INHIBITION AND RELAXATION OF SEA URCHIN SPERM FLAGELLA BY VANADATE

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ABSTRACT

Direct measurements of the stiffness (elastic bending resistance) of demembranated sea urchin sperm flagella were made in the presence of $MgATP^{2-}$ and vanadate. Under these conditions, the flagellum is in a relaxed state, with a stiffness of $\sim0.9 \times 10^{-21}$ N m^2, which is $\sim5\%$ of the stiffness obtained in the rigor state in the absence of $MgATP^{2-}$. $MgADP^-$ does not substitute for $MgATP^{2-}$ in producing the relaxed state. A progressive inhibition of movement is observed after addition of $MgATP^{2-}$ to flagella preincubated with vanadate, in which new bend generation, propagation, and relaxation by straightening are distinguished, depending on the ratio of $MgATP^{2-}$ and vanadate. At appropriate concentrations of vanadate, increase of the velocity of bend propagation is observed at a very low concentration of $MgATP^{2-}$ that is not enough to induce spontaneous beating. Vanadate enhances competitive inhibition of beat frequency by $MgADP^-$ but not by ADP^{3-}, ATP^{4-}, or P_i. These observations, and the uncompetitive inhibition of beat frequency by vanadate, indicate that vanadate can only bind to dynein-nucleotide complexes induced by $MgATP^{2-}$ and $MgADP^-$. The state accessible by $MgATP^{2-}$ binding must be a state in which the cross-bridges are detached and the flagellum is relaxed. The state accessible by $MgADP^-$ binding must be a cross-bridged state. Bound vanadate prevents the transition between these two states. Inhibition and relaxation by vanadate in the presence of $MgATP^{2-}$ results from the specific affinity of vanadate for a state in which nucleotide is bound, rather than a specific affinity for the detached state.

The sliding microtubule hypothesis for flagellar and ciliary movement has been firmly established by a series of experimental (42, 49, 52) and theoretical works (4, 5, 31, 40), since the first important observations by P. Satir (43, 44). This hypothesis predicts that the dynein arm plays a vital role in generating the bending wave of flagella, because the dynein arm is involved in the transduction of the chemical energy provided by ATP dephosphorylation into mechanical energy (15, 16). The conformational change of the dynein arm is assumed to involve the cyclic change of its orientation, including association and dissociation with the adjacent microtubule, as in the actomyosin system (26). This mechanochemical behavior of the dynein arm is thought to be tightly coupled with the cyclic dephosphorylation of ATP, because the rate of ATP hydrolysis is tightly coupled with flagellar movement and beat frequency (7, 10, 11), and because dynein ATPase not only has a latency (1, 21, 32, 47, 48) but also can be increased in activity by microtubular protein (1, 39) and tubulin (25).

The dynein microtubule coupling mediated with ATP has also been suggested by some physiologi-

cal observations, such as the formation of rigor bends (13) of flagella in the absence of ATP, and the change in stiffness of flagella, depending on ATP (29, 35, 38). Meanwhile, recent electron micrography has provided visible evidences of the cross-bridged and unbridged forms of dynein arms (12, 17, 54, 55, 57). However, we have not enough evidence yet to provide the exact correspondence between the steps of ATP dephosphorylation and the conformational changes of dynein arms.

Recently, vanadate has been found to be a potent inhibitor of dynein ATPase (14, 19, 27), as well as of the movement of the reactivated demembranated flagella (19). In the present paper, the effect of vanadate on flagellar stiffness was investigated, and correlated with observations on rigor bend relaxation and beat frequency of demembranated sea urchin sperm flagella.

MATERIALS AND METHODS

Spermatozoa were obtained from the sea urchins, *Lytechinus pictus* and *Strongylocentrotus purpuratus*, as described in earlier papers (8, 36). *S. purpuratus* spermatozoa were used only for pH-stat measurements and a few observations on motility. Spermatozoa demembranated with Triton X-100 were used for all of the experiments.

Sea urchin semen was diluted with 2–50 vol of 0.5 M cold NaCl, depending on the experiments, and stocked on ice. Demembranation of spermatozoa was made according to the method developed by Gibbons and Gibbons (11) with some modifications. A volume of sperm suspension was mixed with 20 vol of the extraction solution and kept for 30 s at room temperature (18°C) followed by mixing with working solution. The extraction solution for most of the experiments, except pH-stat experiments, contained 0.15 M KCl, 1 mM dithiothreitol (DTT), 2 mM Tris buffer, 0.1 mM CaCl₂, and 0.04% (vol/vol) Triton X-100, pH 8.2. This is the same extraction solution referred to as LES in a previous paper (36).

Stiffness Measurements

Extraction solution containing 2 μM vanadate was used to obtain a higher percentage of initially straight flagella. Preliminary experiments showed that this amount of vanadate in the extraction solution had little effect on subsequent stiffness or motility. We transferred <1 μl of the extracted sperm suspension to 1 ml of working solution containing RBASE (20 mM Tris buffer, 2 mM DTT, 2% [wt/vol] polyethylene glycol [PEG], and KCl at 0.2 M or a lesser amount as required to maintain constant ionic strength when other components were added) and other constituents required for particular experiments. Ca²⁺ concentration was buffered to be 10⁻⁹ M or less, and pH was adjusted to 8.2.

The working solution containing spermatozoa was poured into a chamber on the microscope stage. The stiffness measurement was carried out according to the method described previously (38). One spermatozoon floating in the medium was caught by the head with a flat microneedle coated with polylysine (34) and inserted through one of the side openings of the chamber. Position and inclination of the flagellum was adjusted to give a

clear image (Fig. 1). Then, the distal region of the flagellum was supported with a hook-shaped microneedle that was fairly rigid and was applied perpendicular to the flagellum. The length of the flagellum between the head of the flagellum and the supporting microneedle was usually ~30 μm.

A very flexible microneedle for measurements, also hook shaped, was applied perpendicular to the middle region of the flagellum. The measurements were repeated at least three times in each direction, as shown in Fig. 1, and averaged. The measurement microneedles were calibrated against stiffer microneedles (56), and had stiffnesses of 0.3–1.0 × 10⁻¹¹ N per 1 μm displacement of the tip, with calibrating error of ±30%.

Observations of Rigor Bend Relaxation

A small portion of the demembranated sperm suspension, usually 50–100 μl, was added to 2–4 vol of reactivation solution containing 10 μM MgATP²⁻, in which the beat frequency of flagella was ~1 Hz. Then, 0.5–1 μl of the reactivated sperm suspension was abruptly diluted with 1 ml of the working solution to obtain rigor bends of flagella. In the experiments in which the working solution contained a high concentration of Mg²⁺ (1 mM or more), the reactivation solution used contained RBASE, 1.8 mM EGTA, 1.8 mM MgCl₂, 0.5 mM CaCl₂, and 0.011 mM ATP, at pH 8.2. In the experiments in which Mg²⁺-free working solution was used, low Mg²⁺ reactivation solution was used, to lessen the contamination of the working solution by Mg²⁺ from the reactivation solution. This low Mg²⁺ reactivation solution contained RBASE, 1.8 mM EGTA, 0.04 mM MgCl₂, 0.5 mM CaCl₂, and 0.11 mM ATP, at pH 8.2. Although Mg²⁺ concentration was as low as 0.01 mM in this reactivation solution, the wave form of the reactivated flagella was quite similar to that obtained in the condition described above, except for a slightly lower bend angle, as reported previously (36). Spermatozoa were observed for a period of at least 20 min after addition to the working solution, to determine whether relaxation occurred. The motility of spermatozoa was also checked by adding MgATP²⁻ before and after the incubation.

When the effect of ADP was examined, the working solution containing ADP and 100 μM diadenosinepentaphosphate was preincubated with 20 μg/ml hexokinase and 10 mM glucose for 5 h at room temperature. Hexokinase was used to remove ATP contamination (23) and diadenosinepentaphosphate to inhibit adenylate kinase (28).

Two other techniques were used to observe MgATP²⁻-induced bend relaxation in the presence of vanadate: An ATP-free working solution containing RBASE, 1.8 mM MgCl₂, 1.8 mM EGTA, 0.5 mM CaCl₂, and vanadate was used to obtain rigor wave flagella (vanadate-rigor solution). A drop of this suspension of spermatozoa in rigor was placed on a slide and covered with a cover glass. While the spermatozoa were observed, a drop of the working solution containing 1 mM MgATP²⁻ and vanadate was added at the edge of the cover glass, so that MgATP²⁻ was supplied by diffusion to the spermatozoa being observed near the center of the preparation. In the second technique, the rigor sperm suspension was poured into the chamber used for stiffness measurements, and MgATP²⁻ was applied by a micropipette brought close to the spermatozoon being observed.

Beat Frequency Measurements

We added 2 μl of extracted sperm suspension to 1 ml of reactivation solution. A drop of the reactivated sperm suspension was placed in a well slide, covered with a cover slip, and examined by dark-field microscopy with stroboscopic illumination, as de-
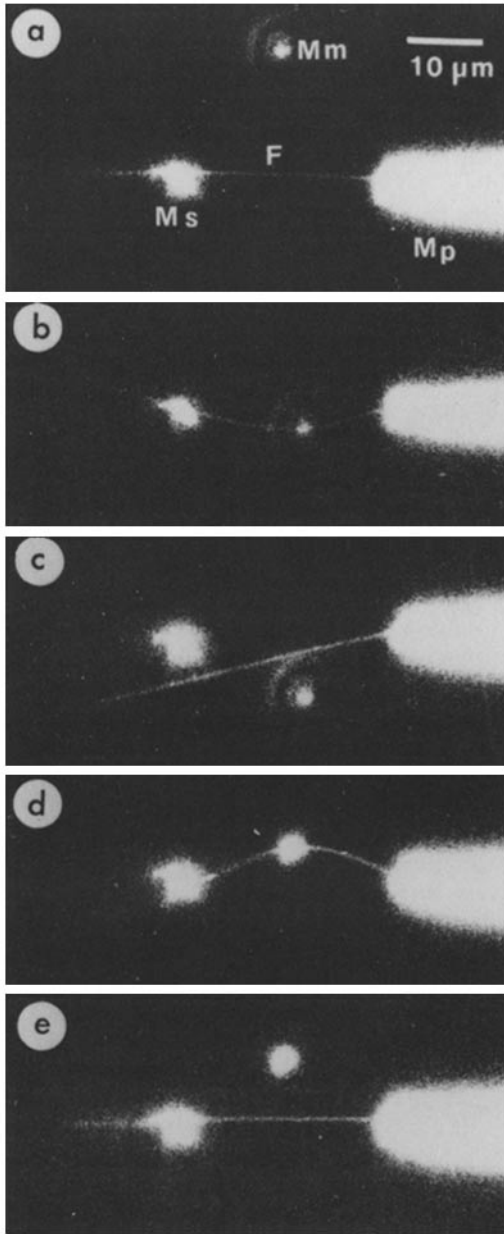
scribed previously (36). The frequency of flagella oscillation was measured on spermatozoa whose heads were tightly attached to the glass surface and beating in a plane parallel to the surface. At least 15 spermatozoa were measured under each condition. Each experiment was repeated four to six times and the results were averaged. The reactivation solution contained RBASE, 1.8 mM EGTA, and variable reagents such as ATP, ADP, $MgCl_2$, $CaCl_2$, KH_2PO_4, and vanadate. Ca^{2+} concentration was buffered to be 10^{-9} M.

ATPase Activity Measurements

ATPase activity of intact and broken spermatozoa was measured according to the methods of Brokaw and Simonick (10). Semen of *S. purpuratus* was diluted with 1-2 vol of cold 0.5 M NaCl, and stocked on ice. The concentration of spermatozoa was checked by optical densitometry at 540 nm. The sperm suspension was added to 20 vol of the extraction solution and kept for 30 s at room temperature. Nonmotile spermatozoa (broken spermatozoa) were prepared by vigorous shaking for 40 s with a Vortex mixer (Scientific Products Co., Evanston, Ill.) before extraction. The extraction solution contained 0.15 M KCl, 2.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 2.0 mM Tris buffer, 2.0 mM DTT, 0.5 mM EDTA, and 0.04% (vol/vol) Triton X-100, pH 8.4. The extracted sperm suspension was added to 40 vol of the reactivation solution, usually 10 ml, which contained 0.15 M KCl, 1.3 mM $MgCl_2$, 0.33 mM ATP, 1.0 mM DTT, 0.2 mM EGTA, 2% (wt/vol) PEG and 8 μg/ml oligomycin. The reactivation solution was titrated at pH 8.4 with 5 mM NaOH during the experiment, using recording pH-stats (Sargent-Welch Scientific Co., Anaheim, Calif.).

Experimental Conditions and Chemicals

All of the observations were carried out by using dark-field microscopy illuminated by stroboscopic light (Chadwick-Helmuth Co., Inc., Monrovia, Calif.) and recorded on Tri-X films with either a Robot camera or a kymograph camera (Grass Instrument Co., Quincy, Mass.). Microneedles and micropipettes were positioned with micromanipulators (Ernst Leitz Wetzlar, Wetzlar, W. Germany, and Narishige Scientific Instrument Laboratory, Tokyo, Japan). All of the experiments were made at room temperature (18° ± 1°C) and, for beat frequency measurements, the stage of the microscope was kept at 18° ± 0.5°C by circulating water.

$Na_2ATP \cdot 3H_2O$ was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., $NaADP \cdot 2H_2O$ from P-L Biochemicals, Inc., Milwaukee, Wis., hexokinase (grade 6) and diadenosinepentaphosphate from Sigma Chemical Co., St. Louis, Mo., oligomycin from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif., and sodium orthovanadate (Na_3VO_4) from Fisher Scientific Co., Pittsburgh, Pa.

The concentrations of Mg^{2+}, $MgATP^{2-}$, etc., were calculated using the following association constants: Mg^{2+} association constants with EGTA, EDTA, ATP, ADP, and P_i at pH 8.2 are 1.6×10^1 M^{-1} (36), 4.2×10^6 M^{-1} (45), 1.0×10^4 M^{-1} (50), 1.0×10^3

FIGURE 1 Dark field micrographs showing the method of determination of the stiffness of flagella. (a) A spermatozoon is fixed by its head with a flat microneedle coated with polylysine (*Mp*), and the flagellum (*F*) is supported with a hook-shaped microneedle (*Ms*) at the distal region. (b) The other flexible hook-shaped microneedle for measurement (*Mm*), with a stiffness of 3.7 × 10^{-12} N (newton) for 1 μm displacement of the tip, is applied vertically at the middle region of the flagellum. (c) Then the flagellum is removed, causing further displacement of *Mm*, and returned to the other side of *Ms*.

(*d* and *e*) The same procedure is made in the opposite direction. The position of the supporting microneedle (*Ms*) is fixed during the experiment. The stiffness determined here is 1.0 ± 0.1 × 10^{-21} N m^2. Bar at top right, 10 μm. The working solution contains RBASE, 1.8 mM EGTA, 2.8 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.1 mM ATP, and 10 μM vanadate, at pH 8.2.

RESULTS

Vanadate concentrations of up to 0.5 μM caused reductions in the beat frequency of *L. pictus* sperm flagella, with little or no change in other wave parameters or in the duration of reactivated motility. However, even with 0.5 μM vanadate, a few nonmotile spermatozoa were noticed in the sample, and the standard deviations of the beat frequency measurements were larger than these observed without vanadate (Figs. 6 and 7).

At vanadate concentrations between 0.5 and 2 μM, there was a gradual deterioration of reactivated motility during the period of observation. Beat frequency, wave amplitude, and symmetry all decreased gradually, and then the movement became erratic and stopped abruptly. Almost all of the spermatozoa stopped beating within 15 min at 1.0 μM vanadate. No flagella with normal beating were observed at vanadate concentrations of 2.5 μM or greater, although with 2.5 μM vanadate some spermatozoa continued to show oscillatory bending limited to the proximal region of the flagellum for periods of up to 5 min. Spermatozoa from *S. purpuratus* required slightly higher vanadate concentrations to obtain the same effect observed with *L. pictus* spermatozoa. The effects of vanadate on *L. pictus* spermatozoa are qualitatively similar to those reported for *Tripneustes* spermatozoa (19), but *L. pictus* spermatozoa appear to be slightly more sensitive to vanadate.

Direct Measurements of Flagellar Stiffness

Calibrated glass microneedles were used as shown in Fig. 1 to make direct measurements of the stiffness (38) of *L. pictus* sperm flagella at various concentrations of MgATP^{2-} and vanadate. At least 2.5 μM vanadate was used, to ensure that the sperm flagella were completely immotile during the measurements. Flagella immobilized by vanadate were sometimes bent slightly, often near the basal end as shown in Fig. 3 (26 s). This bend plane was observed to coincide with the plane of normal bending waves, when inhibition by vanadate was observed in experiments where vanadate reached the flagellum by diffusion from the edge of the preparation. In the stiffness measurements, the force for measurement was applied to the midregion of the flagellum, parallel to the bending plane when it was recognized. A typical experi-

ment is shown in Fig. 1, where the spermatozoon was in a solution containing 1 mM MgATP^{2-} and 10 μM vanadate. The basal joint of the flagellum to the head seemed flexible, because the flagellar axis near the basal region deviated sharply from the head axis, depending on the direction of the applied force. Usually, the stiffness values obtained for the two opposite directions agreed with each other within experimental error.

The effect of MgATP^{2-} concentration on stiffness in the presence of 10 μM vanadate is shown in Fig. 2 a. In the absence of ATP, at 1 mM Mg^{2+}, the mean stiffness was 15.1 × 10^{-21} N m^2. In the absence of vanadate, the mean stiffness in the absence of ATP was 15.6 × 10^{-21} N m^2; these values are not significantly different. With only 1 μM MgATP^{2-}, the stiffness was greatly reduced, but the time required for reaching the equilibrium position of the microneedles was much longer than at higher MgATP^{2-} concentrations. At higher MgATP^{2-} concentrations, the stiffness falls to a value of ~0.9 × 10^{-21} N m^2 and there is no detectable change in stiffness between 0.1 and 5 mM MgATP^{2-}. Fig. 2 b shows the effect of vanadate concentration on stiffness in the presence of 1 mM MgATP^{2-}. Over the range of vanadate concentrations from 2.5 μM to 1 mM, the stiffness values are distributed in the range of 0.7–1.3 × 10^{-21} N m^2, which is probably not significantly different from the result obtained in the experiments in Fig. 2 a.

Some of these measurements are summarized in Table I, along with stiffness measurements in the absence of Mg^{2+}, using working solutions containing 10 mM EDTA. In these measurements, the stiffness was 13.7 × 10^{-21} N m^2 in the absence of ATP (*sol 1*) and 17.3 × 10^{-21} N m^2 in the presence of 10 mM ATP^{4-} (*sol 6*). In contrast to earlier results, when Mg^{2+} was not completely excluded from the working solutions (35), flagella remained in the rigor state in the presence of high concentrations of ATP^{4-}.

Rigor Bend Relaxation

Relaxation of rigor bends was examined in several kinds of working solution, and the results are included in Table I. As might be expected, rigor bends are stable (for >20 min in my experiments) under conditions where high stiffness values, in the neighborhood of 15 × 10^{-21} N m^2, are measured, such as in the working solution containing neither Mg nor ATP, and in the working solutions containing either ATP or Mg. Flagella were also
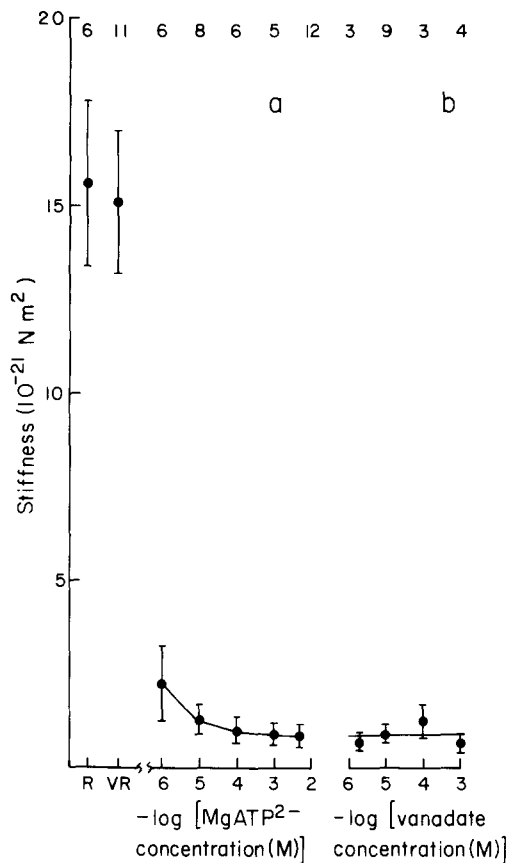
FIGURE 2 (a) Change in stiffness of flagella with MgATP^{2-} concentration in the presence of 10 μM vanadate. (b) Effect of vanadate on flagellar stiffness in the presence of 1 mM MgATP^{2-}. The working solution consisted of the rigor solution (RBASE, 1.8 mM EGTA, 1.8 mM MgCl$_2$, and 0.5 mM CaCl$_2$), ATP, extra MgCl$_2$ (keeping Mg^{2+} concentration at 1 mM), and vanadate. Two values at the left represent those obtained in the rigor solution (R) and in the rigor solution in the presence of 10 μM vanadate (VR). The numbers at the top represent the number of flagella measured, and vertical bars, SD.

stiff when the working solutions described above contained vanadate, because the rigor bends were maintained for >20 min, as shown by conditions 2, 4, and 7 in Table I. Relaxation of rigor bends, and low stiffness values, are only observed in the presence of MgATP^{2-}. Some earlier reports (13, 29, 35) suggested that the relaxed state could be obtained with ADP; however, in my experiments, no relaxation was obtained with ADP if the adenylate kinase activity was inhibited and ATP contamination was excluded by diadenosinepenta-

phosphate and by hexokinase, respectively. The possibility that diadenosinepentaphosphate could inhibit the relaxation was examined by using the working solutions containing RBASE, 2.8 mM MgCl$_2$, 1.8 mM EGTA, 0.5 mM CaCl$_2$, 100 μM diadenosinepentaphosphate, and either 3 μM ATP or 2 mM unpurified ADP (which may contain several percent of ATP; purchased from Boehringer Mannheim Biochemicals). In both cases, the rigor bends relaxed within 2–3 min. An inhibitory effect of hexokinase in the present study was also eliminated, because adding an appropriate amount of MgATP^{2-} to the working solution containing hexokinase after a 20-min incubation induced relaxation or activation of rigor flagella.

P$_i$ or both P$_i$ and ADP did not maintain the relaxed state of flagella, because the rigor bends were preserved during the incubation. Flagella were also stiff under the condition combined vanadate and ADP or P$_i$ or both ADP and P$_i$ as summarized in Table I.

Rigor bend relaxation, terminating with the flagellum in a quiescent, approximately straight state, could be observed with MgATP^{2-} concentrations of 1–5 μM, which were too low to induce spontaneous beating as reported by Gibbons and Gibbons (13), whereas >5 or 6 μM MgATP caused spontaneous beating, under the conditions of these experiments. In the present experiments, relaxation occurred slowly enough that the behavior of individual sperm flagella could be followed. The preexisting bends typically propagated slowly to the distal end of the flagellum, maintaining nearly the original curvature and bend angle. After these bends had propagated past the distal end, the flagellum retained its approximately straight, relaxed shape. Bends that initially had angles less than ~1 rad usually relaxed by simple straightening of the flagellum, without observable propagation.

Vanadate did not inhibit relaxation (14, 41), and actually accelerated bend propagation during relaxation under some conditions. In the presence of 10 μM vanadate, 1 μM MgATP^{2-} caused relaxation by bend propagation to occur at about the same velocity observed in the presence of 3 μM MgATP^{2-} without vanadate. However, at high (100 μM or more) vanadate concentrations, the propagation velocity was reduced and the amplitude of the bends decreased as they propagated.

The addition of vanadate to inhibit spontaneous beating made it feasible to observe relaxation initiated by supplying higher concentrations of

TABLE I

The Rigor Bend Relaxation and Stiffness of Flagella

Solution (sol) no.	Condition of the working solution	Rigor bend relaxation	Stiffness $10^{-21}\ N\ m^2$	No. of flagella measured
1.	0 Mg, 0 ATP*	−	13.7 ± 2.6	6
2.	sol 1 + 10 μM Vanadate*	−		
3.	1 mM Mg^{2+}	−	15.6 ± 2.2	6
4.	sol 3 + 10 μM Vanadate	−	15.1 ± 1.9	11
5.	sol 3 + 1 mM Vanadate	−		
6.	0 Mg, 10 mM ATP^{4-}*	−	17.3 ± 2.3	7
7.	sol 6 + 10 μM Vanadate*	−		
8.	1 mM Mg^{2+}, 1 μM MgATP^{2-}	+‡		
9.	sol 8 + 10 μM Vanadate	+‡	2.3 ± 1.0	6
10.	1 mM Mg^{2+}, 1 mM MgATP^{2-}	Reactivated		
11.	sol 10 + 10 μM Vanadate	+	0.89 ± 0.26	14
12.	0 Mg^{2+}, 10 mM ADP^{3-}*	−		
13.	sol 12 + 10 μM Vanadate*	−		
14.	1 mM Mg^{2+}, 1 mM MgADP$^-$§	−		
15.	sol 14 + 10 μM Vanadate§	−		
16.	1 mM Mg^{2+}, 30 mM P$_i$	−		
17.	sol 16 + 10 μM Vanadate	−		
18.	1 mM Mg^{2+}, 30 mM P$_i$, 1 mM MgADP$^-$§	−		
19.	sol 18 + 10 μM Vanadate§	−		

The rigor bend relaxation was examined after 20 min of incubation with working solutions. Each working solution basically contains RBASE, 1.8 mM EGTA, and 0.5 mM CaCl₂ at pH 8.2. (+) Relaxation was observed; (−) relaxation was not observed.

* The working solution contained 10 mM EDTA.

‡ It took ~20 min to reach a reasonably straight shape. At condition 9, relaxation was completed within a few minutes.

§ The solution was preincubated with 10 mM glucose, 20 μg/ml hexokinase, 100 μM diadenosinepentaphosphate for 5 h at room temperature.

MgATP^{2-} from the edge of a preparation or by micropipette, and to obtain photographs showing the relaxation of individual flagella. Fig. 3 illustrates the results obtained after preincubation of a rigor flagellum for 5 min in the presence of 5 μM vanadate at the room temperature, with relaxation initiated by MgATP^{2-} diffusing from the edge of the preparation. In this example, the rigor bends present at time 0 propagated to the distal end, leaving most of the flagellum straight. This was followed by slow development of a new bend at the basal end, which resembled the initiation of bending in quiescent flagella observed by Goldstein (22) and by Gibbons (18). This new bend then propagated rapidly along the flagellum, but the bend in the opposite direction, which began to form at the basal end as the preceding bend propagated, remained at the base and did not lengthen or propagate. (In interpreting the relative rates of these events, it must be remembered that the MgATP^{2-} concentration was not constant, and

was probably steadily increasing, during the time period of these observations.) At slightly lower vanadate concentrations, or in experiments with micropipette application of MgATP^{2-}, development and propagation of several new bends before the flagellum became quiescent was typically observed.

Results of similar experiments in the presence of 10 μM vanadate are shown in Fig. 4. At this concentration, relaxation usually involved only the propagation of fully established bends, as shown in Figs. 4a and b; growth and propagation of incompletely formed rigor bends, as shown in Fig. 4c, was infrequent. In the presence of 100 μM vanadate, relaxation occurred by a simple straightening of the bends, without propagation, as shown in Fig. 5. However, with more rapid application of MgATP^{2-} by micropipette, bends were sometimes observed to propagate even in the presence of 100 μM vanadate, although usually their amplitude decreased before they reached the
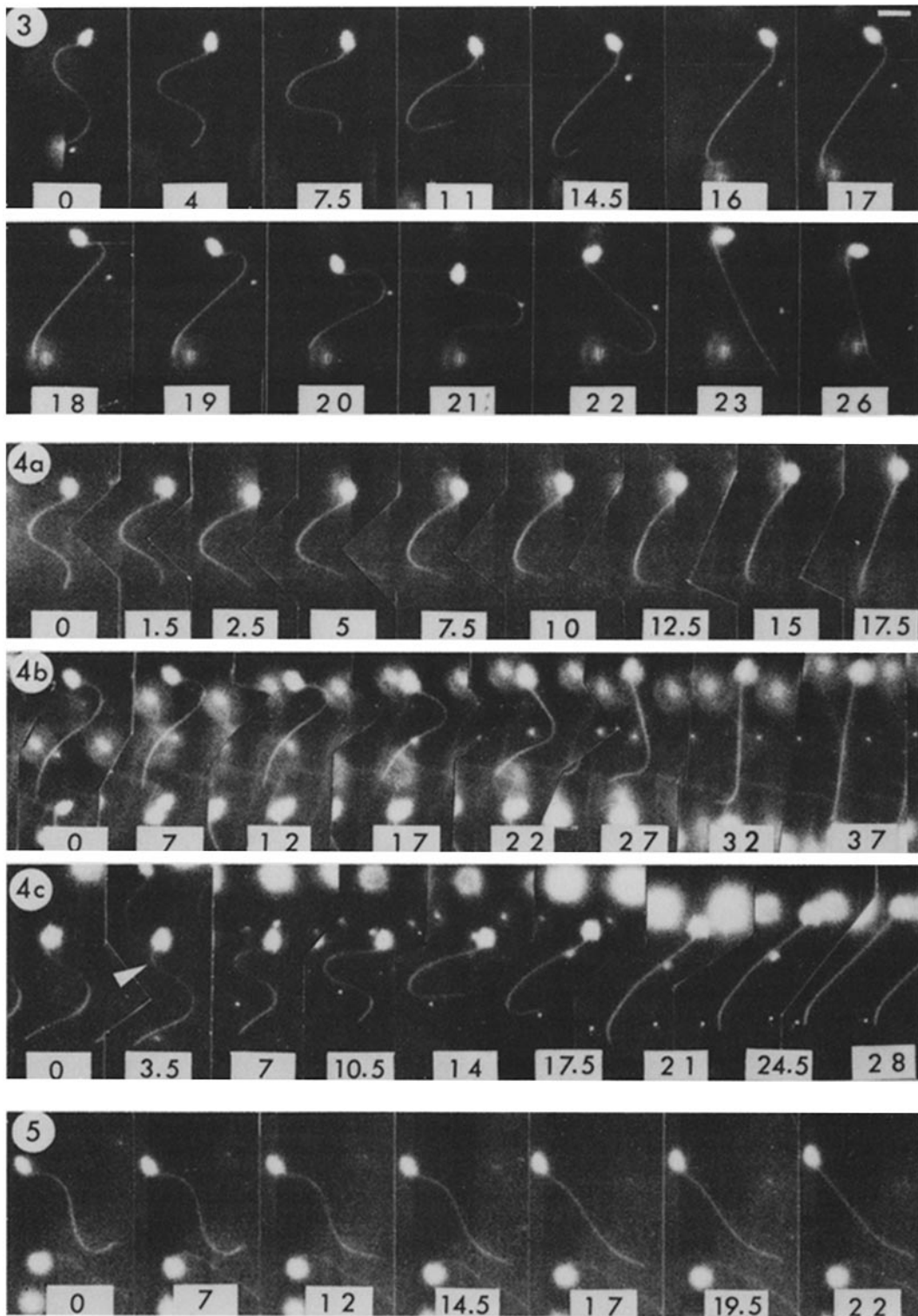
distal end of the flagellum.

Increase in the time of preincubation with 5 or 10 µM vanadate to 30 min or longer did not alter the results. However, in experiments with 50 µM vanadate, relaxation by bend propagation was typically observed after 5-min incubations with vanadate, while after 30-min or longer incubations, the capability for bend propagation was reduced, and bends relaxed by straightening.

Propagation of Mechanically Induced Bends

Bends were induced in the flagella of spermatozoa attached by their heads to the tip of a polylysine-coated microneedle, by tapping the microneedle holder. In solutions containing 1–5 µM MgATP^{2-}, this procedure easily induced bends near the basal region of the flagellum, and these passively formed bends then propagated to the distal end, as described above for propagation of rigor bends; similar results have been reported previously (49). Similar experiments were attempted in solutions containing 10 µM vanadate, at MgATP^{2-} concentrations over the range of 1–10 mM. Under these conditions, it appeared to be very difficult to induce bends in the flagellum by this method, and bends that were induced always relaxed by straightening rather than by propagation.

Beat Frequency Measurements

Fig. 6a shows the effect of vanadate concentration on beat frequency of attached spermatozoa in reactivation solutions containing 0.2 mM MgATP^{2-} and 1.0 mM Mg^{2+}. The measurements were made within 5 min of exposure to vanadate to avoid complications resulting from decrease in frequency with time at the higher vanadate concentrations. Fig. 6b shows the effect of 0.5 µM vanadate on the double reciprocal plots of beat frequency and MgATP^{2-} concentration at 1.0 mM
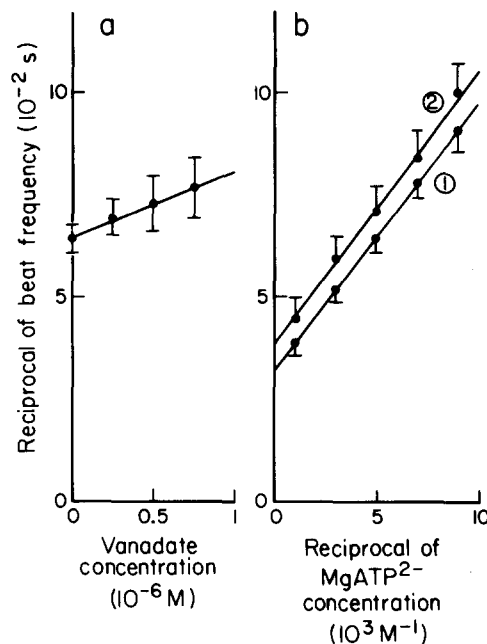

FIGURE 6 Effect of vanadate on beat frequency of flagella of attached spermatozoa. The reactivation solution contained RBASE, 1.8 mM EGTA, 0.5 mM CaCl$_2$, MgCl$_2$ (keeping Mg^{2+} constant at 1 mM), and ATP. Combined data from four experiments. (a) Reciprocal of beat frequency is plotted against vanadate concentration. MgATP^{2-} concentration was 0.2 mM. (b) Double reciprocal plots of beat frequency and MgATP^{2-} concentration in the absence (line 1) and presence (line 2) of 0.5 µM vanadate. Each straight line is obtained by least-squares method. Vertical bars represent standard deviations.

Mg^{2+}. These results confirm previous reports that vanadate is a simple uncompetitive inhibitor of beat frequency (19) and indicate a K_i of 2.6 µM for vanadate under the conditions of these experiments.

The uncompetitive inhibition of beat frequency

FIGURE 3–5 Dark field micrographs showing rigor bend relaxation. In each figure, one of the spermatozoa with rigor bends floating in the vanadate-rigor solution (RBASE, 1.8 mM EGTA, 1.8 mM MgCl$_2$, 0.5 mM CaCl$_2$, and vanadate) is followed. The solution consisting of the vanadate-rigor solution and 1 mM MgATP^{2-} at 1 mM Mg^{2+} is placed at the edge of the slide. The numbers at the bottom represent the time in seconds from each first frame. Bar at top right, 10 µm. Fig. 3 shows flagella in the presence of 5 µM vanadate. After the preexisting bends propagated out, a new bend was initiated at the proximal region of the flagellum (16–18 s) and propagated to the distal end. No new propagating bend was generated. Fig. 4 shows flagella in the presence of 10 µM vanadate. (a) Preformed bends exist in the middle and distal region of the flagellum. These bends propagate to the distal end. (b) A preformed bend exists in the proximal region, which propagates to the distal end. (c) A preformed small bend at the basal region shown by arrow head grows (3.5–7 s) and then propagates. Fig. 5 shows flagella in the presence of 100 µM vanadate. Preexisting bends are relaxed by straightening.

by Mg^{2+} and the competitive inhibition by ATP^{4-}, ADP^{3-}, $MgADP^-$, and P_i were examined in a previous paper (36). I have now examined the effect of 0.5 μM vanadate on inhibition by each of these inhibitors. Results obtained with ADP are shown in Fig. 7. Fig. 7a shows results obtained with a Mg^{2+} concentration of 0.1 mM, where $MgADP^-$ is calculated to be only 9% of the total ADP concentration, so that the inhibitory effect of added ADP is predominantly attributable to ADP^{3-}. In this case, the nearly parallel lines obtained in the presence and absence of vanadate indicate that there is no enhancement of vanadate inhibition by ADP^{3-}. Fig. 7b shows results obtained with an Mg^{2+} concentration of 2.0 mM; $MgADP^-$ is calculated to be 67% of the total ADP concentration, so that the inhibitory effect of added ADP is predominantly attributable to $MgADP^-$. In this case, in addition to the intrinsic inhibitory effects of higher concentration of Mg^{2+} and $MgADP^-$, the lines obtained in the presence and absence of vanadate are not parallel, indicating that the inhibition by vanadate is enhanced by $MgADP^-$ (and vice versa). A K_i of 2.1 μM for the $MgADP^-$-dependent inhibition by vanadate can be calculated from these results in combination with the earlier results (36).

Similar experiments with inhibition by Mg^{2+}, ATP^{4-}, and P_i all gave nearly parallel lines in the presence and absence of vanadate, indicating that there is no enhancement of vanadate inhibition by these inhibitors.

ATP Dephosphorylation Measurements

Results of pH-stat measurements of ATP dephosphorylation rates by suspensions of demembranated spermatozoa of *S. purpuratus* are shown in Fig. 8. 5 μM Vanadate is sufficient to inhibit almost completely the ATP dephosphorylation of broken, nonmotile sperm preparations as well as the movement-coupled ATP dephosphorylation represented by the difference between the rates for unbroken and broken sperm preparations.

DISCUSSION

Rigor and Relaxed States of Flagella

The existence of distinct rigor and relaxed states of flagella was originally indicated by the observations of Gibbons and Gibbons (13) on rigor wave relaxation in sea urchin spermatozoa and the indirect stiffness measurements on bull spermato-
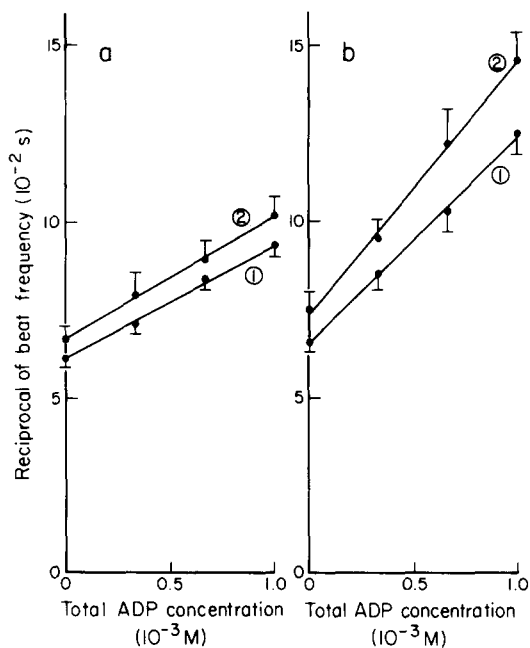

FIGURE 7 Effect of vanadate on beat frequency of flagella of attached spermatozoa. The reactivation solution contained RBASE, 1.8 mM EGTA, $MgCl_2$, ATP, and ADP. $MgATP^{2-}$ was kept at 0.2 mM. Line 1 in each figure represents results obtained without vanadate and line 2, those obtained in the presence of 0.5 μM vanadate. The results shown in a were obtained in the presence of 0.1 mM Mg^{2+} so that 9.1% of total ADP was $MgADP^-$ and 91% ADP^{3-}. In b, Mg^{2+} concentration was 2 mM; thus 67% of total ADP was $MgADP^-$ and 33% ADP^{3-}. Vertical bars represent standard deviations. Data from six experiments. Each straight line is obtained by least-squares method.

zoa by Lindemann et al. (29). Direct stiffness measurements on echinoderm sperm flagella (38) indicated that the relaxed state could be obtained with live spermatozoa immobilized by CO_2, and that the rigor state could be obtained with demembranated spermatozoa in the absence of ATP. In that work, a low stiffness value was measured for demembranated spermatozoa at high ATP concentrations and very low Mg^{2+} concentrations, suggesting that free ATP (ATP^{4-}), which binds to flagella as a competitive inhibitor of beat frequency (36), might be able to maintain the relaxed state. Subsequently, it was shown that stable rigor wave flagella could be obtained by abrupt removal of Mg^{2+} in the presence of ATP (14). My stiffness measurements, using more thorough removal of
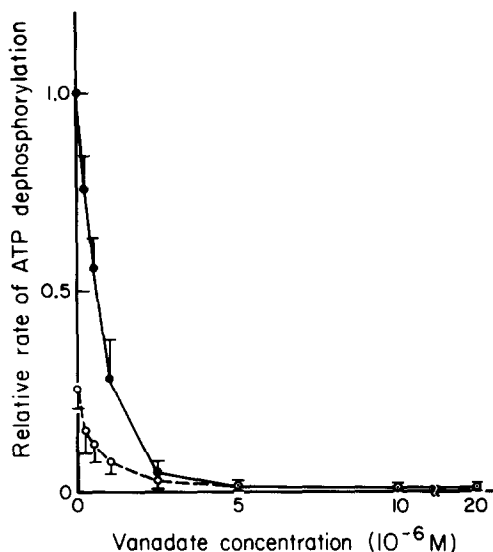
FIGURE 8 Effect of vanadate on ATP dephosphorylation by demembranated spermatozoa. Solid circles represent the intact spermatozoa, open circles the broken spermatozoa. ATP dephosphorylation by intact spermatozoa measured at 0 vanadate concentration is normalized to be 1.0. Data from three experiments.

Mg^{2+} from the preparations, now indicate that ATP^{4-} cannot maintain the relaxed state, and that $MgATP^{2-}$ is required both for relaxation of rigor waves and low values of stiffness. Earlier suggestions that ADP could maintain the relaxed state (13, 29, 38) now appear to be incorrect; no relaxation is obtained with ADP if the presence of ATP is rigorously excluded.

Relaxation of rigor bends can occur in the presence of vanadate (14, 41), and direct measurements of stiffness now show that the combination of vanadate (>2.5 μM) and $MgATP^{2-}$ is a reliable and convenient condition for maintaining flagella in a relaxed state. The best values for the stiffness of the two states now appear to be about 15 × 10^{-21} N m^2 for the rigor state and 0.9 × 10^{-21} N m^2 for the relaxed state. Because the latter value shows no significant trends with increasing vanadate concentration or with increasing $MgATP^{2-}$ concentration above 0.1 mM, it may be an accurate reflection of the intrinsic stiffness of the axonemal structures in the complete absence of dynein cross-bridging. However, it is not clear why the flagella often fail to become completely straight in the presence of vanadate and $MgATP^{2-}$.

The rigor state is believed to result from the presence of stable cross-bridges between the outer doublet microtubules of the axoneme, maintained by attachment of the dynein arms to the B tubule of the adjacent doublet (13). Several electron microscope studies have provided confirmation of this interpretation (17, 53), and a $MgATP^{2-}$ concentration of 1 μM has been shown to be sufficient for detachment of dynein from the B tubules of Tetrahymena axonemes (53). However, other studies report electron microscope observation of cross-bridges between outer doublet microtubules when cilia are fixed in the presence of Mg^{2+}, without added ATP, but no cross-bridging when cilia are fixed in the presence of ATP, without added Mg^{2+} (54, 55, 57). There may be subtle differences between the attached cross-bridges in these two rigor states, such that they respond differently to the fixation procedures for electron microscopy.

Bend Propagation

At $MgATP^{2-}$ concentrations below the level required to support spontaneous beating, bend propagation can still be observed, either by taking advantage of preexisting bends in rigor wave flagella or, as noted previously (49), by inducing bends by means of mechanical stimuli. The $MgATP^{2-}$ concentration required for bend propagation appears to be less than that required for bend initiation and sustained oscillation.

Bend propagation can be observed during relaxation of rigor wave flagella in the presence of $MgATP^{2-}$ at concentrations of vanadate sufficient to completely suppress oscillatory beating and to almost completely inhibit ATP dephosphorylation. At first glance, this might suggest that bend initiation is an energy-dependent process driven by active sliding, and that bend propagation can occur passively. However, the failure to induce bend propagation mechanically after incubation of flagella in both $MgATP^{2-}$ and vanadate, and observation of the initiation of one or a few bends before complete quiescence at slightly lower vanadate concentrations or when the $MgATP^{2-}$ concentration is increased rapidly in the micropipette experiments, suggests a different interpretation. It seems more likely that formation of an inhibitory dynein-$MgATP^{2-}$-vanadate complex is a relatively slow process, so that the relative concentrations of vanadate and $MgATP^{2-}$ determine how much bend propagation and bend initiation can occur before enough vanadate has bound to give complete inhibition. A slow time-course has been mea-

sured for vanadate inhibition of Na,K-ATPase, but was not detected with dynein ATPase (19). However, those measurements would not have been sensitive enough to detect changes occurring within a few seconds after addition of $MgATP^{2-}$ and vanadate, which is the time scale relevant to the observations on bend propagation. It is implicit in this interpretation that vanadate cannot form an inhibitory complex with dynein in the absence of $MgATP^{2-}$.

When inhibition occurs by formation of an inhibitory complex with vanadate, the flagellum frequently stops with a partial bend formed at the basal end of the flagellum (cf. Fig. 3). This bend was presumably formed by active sliding between tubules in the more distal region of the flagellum, and the direction of this active sliding would be the same as required within the preceding bend for propagation of that bend. The flagellum stops just before active sliding in the opposite direction would begin in the newly formed bend near the base of the flagellum to complete its growth and initiate its propagation. The mechanism for reversing the direction of sliding in new bends near the base of a flagellum is probably intimately involved in spontaneous oscillation and the determination of beat frequency, but remains poorly understood.

When bend propagation is resumed after rigor, it always occurs in the normal base-to-tip direction, even in cases such as in Fig. 4a, where the bend is near the midregion of the flagellum. The direction of the propagation must be "remembered" by some control mechanism that ensures that the correct subset of dynein arms becomes force-producing cross-bridges when $MgATP^{2-}$ is added. It is not known whether this information is stored in the form of the distribution of rigor cross-bridges or simply the bent configuration of the flagellum, or in some other manner. As mentioned previously, the mechanically generated bends also propagate from base-to-tip in the amputated starfish sperm flagella (37). However, it is not known yet whether they are caused by the same mechanism in flagella.

A Kinetic Model

Flagellar beat frequency may be equal, or at least proportional, to the frequency of cyclic dephosphorylation of ATP by dynein (3, 8, 10, 24). A simple kinetic scheme for an ATP dephosphorylation cycle is depicted by states 1, 2, and 3 in
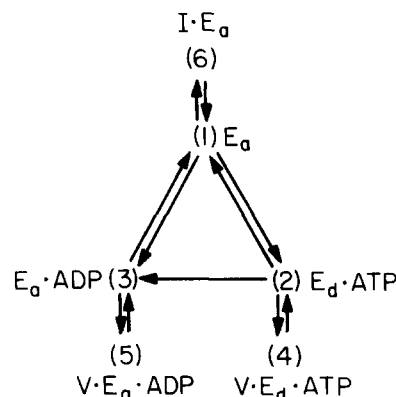

$$I \cdot E_a$$
$$(6)$$

$$(1) E_a$$

$$E_a \cdot ADP\ (3) \longleftarrow (2)\ E_d \cdot ATP$$

$$(5) \qquad\qquad (4)$$
$$V \cdot E_a \cdot ADP \qquad V \cdot E_d \cdot ATP$$

Figure 9 A kinetic model for the flagellar ATP-dephosphorylation cycle. E_a, dynein ATPase of attached state; E_d, detached state; ATP, $MgATP^{2-}$; ADP, $MgADP^-$; V, vanadate; I, any of several competitive inhibitors, such as ATP^{4-}, ADP^{3-}, or P_i.

Fig. 9. Three additional states are shown to indicate the mode of action of inhibitors. The frequency of cyclic ATP dephosphorylation by this cycle is given by:

$$\frac{1}{f} = \frac{1}{k_{23}[E \cdot ATP]} = \frac{1}{k_{23}} + \frac{1}{k_{31}}$$

$$+ \left(\frac{k_{24}}{k_{23}k_{42}} + \frac{k_{35}}{k_{31}k_{53}}\right)[V] + \frac{1}{k_{12}[ATP]}\left(1 + \frac{k_{21}}{k_{23}}\right)$$

$$\cdot \left(1 + \frac{k_{13}}{k_{31}}[ADP]\left(1 + \frac{k_{35}}{k_{53}}[V]\right) + \frac{k_{16}}{k_{61}}[I]\right) \quad (1)$$

in which $[ATP]$ represents the concentration of $MgATP^{2-}$, $[ADP]$ represents the concentration of $MgADP^-$, $[V]$ represents the concentration of vanadate, and $[I]$ represents the concentration of a competitive inhibitor, such as ADP^{3-}, P_i, or ATP^{4-}. If this frequency is equated to flagellar beat frequency, all of the measurements of flagellar beat frequency as a function of concentrations of $MgATP^{2-}$, $MgADP^-$, vanadate, and competitive inhibitors such as ATP^{4-}, ADP^{3-}, and P_i obtained in this and the previous paper (36) can be summarized by Eq. 1. The existence of state 5, in which vanadate is bound to an enzyme-$MgADP^-$ complex accessible by binding of $MgADP^-$, is demonstrated by the results in Fig. 7. The slope of line I in Fig. 7a and b gives a value of 0.4 mM for k_{31}/k_{13}, and the ratio of lines I and 2 in Fig. 7b gives a value of 2.1 μM for k_{53}/k_{35}. State 5 is sufficient to explain the uncompetitive inhibition of beat frequency by vanadate, if $k_{23}/$

k_{31} is approximately 3. However, state 4 could also contribute to uncompetitive inhibition. If state 4 exists, it could be completely responsible for uncompetitive inhibition of beat frequency by vanadate, if in fact the state accessible by MgADP$^-$ binding is not the state (in the normal ATP dephosphorylation cycle) preceding MgADP$^-$ release. Because vanadate does not act as a competitive inhibitor, it does not bind to state 1, and because it does not enhance the competitive inhibition by ATP^{4-}, ADP$^-$, and P_i, it does not bind to state 6. According to this scheme, inhibition by vanadate is the result of reducing the amount of enzyme in the useful states 1, 2, and 3.

Measurements of the elastic bending resistance of flagella and observations of relaxation of rigor bends allow an expanded interpretation of the model shown in Fig. 9. Existence of a rigor state in the absence of MgATP^{2-} indicates that state 1 is a "cross-bridged" or "attached" state in which dynein arms form stable attachments on the opposite B tubule. The observations that rigor bend flagella can be made by sudden removal of Mg^{2+} (14) and that flagella retain a high bending resistance in the presence of high concentrations of ATP^{4-} if Mg^{2+} is excluded indicate that ATP^{4-} binding, leading to state 6, does not cause relaxation. Formation of state 6 by binding of ADP^{3-} or P_i has also been shown to lead to maintenance of the rigor state.

State 3 is an attached state, as is state 5, because MgADP$^-$ or MgADP$^-$ and vanadate together do not cause relaxation.

Addition of 1–5 μM MgATP^{2-} causes relaxation of rigor bends (13) and dissociation of dynein from B tubules (53) and 0.1 mM ATP inhibits recombination of dynein to microtubules (46), indicating that state 2 is a "detached state". Because vanadate does not inhibit but in fact can enhance relaxation by MgATP^{2-}, state 4 must exist and also must be a detached state. These facts also indicate that the reaction path from state 4 to state 5 must be negligible. The ability of vanadate to accelerate relaxation by low MgATP^{2-} concentrations indicates that binding of vanadate to produce state 4 accelerates the transition from state 1 to state 2, thus reducing the number of attached cross-bridges that resist bend propagation. The transition from state 1 to state 2 that accompanies MgATP^{2-} binding must result in the preferential detachment of cross-bridged arms that have completed their working stroke and are in a position

to resist sliding. The remaining cross-bridged arms are in force-producing positions. This type of position-dependent cross-bridge detachment is a useful feature of models for cross-bridge action in muscle and flagella, beginning with the model of A. F. Huxley (26). The observation of bend propagation during relaxation, under conditions where the complete ATP dephosphorylation cycle becomes inhibited, provides some evidence that this feature is a real part of the MgATP^{2-}-concentration-dependent step in flagellar oscillation.

The simple kinetic scheme in Fig. 9 is not adequate to explain one important feature of flagellar oscillation: although the MgATP^{2-} concentration required for half-maximal beat frequency is ~0.2 mM (6, 11, 36), much lower MgATP^{2-} concentrations are adequate for relaxation, for dissociation of solubilized dynein arms from B-tubule binding sites of the outer doublet microtubules (53) and for half-maximal ATPase activity of uncoupled dynein (2, 8, 20, 32). This discrepancy probably indicates that the control of beat frequency by ATP concentration is not brought about simply by determining the concentration of the enzyme-MgATP^{2-} complex (state 2). Examples of mechanochemical models for flagellar oscillation in which the ATP concentration for half-maximal beat frequency is much greater than the equilibrium concentration for the ATP-concentration-dependent reaction step have been described by Brokaw and Rintala (9). There may be other possible ways to compensate the discrepancy. For example, a four-state model in which state 2 in Fig. 9 is divided into two substates can reproduce this behavior if the rate constant from state 2 to state 3, k_{23}, is much smaller than the others.

Recent studies have shown the existence of inhibitory vanadate-enzyme-ADP complexes in myosin (23) and alkaline phosphatase (30), in which vanadate is suggested to occupy a position analogous to phosphate. It would be reasonable to suggest that in flagella the inhibitory complex accessible by MgATP^{2-} binding (state 4 in Fig. 9) is also a vanadate-enzyme-MgADP$^-$ complex that, in contrast to state 5, remains in a detached form. If state 2 in Fig. 9 is an enzyme-MgADP$^-$ complex, an additional state in which MgATP^{2-} is bound must be inserted between state 1 and state 2 of Fig. 9. However, my present data do not provide any evidence to decide whether the bound nucleotide in the inhibitory complex accessible by MgATP^{2-} binding is ATP or ADP or to decide whether the

enzyme-MgATP^{2-} complex, introduced between state 1 and state 2, would be an attached or detached state.

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