Mechanochemical Coupling in the Relaxation of Rigor-wave Sea Urchin Sperm Flagella

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ABSTRACT The relaxation (straightening) of flagellar rigor waves, which is known to be induced by micromolar ATP concentrations, was investigated with respect to its dependence on the binding and hydrolysis of ATP. Flagellar rigor waves were formed by the dilution of demembranated, reactivated sea urchin (*Lytechinus pictus*) spermatozoa into ATP-free buffer. Relaxation in response to nucleotide was quantitated by measuring $\bar{\Theta}$, the mean flagellar bend angle per sperm; this novel assay permitted determination of the rate of relaxation. It was found that (*a*) the rate of flagellar relaxation induced by 4×10^{-6} M ATP was inhibited 80% by vanadate concentrations of 3×10^{-5} M and above; (*b*) of 16 hydrolyzable and nonhydrolyzable nucleotide di-, tri-, and tetraphosphates tested, only three, each of which was hydrolyzed by the flagellar axonemal ATPase activity (ATP, dATP, and ϵ-ATP), were also capable of effecting relaxation; (*c*) several hundred ATP molecules were estimated to be hydrolyzed by each dynein arm in the course of flagellar relaxation; and (*d*) the ratio of the rate of relaxation to the rate of ATP hydrolysis, which defines the efficiency of ATP utilization, increased 30-fold as the ATP concentration was raised from 2×10^{-6} to 9×10^{-6} M. It is concluded that (*a*) flagellar relaxation depends on ATP hydrolysis; (*b*) because it depends on ATP hydrolysis, flagellar relaxation is an inappropriate model system for investigating the role of ATP binding in the mechanochemical cycle of dynein; and (*c*) the efficiency of mechanochemical coupling in flagellar motility is an ATP-dependent phenomenon. A general model of relaxation is proposed based on active microtubule sliding.

The relaxation (straightening) of rigor-wave sea urchin sperm flagella is currently favored as a model system for investigating the mechanochemical cycle of flagellar motility (18, 19, 23, 24). This is due to the view that relaxation results from the release of dynein cross-bridges in the flagellar axoneme in response to ATP binding. A number of observations have contributed to this view. Dynein arms were shown to be in the cross-bridged configuration in rigor-wave flagella (11), and this configuration correlated with mechanical indices of flagellar rigidity (10, 11) and stiffness (18). Addition of micromolar concentrations of ATP induced rigor-wave flagella to relax (10). Straightening was accompanied by a 15-fold decrease in flagellar stiffness (18); images of dynein cross-bridges were not reported in relaxed flagella (11). The nonhydrolyzable ATP analogue, adenyl-5′-yl imidodiphosphate (AMP-PNP), was reported to act as a competitive inhibitor of the beat frequency of reactivated sea urchin sperm (19, 23) and to effect the relaxation of flagellar rigor waves (23). This and other reports that AMP-PNP also caused the dissociation of dynein arms from isolated outer-doublet microtubules (26), and that relaxation was not blocked (24) by the potent dynein ATPase inhibitor vanadate (12, 16), were reasonably accommodated by a model that interpreted flagellar relaxation as corresponding to the first step of the mechanochemical cross-bridge cycle of dynein (24). This mechanochemical interpretation of relaxation appears to have remained prevalent despite recent reports by several groups that AMP-PNP does not effect flagellar relaxation (19, 20, 22, 25).

The relationship of ATP binding to flagellar relaxation is important to investigations of dynein mechanochemistry (18, 19, 24) as well as to an understanding of the role(s) in motility of putative elastic components in the axoneme, since a simple dynein cross-bridge release mechanism implies that flagellar straightening is driven by forces stored in elastic components, such as the nexin links or radial spokes.

We have reinvestigated the roles of ATP binding and hydrolysis in the relaxation of rigor-wave sea urchin sperm flagella by using a novel relaxation assay that has permitted the quantitation of the rate as well as the extent of flagellar straightening. Evidence is presented indicating that flagellar relaxation depends on ATP hydrolysis. We also report the unexpected finding that the efficiency of mechanochemical

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coupling in relaxation increases as a function of ATP concentration. A model of relaxation is discussed which is based on active microtubule sliding.

MATERIALS AND METHODS

Nucleotides and Enzymes

Adenylyl (β,γ-methylene) diphosphonate (AMP-PCP), adenosine 5′-*O*-(3-thiotriphosphate) (ATP-γ-S), adenosine-5′-*O*-(2-thiodiphosphate) (ADP-β-S), and hexokinase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); 1,N^6-ethenoadenosine 5′-triphosphate (ϵ-ATP) and 1,N^6-ethenoadenosine 5′-diphosphate (ϵ-ADP) were from P-L Biochemicals, Inc. (Milwaukee, WI); and ATP (vanadium free), ADP, adenosine tetraphosphate, GTP, ITP, UTP, CTP, firefly extract, luciferin, diadenosine pentaphosphate, and alkaline phosphatase (type VII) were from Sigma Chemical Co. (St. Louis, Mo). AMP-PNP was obtained from Sigma Chemical Co. and ICN Nutritional Biochemicals (Cleveland, OH); and orthovanadate (VO_4^{3-}) was from Fisher Scientific Co. (Springfield, NJ). Other chemicals were reagent grade.

Preparation of Rigor-wave Sperm

Sea urchins were purchased from Pacific Biomarine Laboratory (Venice, CA) and maintained in an aquarium in artificial seawater (Instant Ocean; Fischer Scientific Co., Chicago, IL) at 14°C. *Lytechinus pictus* was used in most experiments; *Strongylocentrotus purpuratus* was substituted during the winter season. No significant differences were noted in the results obtained with the two species.

Sperm were collected in artificial seawater after injection of 2–3 ml of 0.5 M KCl into the body cavity of the sea urchin, stored on ice, and used within 6 h. To prepare rigor-wave sperm, 25 μl of packed sperm were dispersed by gentle agitation into 375 μl of rigor buffer (20 mM Tris-HCl, pH 8.1 adjusted at 21°C, 6 mM $MgSO_4$, 0.5 mM EDTA, 0.15 M KCl, 1 mM dithiothreitol [DTT]) containing 0.1% Triton X-100 and 20 μM ATP. After 10–15 s, a 20-μl aliquot of the demembranated, reactivated sperm was dispersed gently into 980 μl of rigor buffer. Concentrations of rigor-wave sperm were generally on the order of 1.5 $\times$ 10^7 cells/ml or 0.056 mg/ml of protein, corresponding to ~30 cells per microscope field.

Assay for Relaxation of Rigor-wave Sperm Flagella

Generally, a 5-μl aliquot of nucleotide was added to 45 μl of rigor-wave sperm and incubated in a 16°C water bath for the desired length of time. The relaxation reaction was quenched by addition of 5 μl of 1% glutaraldehyde. This concentration of glutaraldehyde was shown in control experiments to arrest ATP-induced relaxation without causing cells to clump; further, it did not cause any noticeable distortion of flagellar wave form, probably because it was 20-fold lower than the glutaraldehyde concentration shown previously to produce irregularities in axonemal geometry (11). Sperm were photographed on ASA 400-Tri-X film (Kodak) under dark-field optics using a Zeiss standard microscope equipped with a 16 $\times$ Neofluar objective and a model C35 attachment camera; exposure time was 8–12 s. Negatives were printed at low contrast at a total magnification of $\times$ 380. Each individual bend angle of each sperm cell in a micrograph, including angles formed by both principal and reverse bends, was measured with a protractor. The sum of the individual bend angles of each sperm axoneme, which is referred to as Θ, was taken as an index of the total amount of bending present in each axoneme. The mean value of Θ averaged over all of the sperm cells in the micrograph is referred to as $\bar{\Theta}$, or the mean bend angle per sperm. It was obtained by summing Θ over the number (n) of sperm axonemes measured in the micrograph and dividing by n:

$$\bar{\Theta} = \left(\sum_{i=1}^{i=n} \Theta\right) \Big/ n .$$

Damaged axonemes (usually ~5% of the total) were excluded from the determination of $\bar{\Theta}$. A correction was made for unreactivated sperm, as follows. Axonemes in the rigor-wave preparation having values of $\Theta \leq 2°$ were defined as having failed to become reactivated and were excluded from the determination of $\bar{\Theta}$ of the rigor-wave sperm preparation. The number of axonemes in nucleotide-treated samples was multiplied by the fraction of unreactivated sperm in the rigor-wave sample to estimate the number of axonemes having $\Theta \leq 2°$ that could be accounted for by a failure to become reactivated. Only axonemes with $\Theta \leq 2°$ in excess of this number were included in the determination of $\bar{\Theta}$ in nucleotide-treated samples. The fraction of unreactivated sperm ($\Theta \leq 2°$) ranged from 0 to 5%.

Reproducibility of $\bar{\Theta}$ as an Index of Flagellar Relaxation

Values of Θ were found to vary considerably among individual cells in a micrograph, resulting in large standard deviations for $\bar{\Theta}$, on the order of 30% (Fig. 1 *a*). The value of $\bar{\Theta}$ itself, however, varied considerably less. The first line of Table I shows the results of an experiment in which 10 micrographs similar to but separate from that in Fig. 1 *a* were made of adjacent microscope fields of rigor-wave sperm cells. Values for $\bar{\Theta}$ were determined for each micrograph, and the standard deviation of the mean value of $\bar{\Theta}$ was found to be on the order of 10%.

Because measuring flagellar bend angles was time-consuming and tedious work, it was desirable, as a practical matter, to determine $\bar{\Theta}$ from a single micrograph. That the experimenter, in selecting cells for inclusion in the micrograph, might bias the determination of $\bar{\Theta}$ was ruled out by the experiment reported in the second line of Table I. In this experiment, the mean value of $\bar{\Theta}$ was determined for the same sample of rigor-wave sperm cells as in the first line of Table I; however, instead of randomly selecting adjacent microscope fields, the experimenter chose fields of sperm which, in his/her judgment, were "representative" of the degree of bending in the sample as a whole. No significant difference in either the mean value or the standard deviation of $\bar{\Theta}$ resulted from this alternative procedure for selecting sperm for inclusion in micrographs, suggesting that the selection of a "representative" set of cells for determination of $\bar{\Theta}$ was equivalent to a random selection. The results in Table I also indicate that an accurate estimate of $\bar{\Theta}$ was provided by as few as 20 cells.

The use of $\bar{\Theta}$ as an assay for the time-course of flagellar straightening is shown in Fig. 2. Duplicate experiments were performed using two separate preparations of rigor-wave sperm; the ATP concentration was 7×10^{-6} M. The decrease in $\bar{\Theta}$ was expressed relative to the value of $\bar{\Theta}$ for each rigor-wave sperm preparation. Thus, for each experiment: $\% \bar{\Theta}_{rigor} = \bar{\Theta}_{relaxed}/\bar{\Theta}_{rigor} \times 100$. Values of $\bar{\Theta}$ were normalized to $\bar{\Theta}_{rigor}$ because of variability in $\bar{\Theta}_{rigor}$ among different rigor-wave sperm preparations (~140°–180°). It should be noted that each point in Fig. 2 corresponds to a micrograph similar (but not identical) to one of those presented in Fig. 1 *a–d*. The good agreement between the duplicate experiments reported in Fig. 2 confirms that $\% \bar{\Theta}_{rigor}$ provided a reasonably reproducible assay for flagellar relaxation. Controls showed that rigor-wave flagella did not straighten spontaneously under the conditions used in these experiments. Furthermore, relaxed sperm could be reactivated, indicating that relaxation did not involve denaturation of the flagellar axoneme.

Analysis of Nucleotides

Nucleotides were analyzed by ascending thin-layer chromatography on polyethyleneimine (PEI)-cellulose thin-layer sheets (Bakerflex; Arthur H. Thomas Co., Philadelphia, PA). Chromatograms were developed in 0.75 M KH_2PO_4, pH 3.4 adjusted at 21°C with phosphoric acid, to a distance of 12–15 cm from the origin (see reference 22 for R_f values of adenosine nucleotides). To resolve ATP from ϵ-ATP, a wick (Whatman no. 1 chromatography paper, 20 cm $\times$ 15 cm) was attached to the top of a PEI-cellulose thin-layer sheet to ensure continuous solvent flow, and the chromatogram was developed overnight. ATP was resolved from AMP-PNP by development in 1.2 M LiCl (22). Nucleotides were visualized under ultraviolet light, and contaminant levels were estimated. Typically, 50 μg of nucleotide was loaded, and as little as 0.2 μg of nucleotide contaminant could be detected. In the case of ATP contamination of AMP-PNP, ϵ-ATP and CTP, the ATP contaminant was purified by thin-layer chromatography (see below) and quantitated by the luciferin-luciferase assay. The luciferin-luciferase assay was performed on a Beckman DB-GT spectrophotometer equipped with a 10-inch potentiometric recorder, as described previously (22).

Purification of Nucleotides and Removal of ATP Contaminants

THIN-LAYER CHROMATOGRAPHY: Nucleotide was scraped from the thin-layer chromatogram, eluted with high ionic strength buffer, and desalted by a cycle of charcoal adsorption and elution. Yield was ~10%. Details of this procedure have been described (22).

ION-EXCHANGE CHROMATOGRAPHY: Columns (0.6 cm $\times$ 11 cm) of DEAE-Sephadex A-25 were loaded with ~15 mg of nucleotide and developed with a linear gradient (2 $\times$ 108 ml) of triethylammonium bicarbonate at 4°C. Gradients were adjusted empirically, depending on the species of nucleotides to be resolved as well as on slight variations in the ionic strength of different batches of triethylammonium bicarbonate. 80 80-drop fractions were collected, nucleotide concentration was determined on the basis of OD_{260}, and peak fractions were pooled and evaporated to dryness on a rotoevaporator. Residual triethylammonium bicarbonate was removed by repeated evaporation with ethanol until the

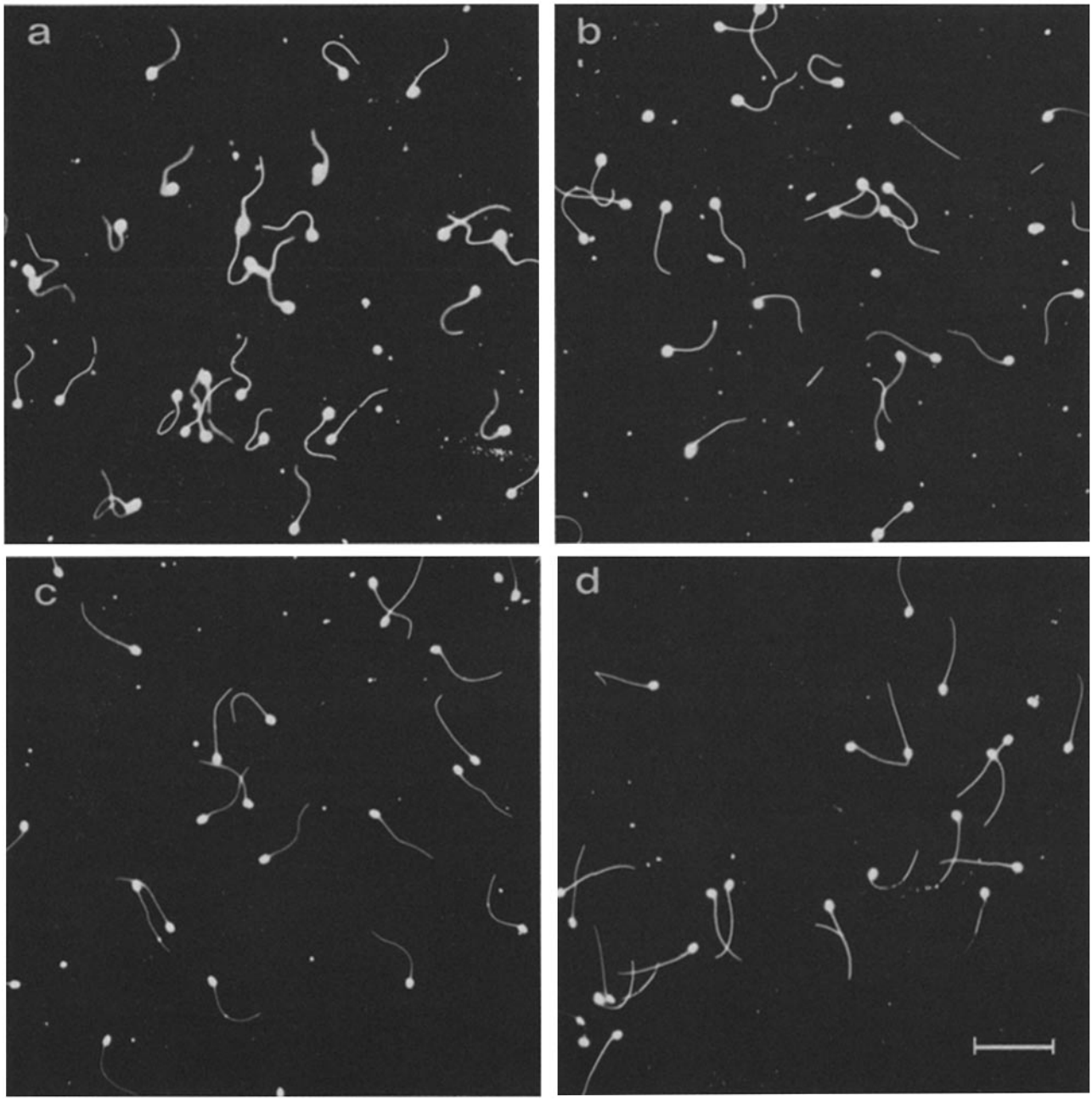

FIGURE 1 *(a–d)* Comparison of decreases in $\bar{\Theta}$, the mean bend angle per sperm in a micrograph, with visual estimates of flagellar straightening. Decreases in $\bar{\Theta}$ are seen to closely parallel decreases in flagellar bending. Values for $\bar{\Theta}$ were determined for each micrograph as described in Materials and Methods. $\bar{\Theta}$ values for *b–d* are expressed relative to $\bar{\Theta}$ (153°) for the rigor-wave sperm sample in *a*, which is taken to be 100%. (*a*) Rigor-wave sperm, $\bar{\Theta} = 153° \pm 51°$; (*b*) $\%\bar{\Theta}_{rigor} = 77\%$; (*c*) $\%\bar{\Theta}_{rigor} = 56\%$; (*d*) $\%\bar{\Theta}_{rigor} = 36\%$. Bar, 50 μm.

pH of the residue was ≤6. Yield was ~50%. The identity of purified nucleotides was verified by comparison with authentic standards on thin-layer chromatograms.

HYDROLYSIS OF ATP CONTAMINANT: Demembranated, washed sea urchin sperm were used as an ATP-discharging system. Adenosine tetraphosphate contaminated with ATP was incubated at a concentration of 4 mM overnight at 16°C with 0.65 mg/ml demembranated, washed sea urchin sperm; 1 μCi carrier-free [^{3}H]ATP was added as a marker. Sperm were removed by centrifugation at 12,000 *g* for 5 min, and hydrolysis of the ATP contaminant was confirmed by separating [^{3}H]ATP, [^{3}H]ADP, and [^{3}H]AMP on thin-layer chromatograms, scraping the spots into scintillation vials, and counting (21). Reduction of [^{3}H]-ATP to <2% of the total counts present in [^{3}H]ADP and [^{3}H]AMP was considered adequate removal of the ATP contaminant. Adenosine tetraphosphate was not hydrolyzed under these conditions, as judged by visual inspection of thin-layer chromatograms.

PREPARATION OF AMP-PN: AMP-PNP was incubated overnight with alkaline phosphatase. Quantitative conversion to AMP-PN was confirmed by thin-layer chromatography, and alkaline phosphatase was removed in the void volume of a Sephadex G-25 gel filtration column (Pharmacia Inc., Piscataway, NJ). Details of this procedure have been described (22).

Assays for Nucleotide Hydrolysis

ATP hydrolysis was determined on the basis of the liberation of $^{32}P_i$ from [γ-^{32}P]ATP, as described previously for GTP hydrolysis (21). Assays were performed at 16°C in a total volume of 0.2 ml; incubation time was 10 min. Because of slight variations in the activities of different preparations of the axonemal ATPase activity (see below), the concentration of cells was adjusted separately for each experiment to keep ATP hydrolysis within the linear range (30% or less hydrolysis); cell concentrations ranged from 8×10^6 to 1.5×10^7 cells/ml (0.030

TABLE I

Accuracy of $\bar{\Theta}$ as an Index of Sperm Flagellar Curvature*

Basis of selection of sperm cells	No. of micro-graphs‡	Average no. of cells per micrograph	Mean $\bar{\Theta}$
			Mean ± SD
Random§	10	23	161° ± 8% (n = 10)
"Representative"‖	9	20	166° ± 12% (n = 9)

* The mean bend angle per sperm flagellum of a micrograph of rigor-wave sperm. See Materials and Methods for a detailed description of the determination of $\bar{\Theta}$.

‡ Since each micrograph was used to make one determination of $\bar{\Theta}$, this number is equal to the number (n) of determinations of $\bar{\Theta}$.

§ Adjacent microscope fields of sperm cells were photographed.

‖ Microscope fields of sperm cells were selected in which flagellar bending was judged to be representative of the sample as a whole.

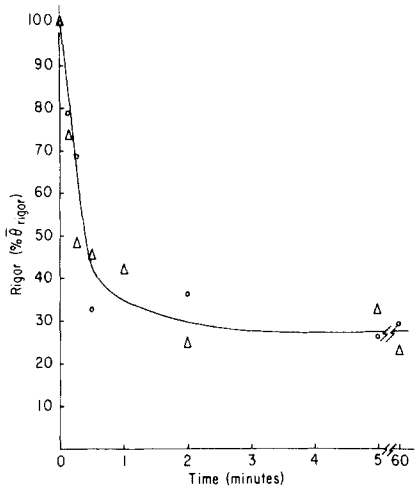

FIGURE 2 Time-course of relaxation of rigor-wave sperm as determined by decreases in $\bar{\Theta}$. Two separate experiments (○ and △) are reported. In each experiment, rigor-wave sperm was incubated with 7×10^{-6} M ATP at 16° C, and the relaxation reaction was quenched at the indicated times by addition of 0.09% glutaraldehyde. Each graph point corresponds to one determination of $\bar{\Theta}$ from a micrograph containing 20–35 sperm (details of the relaxation assay are described in Materials and Methods). The good agreement between two separate experiments indicates that $\bar{\Theta}$ provided a reasonably reproducible assay for flagellar relaxation.

to 0.056 mg/ml protein). Background averaged 2.5% of total counts. Hydrolysis of nonradioactive nucleotides was estimated visually from thin-layer chromatograms. In the case of ε-ATP, hydrolysis was quantitated by densitometry (Joyce Loebl model 3CS microdensitometer) of photographic negatives (Polaroid) of thin-layer chromatograms.

Isolation of Axonemal ATPase Activity

It was important to remove possible nonaxonemal ATPases (9, 28) to measure the rate of ATP hydrolysis intrinsic to the flagellar axoneme. The ATPase activity of rigor wave sperm was not inhibited by oligomycin at concentrations up to 5 μg/ml, possibly because the concentration of Triton X-100 used for demembranation of sperm cells (0.1%) either destroyed the mitochondrial ATPase or rendered it insensitive to oligomycin (9). Enzyme kinetic analysis of the ATPase activity of rigor-wave sperm yielded a biphasic Hofstee plot, indicating the presence of at least two species of ATPase with K_m values of 6.4×10^{-5} and 17.2×10^{-5} M (Fig. 3*a*). In an effort to remove possible nonaxonemal ATPases, sperm were washed twice following demembranation, as described previously (1). When this procedure was followed, a homogeneous Hofstee plot was obtained with a K_m of 5.9×10^{-5} M (Fig. 3*b*). This result suggested that the lower-affinity ATPase activity present in unwashed rigor-wave sperm preparations was not intrinsic to the axoneme. Alternatively, it is conceivable that dynein existed in two forms in the presence of Triton X-100, and that removal of detergent by washing resulted in the disappearance of the Triton-activated form (13). We demonstrated that the fraction of washed sperm that could be reactivated by ATP was similar to that of unwashed sperm (95–100%), indicating that washing did not destroy a movement-coupled ATPase activity (9). The activity present in demembranated, twice-washed sperm is referred to as the axonemal ATPase activity.

Other Methods

Crude dynein 1 was prepared by extracting demembranated, twice-washed sperm tails with extraction buffer (20 mM Tris-HCl, pH 8.1, 0.6 M KCl, 5 mM EDTA, 1 mM DTT) (1). Protein concentration was determined by the method of Lowry et al. (17) using bovine serum albumin as a standard. Cell counts were performed with a hemocytometer. Nucleotide concentrations were determined on the basis of extinction coefficients from Cohn (6).

RESULTS

Dependence of the Rate and Extent of Flagellar Relaxation on ATP Concentration

The time-course of relaxation (cf. Fig. 2) was determined at several ATP concentrations ranging from 2×10^{-6} to 9×10^{-6} M. These time-course curves were then used to determine: (*a*) the initial rate of relaxation, defined as the slope of the intial linear portion of each time-course curve (slope fitted by the method of least squares); and (*b*) the total extent of relaxation, defined as the plateau level of $\%\bar{\Theta}_{rigor}$ at each ATP concentration (cf. Fig. 2). Initial rate and total extent of relaxation are plotted as functions of ATP concentration in Fig. 4. The results in Fig. 4 show a striking difference between the ATP dependence of the initial rate of relaxation, on the one hand, and that of the total extent of relaxation, on the other. Initial relaxation rate did not become saturated with respect to ATP, while total relaxation did. Initial relaxation rate increased several hundredfold when the ATP concentration was raised from 2×10^{-6} to 9×10^{-6} M ATP, whereas total relaxation increased only threefold, indicating that initial relaxation rate was about

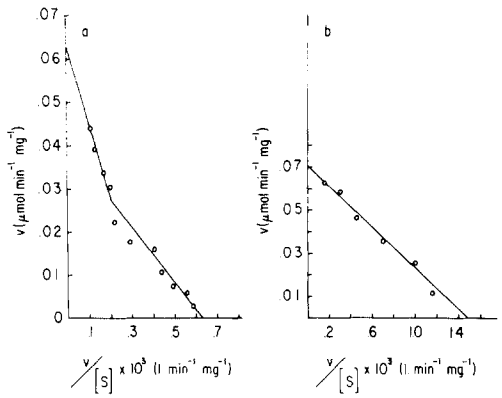

FIGURE 3 Removal of nonaxonemal ATPase activity from preparations of demembranated sperm by washing. (*a*) ATP concentration dependence of the ATPase activity of demembranated rigor-wave sperm. The Hofstee plot is biphasic, indicating the presence of two classes of ATPase activities having K_m values of 6.5×10^{-5} M and 17.2×10^{-5} M. (*b*) ATP concentration dependence of the ATPase activity of demembranated sperm which were washed two times by pelleting and resuspending in rigor buffer (see Materials and Methods). The Hofstee plot is homogeneous, indicating the presence of a single ATPase activity having a K_m of 5.9×10^{-5} M. The absence of the lower-affinity ATPase in the twice-washed sperm preparation suggests that it was removed by washing and therefore was not an integral component of the flagellar axoneme (see Materials and Methods). The ATPase which was resistant to removal by washing is referred to as the axonemal ATPase. Its K_m suggests that axonemal ATPase activity is comprised primarily of dynein 1; the contribution, if any, of dynein 2 to the axonemal ATPase activity cannot be determined from these data (14).

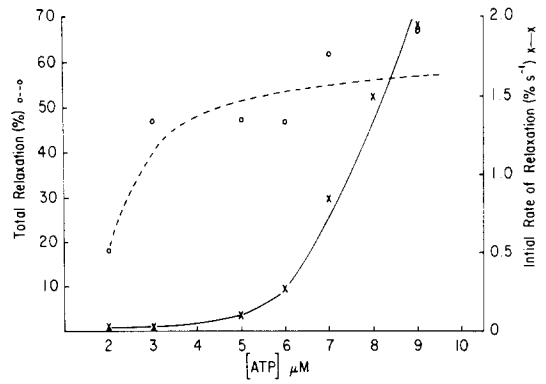

FIGURE 4 Dependence of the initial rate and the total extent of flagellar relaxation on ATP concentration. Data points at 7×10^{-6} M ATP were derived from an analysis of the time-course of relaxation in Fig. 2; data points at other ATP concentrations were derived from analogous time-course curves (data not shown). (×——×) Initial rate of relaxation, determined as the initial slope of the time-course curve at each ATP concentration (e.g., Fig. 2); (O- - -O) total relaxation, determined as the plateau level of the time-course curve at each ATP concentration (e.g., Fig. 2). The units of relaxation, %, refer to the change in $\bar{\Theta}$ in ATP-treated samples compared with untreated, rigor-wave controls (cf. Fig. 2).

two orders of magnitude more sensitive to ATP than was total relaxation. These results suggest that the rate of relaxation is governed by an ATP-dependent process that is different from the ATP-dependent process governing the extent of relaxation.

Sensitivity of Flagellar Relaxation Rate to Inhibition by Vanadate

The effects of increasing concentrations of vanadate on the initial rate of flagellar relaxation was determined in the following experiment. Paired samples from the same rigor-wave sperm preparation were relaxed with 4×10^{-6} M ATP; one sample contained vanadate, the other did not. The initial relaxation rate was determined for each of the paired samples. The ratio of the relaxation rates of the paired samples is plotted as a function of the vanadate concentration in Fig. 5. The initial rate of flagellar relaxation was inhibited by vanadate in a dose-dependent fashion, with inhibition reaching ~80% at vanadate concentrations of 3×10^{-5} M and above. Relaxation was stimulated slightly at a low (1×10^{-6} M) vanadate concentration; a similar effect was reported previously by Okuno (18). The total extent of relaxation was not decreased by vanadate (data not shown), in agreement with Sale and Gibbons (24). The axonemal ATPase activity measured at an ATP concentration of 4×10^{-6} M ATP was found to be significantly less sensitive to inhibition by vanadate than the flagellar relaxation rate measured at 4×10^{-6} M ATP (Fig. 5).

Dependence of the Axonemal ATPase Activity on ATP Concentration

ATPase activity was determined as a function of ATP concentration from 1×10^{-6} to 9×10^{-6} M and normalized to the number of dynein arms in the flagellar axoneme. Normalization was based on a dynein periodicity of 225 Å (27) and an average axonemal length of 47.9 μm (± 7%) ($n = 20$). The rate of ATP hydrolysis was found to increase from about one to five molecules of ATP hydrolyzed per dynein arm per second (Fig. 6).

Knowing the rate of ATP hydrolysis and the total time required for relaxation at each ATP concentration (see Fig. 2 and text), it was possible to estimate the number of ATP molecules hydrolyzed per dynein arm in the course of the relaxation reaction. For example, at 7×10^{-6} M ATP, 4.8 molecules of ATP were hydrolyzed per second per dynein arm (Fig. 6), and ~120 s were required for relaxation (Fig. 2), so that relaxation was accompanied by the hydrolysis of an estimated 576 ATP molecules per dynein arm. Similar large stoichiometries of ATP hydrolysis were observed at other ATP concentrations (data not shown). These results suggest that flagellar relaxation is an energy-inefficient process compared with flagellar beating, in which approximately one molecule of ATP is hydrolyzed per dynein arm per flagellar beat (2, 3, 5). The precise relationship of the relaxation rate, determined on the basis of fractional changes in $\bar{\Theta}$ (see Materials and Methods), to the beat frequency is not clear.

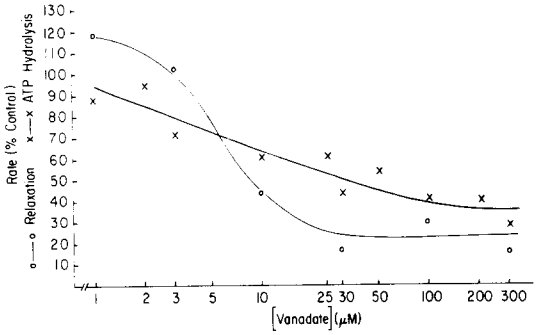

FIGURE 5 Effects of increasing doses of vanadate on the rate of flagellar relaxation and on the rate of ATP hydrolysis by the axonemal ATPase activity. Each point on the relaxation rate curve (O) represents a separate experiment in which the initial phase of the time-course of relaxation was determined at 4×10^{-6} M ATP in the presence of the indicated concentration of vanadate (experimental) and in the absence of vanadate (control). The rate of relaxation in experimental and control samples was determined as the slope (calculated by the method of least squares) of the time-course curve. Plotted is the ratio of the relaxation rates in experimental and control samples (average of two separate experiments at each vanadate concentration). The rate of ATP hydrolysis by the axonemal ATPase (×) was determined separately by incubating demembranated, twice-washed sperm with 4×10^{-6} M [γ-^{32}P]ATP in the presence of the indicated concentrations of vanadate. ATPase activity is expressed relative to a control containing no vanadate. Data from two separate sets of measurements of ATPase activity are included.

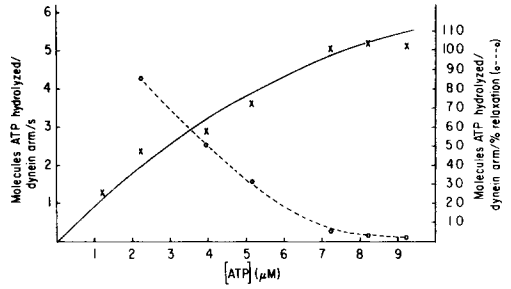

FIGURE 6 Rate of ATP hydrolysis by the axonemal ATPase activity at micromolar concentrations of ATP and its relationship to the rate of flagellar relaxation. (×) ATP hydrolysis by demembranated, twice-washed sperm. ATPase activity is expressed relative to the concentration of dynein arms (see text). (O) The ratio of ATP hydrolysis rate to the relaxation rate (Fig. 4); this ratio is, by definition, the inverse of the efficiency of ATP utilization in relaxation.

Increase in the Efficiency of Mechanochemical Coupling as a Function of ATP Concentration

The ratio of the rate of ATP hydrolysis by the axonemal ATPase activity (Fig. 6) to the rate of relaxation (Fig. 4) was plotted as a function of ATP concentration (Fig. 6). Because the relaxation rate was much more sensitive to ATP concentration than the rate of ATP hydrolysis, the turnover of ATP relative to the rate of flagellar straightening decreased as a function of ATP concentration (Fig. 6). The fact that fewer molecules of ATP were hydrolyzed to achieve the same degree of relaxation at higher ATP concentrations means that the efficiency of mechanochemical coupling increased as the ATP concentration was raised. The results in Fig. 6 indicate that the efficiency of mechanochemical coupling in flagellar relaxation increased on the order of 30-fold between 2×10^{-6} and 9×10^{-6} M ATP. In separate experiments on reactivated sea urchin sperm, the ratio of the rate of ATP hydrolysis to flagellar beat frequency was found to decrease fourfold as the ATP concentration was increased from 1.5×10^{-5} to 4×10^{-4} M (data not shown).

Effects of ATP Analogues on Flagellar Relaxation

A variety of nucleotides were investigated with respect to their competency to relax flagellar rigor waves, on the one hand, and their susceptibility to hydrolysis by the axonemal ATPase, on the other. The results of relaxation competency tests on 15 nucleotide di-, tri-, and tetraphosphate preparations are presented in Table II, columns 1–3. A total of eight different nucleotide preparations (ATP, dATP, ϵ-ATP, AMP-PNP, adenosine tetraphosphate, ADP, ADP-β-S, and CTP), which were used as obtained from the suppliers without further purification, showed significant competency to relax rigor-wave flagella ($\bar{\Theta}$ reduced to 60% or less of rigor-wave values (cf. Fig. 1 *a–d*)). We suspected that ATP contamination might account for relaxation with some or all of these preparations (22). Analysis by thin-layer chromatography and/or the firefly assay confirmed that ϵ-ATP, AMP-PNP, adenosine tetraphosphate, ADP, and CTP contained significant levels of ATP contamination (Table II, fourth column). ADP-β-S may have contained ATP that went undetected on thin-layer chromatograms.

TABLE II

Relaxation of Rigor-wave Sperm by Unpurified Nucleotides *

Unpurified nucleotide preparation	Relaxation: $\%\bar{\Theta}_{rigor}$‡	Relaxation: Nucleotide concentration	Impurities identified
		M	
ATP	44	7.0×10^{-6}	None§
dATP	50	7.0×10^{-6}	None§
ϵ-ATP	55	1.0×10^{-3}	ATP, 2.0%‖; ϵ-ADP, 11.5%¶
ATP-γ-S	79	4.0×10^{-3}	ADP, 5%§
AMP-PCP	89	4.0×10^{-3}	None**
AMP-PNP‡‡	90	4.0×10^{-3}	ATP, 0.018%**; AMP-PN, 2%§
AMP-PNP§§	41	2.0×10^{-3}	ATP, 0.29%‖; AMP-PN, 10%§; AMP-PNP-P, 5%§
Adenosine tetraphosphate	40	8.3×10^{-5}	ATP, 5%§
ADP	51	1.0×10^{-3}	ATP, 1%§
ADP-β-S	52	4.0×10^{-3}	None§
ϵ-ADP	74	4.0×10^{-3}	ϵ-AMP, 5%§; ϵ-ATP, 2%§
GTP	80	4.0×10^{-3}	None§
ITP	82	4.0×10^{-3}	None§
UTP	77	4.0×10^{-3}	None§
CTP	41	4.0×10^{-3}	ATP, 0.15%‖

* Each result is an average of two or more experiments using different preparations of rigor-wave sperm.
‡ Rigor-wave sperm was incubated with nucleotide for 10 min at 16°C. Relaxation was quenched by addition of 0.09% glutaraldehyde (see Materials and Methods).
§ Estimated visually from thin-layer chromatogram (see Materials and Methods).
‖ Determined by firefly assay on ATP purified by thin-layer chromatography (see Materials and Methods).
¶ Determined by densitometry on photographic negative of thin-layer chromatogram (see Materials and Methods).
** Determined by firefly assay directly on nucleotide preparation (see Materials and Methods).
‡‡ Sigma lot #98C-0027.
§§ ICN lot #1253.

TABLE III

Relaxation of Rigor-wave Sperm by Purified Components of Commercially Obtained Nucleotide Preparations

Unpurified nucleotide preparation	Purified component	Relaxation by purified component: $\%\bar{\Theta}_{rigor}$*	Relaxation by purified component: Conditions
			M
ϵ-ATP	ϵ-ATP‡	47	2.0×10^{-3}
	ATP‡	62	7.0×10^{-6}
ATP-γ-S	ATP-γ-S§	86	4.0×10^{-3}
AMP-PNP‖	AMP-PNP‡	97	4.0×10^{-3}
	AMP-PNP§	89	4.0×10^{-3}
	AMP-PN§	72	4.0×10^{-3}
	AMP-PN¶	82	2.2×10^{-3}
	AMP-PNP-P‡	84	5.7×10^{-4}
	AMP-PNP-P§	95	2.1×10^{-4}
	ATP‡	34	8.0×10^{-6}
Adenosine tetraphosphate	Adenosine tetraphosphate**	106	1.1×10^{-3} (20 min)
ADP	ADP§	85	4.0×10^{-3} (30 min)‡‡
ADP-β-S	ADP-β-S§	91	4.0×10^{-3}
CTP	CTP‡	86	4.1×10^{-3}
	ATP‡	31	8.0×10^{-6}

* Rigor-wave sperm was incubated with purified nucleotide for 10 min at 16°C, unless otherwise stated. Relaxation was quenched by addition of 0.09% glutaraldehyde (see Materials and Methods).
‡ Purified by thin-layer chromatography (see Materials and Methods).
§ Purified by ion-exchange chromatography (see Materials and Methods).
‖ ICN lot #1253 (cf. Table II).
¶ Prepared by digestion of AMP-PNP with alkaline phosphatase (see Materials and Methods).
** ATP removed by treating overnight with demembranated sea urchin sperm (see Materials and Methods).
‡‡ Sample included 0.5 mM diadenosine pentaphosphate, 62.5 μg/ml hexokinase, and 10 mM glucose.

Except for ATP and dATP, each of the nucleotides which effected relaxation and, in some cases, their contaminants as well (Table II), were purified and retested for their relaxation competency. The results of these experiments are presented in Table III. Purified AMP-PNP, adenosine tetraphosphate, ADP-β-S, and CTP failed to relax rigor-wave flagella. Further, of the three major contaminants of AMP-PNP preparations (AMP-PN, phosphorylated AMP-PNP [AMP-PNP-P], and ATP), only ATP effected relaxation. This result provided direct evidence that ATP was the active principle responsible for the relaxation of flagellar rigor waves observed previously with unpurified AMP-PNP preparations (23). Purified ADP effected relaxation (data not shown). However, an adenylate kinase activity is known to be present in sea urchin sperm axonemes (4; A. Cheung, unpublished data); inclusion of diadenosine

pentaphosphate (a specific adenylate kinase inhibitor) and hexokinase plus glucose (an ATP-discharging system) in the relaxation mixture prevented relaxation with purified ADP (Table III). As controls, we showed that relaxation was not inhibited by diadenosine pentaphosphate, and that reactivation with 50 μM ATP was not blocked by a mixture of diadenosine pentaphosphate, hexokinase, and glucose. These results are similar to those of Okuno (18), except that we used a fourfold higher ADP concentration in our experiments.

Of the six commercial nucleotide preparations other than ATP and dATP that induced relaxation, only ϵ-ATP was found to be effective after purification (Table III).

A variety of controls indicated that the failures of purified nucleotides to effect relaxation reported in Table III could not have arisen as artifacts of our methods for purifying nucleotides. These controls are enumerated below for each purification method: (*a*) Thin-layer chromatography (TLC): (*i*) ATP purified by TLC from ϵ-ATP, AMP-PNP, and CTP, as well as TLC-purified ϵ-ATP itself, effected relaxation (Table III); (*ii*) "dummy" TLC scrapings containing no nucleotide were taken through the purification procedure and shown not to inhibit relaxation induced by ATP (data not shown). (*b*) Ion-exchange chromatography: (*i*) ion-exchange-purified ADP effected relaxation (data not shown); (*ii*) ATP-induced relaxation was not inhibited by concentrations of triethylammonium bicarbonate up to 25 mM (data not shown). (*c*) Overnight incubation of adenosine tetraphosphate with demembranated, washed sperm: (*i*) Adenosine tetraphosphate preparations treated overnight but in which ATP was only partially digested retained their abiliity to effect relaxation (data not shown).

Hydrolysis of ATP Analogues by the Axonemal ATPase

The susceptibility of 10 nucleotide triphosphates and adenosine tetraphosphate to hydrolysis by the axonemal ATPase activity was investigated. Under the conditions chosen, ATP and dATP were quantitatively hydrolyzed to mixtures of their respective di- and monophosphate homologues; ϵ-ATP, GTP, ITP, and CTP were hydrolyzed to varying extents ranging from 10 to 60%; and no hydrolysis of ATP-γ-S, AMP-PNP, AMP-PCP, adenosine tetraphosphate, or UTP by the axonemal ATPase was detected (Table IV). A similar experiment substituting crude dynein 1 for the axonemal ATPase was performed on ATP, ϵ-ATP, ATP-γ-S, AMP-PNP, and AMP-PCP. ATP was converted to ADP, suggesting that the AMP resulting from incubation of ATP with the axonemal ATPase was due to the axonemal adenylate kinase activity. ATP-γ-S, AMP-PCP, and AMP-PNP were not hydrolyzed by dynein 1 (Table IV). The results indicate that each of the three nucleotides shown (Table III) to be capable of inducing relaxation, ATP, dATP, and ϵ-ATP, was also hydrolyzed by the axonemal ATPase activity and by dynein 1. Of the nucleotides which did not effect relaxation (Table III), some were hydrolyzed by the axonemal ATPase and others were not (Table IV).

Affinity of ATP Analogues for the Axonemal ATPase

ATP-γ-S, ϵ-ATP, AMP-PCP, AMP-PNP, and ADP acted as competitive inhibitors of the axonemal ATPase activity (Fig. 7; data for ADP omitted). Their inhibition constants (Table V) indicate that ϵ-ATP, ATP-γ-S, and ADP bound to the active site of the axonemal ATPase with dissociation constants ranging from 0.2 to 0.3 mM; AMP-PCP and AMP-PNP had ~10-

TABLE IV

Hydrolysis of Nucleotides by Axonemal Enzymes

Nucleotide	Concentration	Hydrolysis products*	
		Axonemal ATPase‡	Crude dynein 1§
	mM		
ATP	2	ADP, 20%; AMP, 80%	ADP, 100%
dATP	3	dADP, 90%; dAMP, 10%	—
ϵ-ATP	4	ϵ-ATP, 68%; ϵ-ADP, 32%‖	ϵ-ATP, 80%; ϵ-ADP, 20%‖
ATP-γ-S	4	No hydrolysis	No hydrolysis
AMP-PCP	4	No hydrolysis	No hydrolysis
AMP-PNP	4	No hydrolysis	No hydrolysis
Adenosine tetraphosphate	2	No hydrolysis	—
GTP	3	GTP, 50%; GDP, 50%	—
ITP	3	ITP, 40%; IDP, 60%	—
UTP	3	No hydrolysis	—
CTP	3	CTP, 90%; CDP, 10%	—

* Estimated from thin-layer chromatograms (see Materials and Methods).
‡ Concentration of demembranated, washed sperm was 0.65 mg/ml protein or 1.8×10^8 cells/ml.
§ Protein concentration of crude dynein 1 was 0.26 mg/ml.
‖ Determined by densitometry on photographic negative of thin-layer chromatogram.

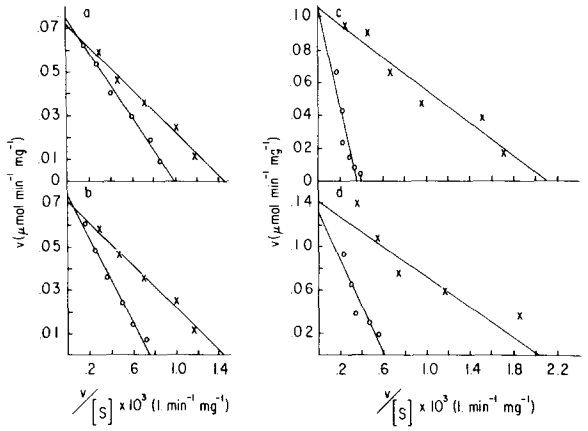

FIGURE 7 Inhibition of the axonemal ATPase activity by analogues of ATP. The ATP concentration dependence of the axonemal ATPase activity was determined in the absence (×) and presence (○) of: (*a*) 1.4 mM AMP-PNP; (*b*) 4.36 mM AMP-PCP; (*c*) 0.98 mM ATP-γ-S; and (*d*) 0.75 mM ϵ-ATP. Intersection of curves on the ordinate indicates competitive inhibition.

TABLE V

Inhibition of the Axonemal ATPase Activity by Analogues of ATP

Analogue	Type of inhibition*	K_i*
		mM
ϵ-ATP	Competitive	0.31
ATP-γ-S	Competitive	0.21
AMP-PCP	Competitive	4.5
AMP-PNP	Competitive	2.7
ADP	Competitive	0.27

* Determined from Hofstee plots in Fig. 7, except ADP, for which the Hofstee plot is not shown.

fold lower affinities (Table V). Therefore, at the nucleotide concentrations used in our relaxation assays (2–4 mM, Table III), the axonemal ATPase was ~90% saturated with respect to ϵ-ATP, ATP-γ-S, and ADP but only ~50% saturated with respect to AMP-PCP or AMP-PNP. The observations that only ϵ-ATP effected relaxation (Table III) and that 2×10^{-3} M was the minimum effective concentration of ϵ-ATP (data not shown) suggest that, in addition to nucleotide hydrolysis, at least 90% occupancy of the axonemal ATPase active sites is required for relaxation to occur.

DISCUSSION

Relaxation of rigor-wave flagella has been postulated to result from the ATP binding-induced detachment of dynein cross-bridges (18, 19, 23, 24, 26). In this paper we have presented several findings that are inconsistent with this view of the mechanochemical basis of relaxation:

(*a*) The rate of relaxation was shown to be sensitive to inhibition by vanadate (Fig. 5). Vanadate is thought to act as a noncompetitive inhibitor of dynein, antagonizing ATP dephosphorylation but not ATP binding (7, 12). Thus, the sensitivity of relaxation rate to vanadate strongly suggests that ATP hydrolysis is a rate-limiting step in the relaxation reaction. The observation that the relaxation rate was more sensitive to vanadate than was the ATP hydrolysis rate (Fig. 5) suggests, but does not prove, that the vanadate-sensitive ATPase that was most important to the relaxation rate was not dynein 1, which comprises ~80% of the ATPase activity of the axoneme (14); rather, a second, minor ATPase may be involved in relaxation.

(*b*) Only nucleotides that were hydrolyzed by the axonemal ATPase activity were also capable of effecting flagellar relaxation. It is possible that the failure of two nonhydrolyzable ATP analogues, AMP-PNP and AMP-PCP, to effect relaxation was due to their low affinity for the dynein ATPase active site (Table V; see also reference 19). However, the significance of nucleotide hydrolysis was confirmed by the observation that of two ATP analogues with similar affinities for dynein, the hydrolyzable one (ϵ-ATP) effected relaxation, while the nonhydrolyzable one (ATP-γ-S) did not (Tables III–V). It is conceivable that relaxation with purified ϵ-ATP resulted from an ATP contaminant that might have arisen spontaneously from the decomposition of purified ϵ-ATP. We did not attempt to rule out this possibility.

(*c*) The rate of relaxation must reflect the rate-limiting step of the relaxation reaction. If relaxation depends on the occupancy of the dynein arm ATPase by unhydrolyzed ATP, three general types of relaxation kinetics can be predicted: (*i*) Assuming that formation of an ATP-dynein complex were rate-limiting, then if, in the binding reaction

$$\text{ATP} + \text{dynein} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ATP} - \text{dynein},$$

the on-rate for ATP, k_1, is much larger than the off-rate, k_{-1}, relaxation rate should be first-order in ATP (i.e., it should double when the ATP concentration is doubled, and so on); (*ii*) assuming, again, that formation of an ATP-dynein complex were rate-limiting but that k_{-1} were significant compared with k_1, then the dependence of relaxation rate on ATP should be identical to an ATP binding curve for dynein (i.e., relaxation rate should be a hyperbolic function of ATP concentration). This prediction implies that the ATP dissociation constant for dynein should lie between 2×10^{-6} and 9×10^{-6} M; (*iii*) assuming that the rate-limiting step of the relaxation reaction were distal to the formation of an ATP-dynein complex, then relaxation rate should be independent of ATP concentration (zero-order kinetics with respect to ATP). An example would be if relaxation did not occur until the dynein arms were 100% saturated with ATP. Fig. 4 shows that the actual increase in relaxation rate is an *accelerating* function of ATP concentration, which is inconsistent with each of the three kinetic predictions derived from the assumption that ATP binding induces relaxation. The acceleration of relaxation rate as a function of ATP concentration (Fig. 4) might be explained, in the context of an ATP binding-dependent model of relaxation, by invoking long-range, positive cooperative interactions among ATP-dynein complexes as they are formed in the axoneme; no evidence for or against such interactions is available, to our knowledge.

(*d*) ATP was shown to be hydrolyzed at superstoichiometric levels, several hundred ATP molecules being consumed per dynein arm during the relaxation reaction (Fig. 6). The mere occurrence of ATP hydrolysis does not prove its role in relaxation; however, because ~80% of the axonemal ATPase activity is thought to reside in the dynein arms (14), most of the ATP hydrolysis observed during relaxation was probably due to the dynein arm ATPase. Therefore, a model of relaxation based on ATP binding alone would imply that ATP hydrolysis by the dynein arms becomes completely uncoupled from the relaxation reaction. While this is possible, it seems somewhat far-fetched.

In summary, the sensitivity of relaxation rate to inhibition by vanadate, the positive correlation between the hydrolyzability and the relaxation competency of nucleotide triphosphates, the kinetics of the relaxation reaction, and the turnover of large amounts of ATP indicate that ATP hydrolysis provides the mechanochemical driving force for flagellar relaxation. This view is consistent with an earlier interpretation of relaxation (10) and inconsistent with more recent work that has suggested that ATP binding alone, not ATP hydrolysis, produces an allosteric effect on the dynein arms that leads to relaxation (18, 19, 23, 24, 27).

Unexpectedly, we found that the relaxation rate increased some 30 times faster than did the ATP hydrolysis rate when the ATP concentration was raised from 2×10^{-6} to 9×10^{-6} M (Figs. 4 and 6), indicating that the *efficiency* of mechanochemical coupling is an ATP-dependent phenomenon. It might be argued that efficiency increased as a result of progressively fewer rigor cross-bridges resisting active sliding as the ATP concentration was increased. This explanation appears improbable, however, for two reasons. First, efficiency was found to increase with ATP concentration not only in relaxing sperm but in reactivated sperm (see above) as well, where rigor cross-bridges presumably were not present. Second, the demonstration of superstoichiometric ATP hydrolysis relative to the concentration of dynein arms (Fig. 6) implies the absence of static cross-bridges that resist relaxation; it suggests instead that each dynein arm hydrolyzes ATP and, as a result, undergoes many cross-bridge cycles during relaxation, albeit for the most part mechanically ineffective ones. It is difficult to compare the ATP-dependence of mechanochemical coupling efficiency in flagellar motility with previous work on muscle, because typically load, not ATP, has been varied to produce changes in the velocity of muscle contraction (8, 15). It would be interesting in this regard to determine whether the thermodynamic efficiency of muscle contraction is reduced significantly at low ATP concentrations.

Our measurements of mechanochemical coupling differ from

those of other authors (2, 3, 5) in that we used separate, washed sperm preparations to measure ATP hydrolysis, while they measured beat frequency and ATP hydrolysis simultaneously. While our data do not permit us to make a direct estimate of the absolute efficiency of energy coupling in relaxation, they reliably indicate a change in energy-coupling efficiency as a function of ATP concentration. Figuratively speaking, raising the ATP concentration had the effect of shifting the relaxation reaction into higher "gears" such that progressively less energy was needed to achieve the same degree of straightening at higher ATP concentrations. It might be argued that the increase in the efficiency of energy coupling was an artifact of relaxation. This seems unlikely, however, because we observed an analogous, though less dramatic, fourfold increase in the ratio of the beat frequency to ATPase activity in reactivated sperm when the ATP concentration was raised from 1.5×10^{-5} to 1.0×10^{-4} M ATP; this ratio reached a plateau at ATP concentrations $>10^{-4}$ M, suggesting saturation of the ATP-dependent mechanochemical coupling function (data not shown). The increase in mechanochemical coupling efficiency reported here is consistent with previous measurements of ATP hydrolysis and beat frequency, which showed a slight (~1.5- to 2-fold) efficiency increase when the ATP concentration was raised from 0.25 to 2.0 mM (2, 3). Comparison of these earlier results with our own suggests that the ATP-dependence of mechanochemical efficiency can be observed more clearly at micromolar than at millimolar concentrations of ATP.

We propose the following general model for flagellar relaxation: Relaxation depends on active microtubule sliding resulting from multiple dynein cross-bridge cycles powered by the binding and hydrolysis of ATP. The ATP-dependent dynein cross-bridge cycles are, themselves, subject to a separate ATP-dependent control mechanism that regulates the efficiency with which dynein cross-bridge cycling is converted into active microtubule sliding. Efficiency increases dramatically at micromolar ATP concentrations and reaches a plateau level at $\sim 10^{-4}$ M ATP. To illustrate this general model with a bicycle analogy, the dynein cross-bridge cycle is analogous to the cyclist pedaling, the sliding microtubules are the wheels, and the ATP-dependent coupling efficiency mechanism acts like a derailleur.

While the function of the energy coupling mechanism may be represented by a derailleur, the anatomical details of its operation in the flagellar axoneme remain speculative. Two possibilities are outlined briefly: (*a*) Some cross-bridge cycles may occur at low ATP concentrations but fail to produce active sliding. Raising the ATP concentration could result in tighter coupling of dynein cross-bridging to active sliding by "turning on" an additional component distinct from the dynein arm ATPase that is required for active sliding, such as a second ATPase (dynein 2?) (14) or a protein kinase. (*b*) Active microtubule sliding dependent on ATP hydrolysis may be subject to long-range, positive cooperative interactions, not only between individual dynein arms but possibly among sets of dynein arms, the composition of the sets changing periodically as a dynamic function of their position in the beating flagellum.

In conclusion, we have presented evidence that flagellar relaxation requires ATP hydrolysis, and that the efficiency of mechanochemical coupling in flagellar motility is, itself, an ATP-dependent function. Flagellar relaxation, therefore, does not, by itself, shed light on the role of ATP binding in the dynein mechanochemical cycle. Elucidation of the exact relationship of the individual steps in the dynein cross-bridge cycle to the binding and hydrolysis of ATP may come, for flagellar relaxation as for flagellar beating, from in vitro kinetic studies using purified dynein and outer-doublet microtubules.

ADDENDUM

Since submission of this article for publication, T. Shimizu (1981, *Biochemistry*, 20:4347–4354) has presented new evidence that vanadate acts as a noncompetitive inhibitor of dynein from *Tetrahymena*. This observation lends support to the view that vanadate reduces flagellar relaxation rate (Fig. 5) by inhibiting ATP hydrolysis, not ATP binding.

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