines drawn through the points in a double reciprocal plot by the formula for linear regression (ignoring the highest ATP concentration) appear to be parallel (Fig. 3), indicating a

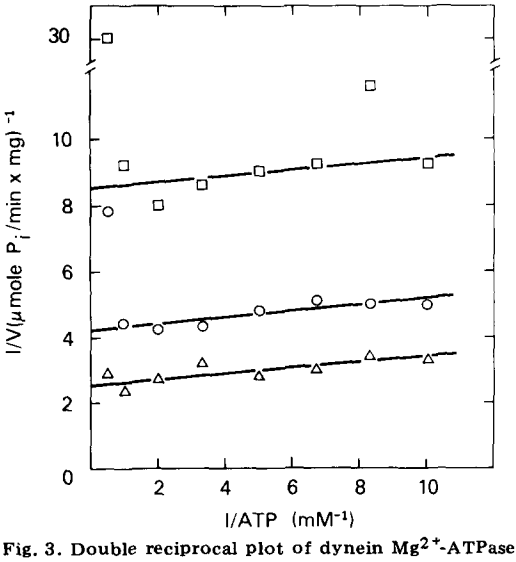

Fig. 3. Double reciprocal plot of dynein Mg^{2+}-ATPase velocity against ATP concentration in the absence of purified inhibitor (△———△), and the presence of 5 μl (○———○) or 50 μl (□———□). The Mg^{2+} concentration was 2.4 mM.

"mixed" or "uncompetitive" type of inhibition [11]. Fig. 3 also shows that with increasing amounts of inhibitor an apparent marked substrate inhibition occurred at the highest concentration of ATP (2 mM). With a higher ratio of Mg^{2+} to ATP substrate inhibition did not appear; with a lower ratio it appeared at even lower concentrations of inhibitor.

Properties of the inhibitor

When ATP(i) was applied to an anion-exchange column (AG1-X10, 100—200 mesh, Cl^- form, Bio-Rad) both ATP and inhibitor activity were retained and not eluted by water, suggesting that the inhibitor has anionic character.

When ATP(i) was eluted from a cation-exchange column as described in Materials and Methods, ATP and inhibitor activity were eluted together in the same fraction, although a small tail of inhibitor was present in later fractions. The recovery of inhibitor was 100%.

An aliquot of inhibitor, purified by Sephadex G-25 filtration and concentrated by lyophilization, was applied to a charcoal column as described in Materials and Methods. The aliquot corresponded to the amount of inhibitor originally present in 220 μmol of ATP and contained 0.07 μmol of nucleotide by absorbance at 260 nm. 85% of the applied inhibitor was not adsorbed by charcoal, and was eluted in a symmetrical peak having no detectable ultraviolet absorbance when the column was washed with water (Fig. 4). The trace of ultraviolet-absorbing material in the inhibitor was recovered in 110% yield when the column was further eluted with ethanol/ammonia/water, (50 : 2 : 48, v/v). The total phosphate in this second peak was 1/3 of the amount of adenine which would correspond to the absorbance at 260 nm. A second peak of "inhibitor" was also eluted by ethanol/ammonia/water (Fig. 4). We believe

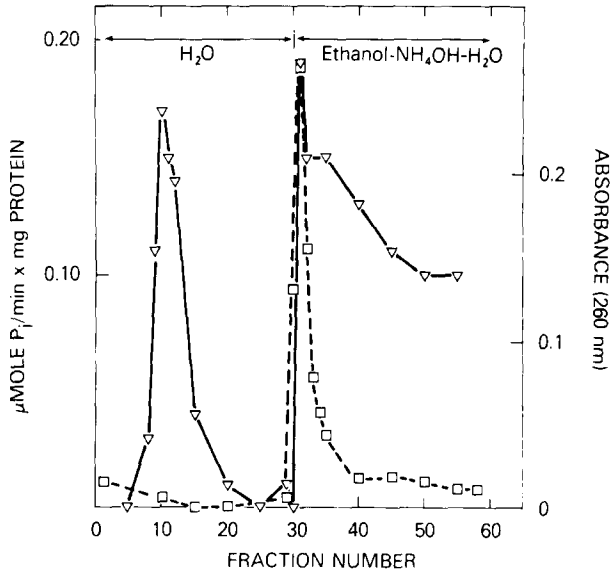

Fig. 4. Charcoal column chromatography of purified inhibitor: absorbance at 260 nm (□- - - - -□); amount of inhibitor per fraction (△———△), calculated as in Fig. 2. The specific activity of the dynein without adding any eluate fraction was 0.30 μmol · min^{-1} · mg^{-1}.

this is not the original inhibitor but something derived from the eluting solvent or the charcoal. The amount of "inhibitor" in the second peak was 140% of the amount applied to the column, over and above the 85% that was not adsorbed to charcoal.

The inhibitor in ATP(i) thus has anionic character and is probably not a purine or pyrimidine derivative since it is not adsorbed to charcoal.

Inhibition of dynein ATPase by metals and nucleotides

At 1 mM concentration Cu^{2+} and Zn^{2+} inhibited Mg^{2+}-activated dynein ATPase 90%, but ATP(i) contained only $2.7 \cdot 10^{-5}$ gramatoms/mol ATP of these metals. Metal concentrations in ATP and ATP(i) were analyzed with atomic absorption spectrophotometer. The two preparations contained about the same amounts, in decreasing order, of calcium, magnesium, strontium, zinc, copper and cadmium, but ATP(i) contained 23 times more iron ($1.3 \cdot 10^{-3}$ gramatoms/mol ATP). However, neither Fe^{2+} nor Fe^{3+} inhibited dynein, at 0.5 mM.

Mg^{2+}-activated dynein is quite specific for ATP [4]. The following did not inhibit when added in addition to ATP: adenosine, AMP, ADP, GTP, ITP, TTP, UTP, and GDP. CTP inhibited 50% at 50 μM and 25% at 10 μM. No CTP was detected in ATP(i).

Discussion

The ATP preparations which contains a dynein inhibitor actually appears to be very pure by metal analysis and chromatography, so the inhibitor must be effective at low concentrations. We have no information about the history of this preparation except that it has been isolated from equine muscle, whereas other preparations may be isolated from yeast or after phosphorylation of adenosine. It seems likely that the presence of inhibitor reflects the origin of the ATP. A wide range of other ATP preparations do not contain the inhibitory material, as indicated by our own experience and the fact that other investigators have not reported this phenomenon in the decade since it was first noted [3].

Our results suggest the inhibitor is slightly smaller in size than ATP, with which it may form a complex, has anionic character, and is not adsorbed to charcoal. Dynein ATPase is not inhibited when Mg^{2+} is replaced by Ca^{2+}. The inhibition is more effective at high (2 mM) ATP and relatively low Mg^{2+} concentration, i.e. appears synergistic with the substrate inhibition observed with excess free ATP [12]. A plot of $1/[S]$ against $1/v$ at several inhibitor concentrations yields parallel lines.

With increasing amounts of inhibitor under standard assay conditions the inhibition plateaus when the rate has been reduced to 20%. This may be related to the accumulating evidence that dynein, as we have isolated it, is a mixture of several different ATPases [2].

Since this paper was submitted for publication a report has appeared [14] that Sigma ATP from equine muscle contains a modifier of $(Na^+ + K^+)$-ATPase. From the common source and properties it seems quite likely that the substance responsible is the same as the dynein inhibitor.

Acknowledgements

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