

## RAPID COMMUNICATIONS

### EFFECTS OF ADENYLYL IMIDODIPHOSPHATE, A NONHYDROLYZABLE ADENOSINE TRIPHOSPHATE ANALOG, ON REACTIVATED AND RIGOR WAVE SEA URCHIN SPERM

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#### ABSTRACT

A nonhydrolyzable ATP analog, adenylyl imidodiphosphate (AMP-PNP), has been used to study the role of ATP binding in flagellar motility. Sea urchin sperm of *Lytechinus pictus* were demembranated, reactivated, and locked in "rigor waves" by a modification of the method of Gibbons and Gibbons (11). Rigor wave sperm relaxed within 2 min after addition of 4  $\mu\text{M}$  ATP, and reactivated upon addition of 10–12  $\mu\text{M}$  ATP. The beat frequency of the reactivated sperm varied with ATP concentration according to Michaelis-Menten kinetics ("K<sub>m</sub>" = 0.24 mM; "V<sub>max</sub>" = 44 Hz) and was competitively inhibited by AMP-PNP ("K<sub>i</sub>"  $\cong$  8.1 mM). Rigor wave sperm were completely relaxed (straightened) within 2 min by AMP-PNP at concentrations of 2–4 mM. The possibilities that relaxation in AMP-PNP was a result of ATP contamination, AMP-PNP hydrolysis, or lowering of the free Mg<sup>++</sup> concentration were conclusively ruled out. The results suggest that dynein cross-bridge release is dependent upon ATP binding but not hydrolysis.

KEY WORDS flagella · motility · dynein · ATP binding · mechanochemistry · microtubules

There is now considerable evidence that the basis for flagellar movement is an ATP-dependent sliding of the outer doublet microtubules relative to one another (25, 27, 28). This sliding appears to be brought about by the action of the outer doublet arms which contain a high molecular weight ATPase, dynein (1, 8, 10, 13, 16, 17, 18, 23, 30). It has been proposed that, concomitant with ATP binding and hydrolysis, the arms undergo an attachment-detachment cycle which causes them to "walk" along the B-tubule of the adjacent outer doublet, resulting in sliding displacement between the outer doublet microtubules (26, 27); the chemical energy of ATP would thus be converted into the mechanical work of interdoubt sliding. Although the broad outlines

of this sliding filament model appear reasonably well established (4, 26), there is at present very little data to connect successive steps in the chemical cycle of ATP hydrolysis to the postulated events in the mechanical cycle of the outer doublet arms.

Reactivated and rigor wave sea urchin sperm appear to be promising systems for studying the relationship between ATP utilization and dynein-microtubule interactions. Gibbons and Gibbons (9) have shown that when demembranated sea urchin sperm are placed in an appropriate buffer solution, they "reactivate" on addition of ATP—that is, their axonemes begin beating with a wave form similar to that of the flagella of intact sperm. Gibbons and Gibbons (11) further showed that abrupt lowering of the ATP concentration in suspensions of reactivated sperm caused the axonemes to become fixed in rigid "rigor waves"

which resembled the wave forms of the reactivated sperm at the time of ATP dilution. Adding back ATP in low concentrations resulted in relaxation (straightening) of the rigor waves; reactivation resumed if the ATP concentration was raised above  $\sim 10 \mu\text{M}$ . It appeared that the rigor waves which formed at low ATP concentrations were maintained by dynein cross-bridges (11, 15), and that relaxation resulting from replenishment of ATP involved the detachment of dynein from the B-tubule. However, it was not possible to distinguish whether this detachment was a result of the binding or the hydrolysis of ATP.

Nonhydrolyzable nucleotide analogs offer a means for identifying biochemical and physiological events which depend on nucleotide binding. Such analogs have been used, for example, to clarify the role of nucleotide binding in muscle contraction (32), protein synthesis (19), microtubule assembly (24), and actin polymerization (6). By using an appropriate nucleotide analog to study dynein-outer doublet interactions, it should be possible to determine which mechanical event(s) in the dynein cross-bridge cycle is solely a result of nucleotide binding.

In this paper, we report the effects on reactivated and rigor wave sea urchin sperm axonemes of a nonhydrolyzable ATP analog, adenylyl imidodiphosphate (AMP-PNP). These observations suggest that ATP binding to the active site of the dynein arm ATPase induces the detachment of dynein cross-bridges from the B-tubule of the adjacent outer doublet microtubule. This study indicates that AMP-PNP should be a powerful tool for analyzing the fine structural changes which occur in dynein during the mechanochemical cycle of flagellar motility.

## MATERIALS AND METHODS

Sea urchins of the species *Lytechinus pictus* were purchased from Pacific Biomarine Laboratories, Inc., Venice, Calif. After injection of a sea urchin with 1–2 ml of 0.5 M KCl, sperm were collected in a small volume of artificial seawater (24.7 g NaCl, 0.7 g KCl, 3.08 g  $\text{MgSO}_4$ , 4.6 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.0 g  $\text{CaCl}_2$ , 0.2 g  $\text{NaHCO}_3$  per liter). The concentrated sperm were stored on ice and used within 5 h of collection. Experiments were performed at room temperature (22–23°C).

Rigor wave sperm were prepared according to a modification of the procedure described by Gibbons and Gibbons (11). A 25- $\mu\text{l}$  aliquot of sperm was added to 0.375 ml of rigor buffer (20 mM Tris-HCl, 0.15 M KCl, 6 mM  $\text{MgSO}_4$ , 1 mM dithiothreitol, 0.5 mM ethylenediaminetetraacetic acid [EDTA], pH 8.1, at 23°C) sup-

plemented with 0.1% Triton X-100 and 20  $\mu\text{M}$  ATP. Within 15 s, 20  $\mu\text{l}$  of the demembrated, reactivated sperm were dispersed by gentle agitation into a final volume of 1.0 ml of rigor buffer. Over 90% of the sperm axonemes were arrested in rigor waves (see Fig. 1a). Rigor wave sperm were stored on ice and used within 1 h of preparation. The concentration of rigor wave sperm in these experiments was  $\sim 8 \times 10^6$  cells/ml, or 0.2 mg/ml of total protein, as determined by the method of Lowry et al. (21) using bovine serum albumin as a standard.

For measurements of beat frequency, rigor wave sperm were reactivated by addition of ATP. Beating sperm which had become attached by their heads to the glass microscope slide, and thus were stationary, were picked at random for beat frequency measurements. The beat frequency was determined as the mean value for 10–15 sperm; standard deviations were less than  $\pm 10\%$  of the means.

Rigor wave and reactivated sperm were observed by dark-field light microscopy using a Zeiss Universal Microscope with an Ultracondenser and a 16 $\times$  Neofluar objective. Dark-field micrographs of rigor wave sperm were taken on Kodak Tri-X film. Beat frequencies of reactivated sperm were determined by stroboscopic illumination from a Chadwick-Helmuth Model 136 stroboscopic flash unit triggered by a General Radio Model 1310-B audio oscillator.

Ascending thin-layer chromatography of nucleotides was performed at ambient temperature on polyethyleneimine (PEI)-cellulose (Baker-flex, J. T. Baker Chemical Co., Phillipsburg, N. J.) thin layer sheets using 1.2 M LiCl as developing solvent (24).  $R_f$  values in this system are reported in Table I. Nucleotides were detected by fluorescence quenching under ultraviolet light. Proportional amounts of nonradioactive nucleotides were estimated from relative spot intensities. Radioisotopically labeled nucleotides, which were identified by comigration with unlabeled standards, were quantitated by scraping spots from the chromatogram into scintillation vials, extracting for 10 min with 0.1 ml of 30 mM Tris-HCl, 1.0 M KCl, pH 8.0, and counting by liquid scintillation spectrometry.

For some experiments, AMP-PNP was pretreated with alkaline phosphatase to remove possible ATP contamination. AMP-PNP at a concentration of 4 mM was incubated with 0.2  $\mu\text{g}/\text{ml}$  ( $\sim 0.004$  U) alkaline phosphatase in rigor buffer supplemented with 1  $\mu\text{M}$   $\text{ZnCl}_2$  in a final volume of 50  $\mu\text{l}$  for 65 h at 23°C. A control sample contained, in addition to the stated components, 50  $\mu\text{M}$  [ $^3\text{H}$ ]ATP. Analysis by thin-layer chromatography showed that, under these conditions, 99.4% of the [ $^3\text{H}$ ]ATP in the control was hydrolyzed to [ $^3\text{H}$ ]AMP and/or [ $^3\text{H}$ ]adenosine, with the remaining 0.6% of the radioactive label distributed between [ $^3\text{H}$ ]ADP and [ $^3\text{H}$ ]ATP. No hydrolysis of AMP-PNP was detected.

ATP, AMP-PNP, ADP, AMP and Type VII alkaline phosphatase were purchased from Sigma Chemical Co.,

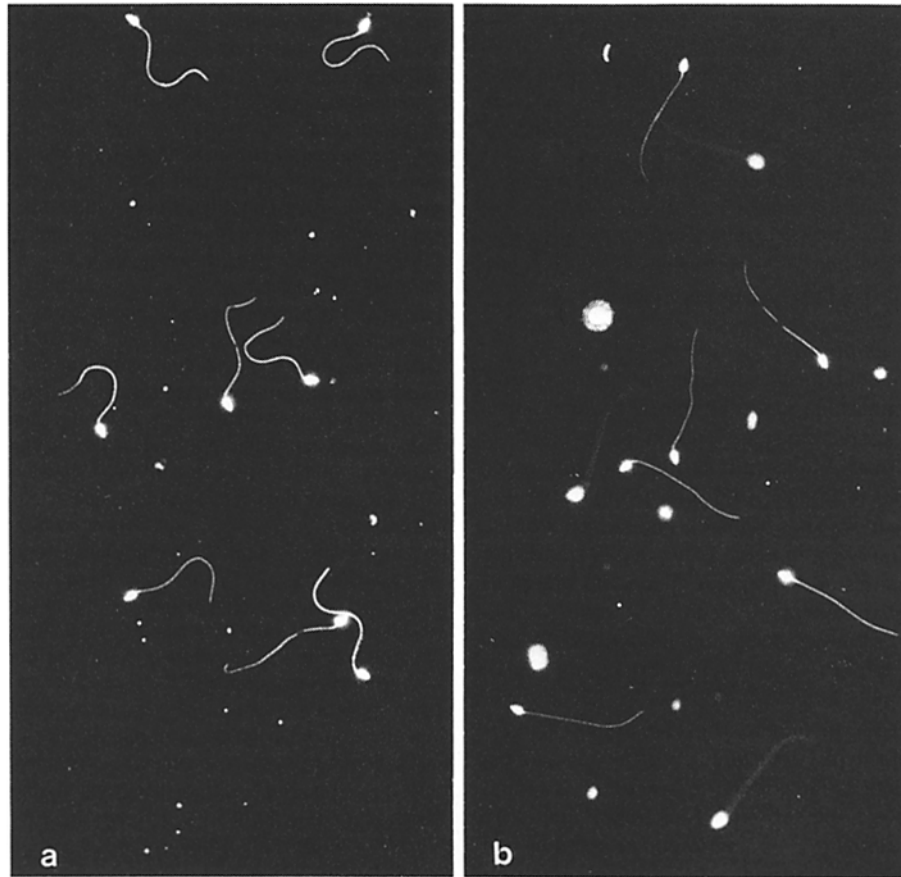


FIGURE 1 Effect of AMP-PNP on rigor wave sea urchin sperm. (a) Dark-field micrograph of rigor wave sperm from *Lytechinus pictus*. (b) Sperm from the preparation shown in a, but after treatment for 10 min at 23°C with 2 mM AMP-PNP. AMP-PNP has induced relaxation of the rigor waves. The AMP-PNP had been pretreated with alkaline phosphatase to ensure that no contaminating ATP was present.  $\times 380$ .

TABLE I  
*R<sub>f</sub> Values of Nucleotides\**

Nucleotide	R <sub>f</sub> value
ATP	0.08
ADP	0.20
AMP	0.52
Adenosine	0.56
AMP-PNP	0.16
AMP-PN‡	0.60
Trailing impurity§	0.03

\* Thin-layer chromatography performed on PEI-cellulose using 1.2 M LiCl as developing solvent.

‡ Identified on the basis of alkaline phosphatase digestion of AMP-PNP.

§ Unidentified trailing impurity in AMP-PNP preparations.

St. Louis, Mo. [<sup>3</sup>H]ATP and [<sup>3</sup>H]AMP-PNP were obtained from New England Nuclear, Boston, Mass. All other chemicals were reagent grade. The purity of ATP and AMP-PNP was estimated by thin-layer chromatography. ATP was over 99% pure, the only visible impurity being ADP; AMP-PNP preparations contained ~80% AMP-PNP, 20% adenylyl phosphoramidate (AMP-PN), and <1% of an unidentified trailing impurity (Table I). Stock solutions of ATP and AMP-PNP were titrated to pH 8.0 (litmus paper) before use in an experiment.

## RESULTS

Axonemes of *Lytechinus* rigor wave sperm were locked in rigid wave forms which resembled the propagated bends of native sperm or reactivated demembrated sperm (Fig. 1a). Rigor sperm

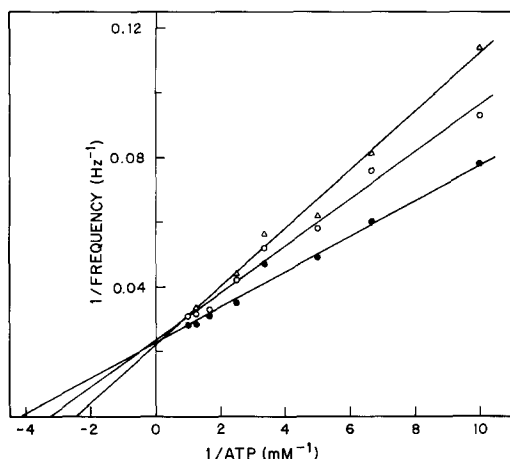


FIGURE 2 Double reciprocal plots of beat frequency vs. ATP concentration for reactivated sperm in the presence and absence of AMP-PNP. Curves were fitted by the least squares method. ●—●, ATP; ○—○, ATP + 2.5 mM AMP-PNP; △—△, ATP + 4 mM AMP-PNP.

neither relaxed nor reactivated in the absence of added nucleotide, even after 1 h. However, rigor wave axonemes relaxed within 2 min after addition of 4  $\mu\text{M}$  ATP and within 1 min after addition of 9  $\mu\text{M}$  ATP. On addition of 10–12  $\mu\text{M}$  ATP, rigor wave sperm reactivated. This behavior agrees well with previous descriptions of rigor wave sperm of the sea urchin *Colobocentrotus* (11).

The beat frequency of sperm that had been reactivated by addition of ATP increased with ATP concentration. This dependence of beat frequency on ATP concentration appeared to follow Michaelis-Menten kinetics, in agreement with earlier studies (2, 9, 20). A double reciprocal plot of the data (Fig. 2) gave a “ $K_m$ ” value of 0.24 mM and a maximum beat frequency of 44 Hz; the highest beat frequency actually observed was 42.5 Hz at 1 mM ATP. These values are slightly higher than those previously reported by Brokaw (2) for *L. pictus* (“ $K_m$ ” of 0.20–0.22 mM and maximum beat frequency of 28 Hz), possibly because our experiments were carried out at a higher temperature, at higher free  $\text{Mg}^{++}$  concentrations, and in the absence of polyethylene glycol.

Previous work has provided evidence that the beat frequency is directly related to the functional activity of the dynein arms under conditions where bend angle remains constant (10, 12, 17). Therefore, to investigate whether AMP-PNP interacted with the dynein arms, we determined the effect of

the analog on the beat frequency. Fig. 2 shows double reciprocal plots of beat frequency vs. ATP concentration in the presence of 2.5 and 4 mM AMP-PNP. The analog appears to be a competitive inhibitor of beat frequency, since it significantly increased the apparent “ $K_m$ ” but did not affect the “ $V_{max}$ ” obtained from the plots. The “ $K_i$ ” for AMP-PNP was calculated from the data in Fig. 2 to be  $\sim 8.1$  mM. AMP-PNP had no noticeable effect on the bend angle or wavelength of reactivated sperm.

Since AMP-PNP appeared to interact with dynein, we reasoned that AMP-PNP should cause relaxation of rigor wave sperm if release of dynein cross-bridges required only ATP binding, but should have no effect if cross-bridge release was dependent upon ATP hydrolysis. Therefore, the effect of AMP-PNP on rigor wave sperm was tested. When AMP-PNP was added to rigor wave sperm to final concentrations of 2–4 mM, the axonemes relaxed completely within 2 min (Fig. 1*b*). Addition of 0.5 mM AMP-PNP resulted in partial relaxation after 5 min; 0.1 mM AMP-PNP had no noticeable effect after 10 min. Sperm did not reactivate in concentrations of AMP-PNP up to 4 mM.

An important consideration was whether relaxation of rigor wave axonemes observed in AMP-PNP might be a result of contamination by low levels of ATP. Contamination by ATP seemed unlikely given the pathway of synthesis of AMP-PNP from AMP and imidodiphosphate (PNP) (31), and analysis of AMP-PNP preparations by thin-layer chromatography revealed no ATP (see Materials and Methods). Nevertheless, to conclusively rule out the possibility of ATP contamination, AMP-PNP was treated with alkaline phosphatase before use in the experiment shown in Fig. 1*b*. Although alkaline phosphatase is known to hydrolyze AMP-PNP at a slow rate (31), the conditions selected were such that a hypothetical ATP contaminant present at a level of 1.25% of the AMP-PNP concentration would have been more than 99% hydrolyzed to AMP and/or adenosine with no effect on AMP-PNP (see Materials and Methods). As can be seen in Fig. 1*b*, AMP-PNP treated in this way induced complete relaxation of rigor wave sperm axonemes. A control showed that alkaline phosphatase and  $\text{ZnCl}_2$ , which were carried over into the relaxation mixture in this experiment, did not relax rigor waves in the absence of the analog.

A further consideration was whether relaxation

might have been a result of hydrolysis of AMP-PNP by dynein or some other axonemal ATPase. To investigate this possibility, [ $^3\text{H}$ ]AMP-PNP at a concentration of 3.5 mM was incubated with rigor wave sperm at 23°C. The rigor waves were completely relaxed within 2 min. Analysis by thin-layer chromatography after a total incubation time of 24 h showed no conversion of [ $^3\text{H}$ ]AMP-PNP to [ $^3\text{H}$ ]AMP-PN, [ $^3\text{H}$ ]AMP, or [ $^3\text{H}$ ]adenosine. In control samples, 9  $\mu\text{M}$  [ $^3\text{H}$ ]ATP was converted to [ $^3\text{H}$ ]ADP (20%) and to [ $^3\text{H}$ ]AMP and/or [ $^3\text{H}$ ]adenosine (80%),<sup>1</sup> and 0.9 mM [ $^3\text{H}$ ]ATP was converted to [ $^3\text{H}$ ]ADP (80%), [ $^3\text{H}$ ]AMP and/or [ $^3\text{H}$ ]adenosine (15%)<sup>1</sup> and [ $^3\text{H}$ ]ATP (5%). It is possible that [ $^3\text{H}$ ]AMP was formed by an adenylate kinase acting on the [ $^3\text{H}$ ]ADP resulting from [ $^3\text{H}$ ]ATP hydrolysis; an axonemal adenylate kinase activity has been reported previously for sea urchin sperm (3).

Recently, Warner (29) has suggested that the stability of dynein cross-bridges may be dependent upon the free  $\text{Mg}^{++}$  concentration. We therefore investigated the possibility that addition of the analog, which binds  $\text{Mg}^{++}$  (31), might have caused relaxation by lowering the free  $\text{Mg}^{++}$  concentration below some critical level. Increasing the  $\text{Mg}^{++}$  concentration by 2 mM (total  $\text{Mg}^{++}$  concentration of 8 mM) did not inhibit relaxation in 2 mM AMP-PNP. Moreover, addition of 2, 5, and 10 mM EDTA to suspensions of rigor wave sperm failed to induce relaxation, even after 15 min. These results indicated that relaxation by AMP-PNP was not simply a result of a lowering of the free  $\text{Mg}^{++}$  concentration.

## DISCUSSION

The experiments described in this report indicate that the nonhydrolyzable ATP analog AMP-PNP inhibits the beat frequency of reactivated sea urchin sperm. The inhibition observed with AMP-PNP conforms to the pattern of a classic competitive inhibitor (7). Double reciprocal plots of beat frequency vs. ATP concentration revealed that, within experimental error, the " $V_{\text{max}}$ " remained constant, while the apparent " $K_m$ " was increased at two different AMP-PNP concentrations (Fig. 2). Since beat frequency is a function of dynein arm activity under conditions of constant bend angle (references 10, 12, 17, and see 14 for

<sup>1</sup> AMP and adenosine were not completely resolved in the thin-layer chromatography system used (see Table I).

review), these results suggest that AMP-PNP competes with ATP for binding to the active site of the dynein arm ATPase. A value of  $\sim 8.1$  mM was obtained for " $K_i$ ", the apparent dissociation constant for the enzyme-inhibitor complex. Assuming that the " $K_m$ " approximates the dissociation constant of the dynein-ATP complex, this value of " $K_i$ " suggests that dynein has a 25- to 50-fold lower affinity for AMP-PNP than for ATP.

AMP-PNP was also found to relax rigor wave sperm. This effect also is probably a result of binding of the analog to the active site of dynein; the possibilities that relaxation in AMP-PNP might have resulted from ATP contamination, AMP-PNP hydrolysis, or lowering of the free  $\text{Mg}^{++}$  concentration as a result of formation of a  $\text{Mg} \cdot \text{AMP-PNP}^-$  complex were ruled out. Rigor waves appear to be maintained by dynein cross-bridges, as evidenced by the fact that rigor waves are unaffected by trypsin treatment under conditions where the radial spokes and the nexin links are digested (11), and that fine structural observations of rigor wave sperm show large numbers of dynein arms attached to the B-tubule (15). If so, then ATP or AMP-PNP must bring about relaxation of rigor waves by causing release of the dynein cross-bridges. The fact that a nonhydrolyzable analog of ATP causes relaxation suggests that nucleotide binding alone is sufficient to induce cross-bridge release; ATP hydrolysis is not required for this step in the mechanochemical cycle. This finding suggests an important point of similarity between the mechanochemistry of muscle contraction (22) and flagellar movement.

Brokaw and Rintala (5) have recently described computer simulations of flagellar behavior based on models having a three-state dynein cross-bridge cycle. Only one of these models responded in a realistic way to changes in ATP concentration and viscosity; a central feature of this model is that ATP binding causes detachment of the dynein cross-bridges. Our observation that AMP-PNP causes relaxation of rigor wave axonemes provides experimental support for such a model. While a three-state model may be an oversimplification of the mechanochemical cycle of dynein, future kinetic schemes for the dynein-B-tubule interaction should include a sequence in which dissociation of dynein from the B-tubule precedes hydrolysis of bound ATP.

Recently, Warner (29) has reported that dynein cross-bridges in *Unio* gill cilia are stabilized by  $\text{Mg}^{++}$  and are selectively released upon addition



of ATP. In our study, the concentration of free  $Mg^{++}$  did not appear to be a critical factor in the relaxation of rigor wave sea urchin sperm axonemes, since addition of EDTA up to a concentration of 10 mM, or approximately twice the  $Mg^{++}$  concentration, had no effect on rigor waves. Nevertheless, our interpretation of rigor wave relaxation as an effect of cross-bridge release because of ATP binding appears to be compatible with Warner's observations.

The findings reported in this paper strongly suggest that, in the mechanochemical cycle of dynein, ATP binding but not hydrolysis induces detachment of the outer doublet arms from the adjacent B-tubule. Direct evidence for this hypothesis must await definitive fine structural studies showing that the number of dynein cross-bridges in rigor wave axonemes is significantly larger than the number of cross-bridges in axonemes that have been relaxed by AMP-PNP.

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