The Pathway of ATP Hydrolysis by Dynein

KINETICS OF A PRESTEADY STATE PHOSPHATE BURST*

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The kinetics of ATP binding and hydrolysis (formation of acid-labile phosphate) by the Tetrahymena 30 S dynein ATPase has been measured by chemical quench flow methods. The amplitude of the ATP-binding transient gave a molecular weight per ATP-binding site of approximately 750,000, suggesting nearly 3 ATP binding sites/2 million M, dynein molecule (Johnson, K. A., and Wall, J. S. (1983) J. Cell Biol. 96, 669-678). ATP binding occurred at the rate predicted from the apparent second order rate constant of $4.7 \times 10^8$ M$^{-1}$ s$^{-1}$ measured by analysis of the ATP-induced dissociation of the microtubule-dynein complex (Porter, M. E., and Johnson, K. A. (1983) J. Biol. Chem. 258, 6582-6587). Hydrolysis was slower than binding and occurred at a rate of 55 s$^{-1}$, at 30 and 50 mM ATP. The rate limiting step for steady state turnover (product release) occurred with a rate constant of 8 s$^{-1}$. These data show that the first two steps of the pathway of coupling ATP hydrolysis to the microtubule-dynein cross-bridge cycle are the same as those described by Lymn and Taylor for actomyosin (Lymn, R. W., and Taylor, E. W. (1971) Biochemistry 10, 4617-4624). Namely, ATP binding induces the very rapid dissociation of dynein from the microtubule and ATP hydrolysis occurs more slowly following dissociation. Moreover, in spite of rather gross structural differences, the kinetic constants for dynein and myosin are quite similar.

Dynein, as originally defined (Gibbons and Rowe, 1965), is a general name describing any high molecular weight ATPase that interacts with microtubules (Gibbons, 1981). The most extensively studied is the outer arm dynein isolated from cilia and flagella. Numerous reports have established that this dynein is permanently attached to the A-tubule of the outer doublet and interacts transiently with the adjacent B-subfiber to produce a force for sliding (Summers and Gibbons, 1971; Satir, 1968; for reviews, see Gibbons, 1981, or Warner and Mitchell, 1980). The interaction of dynein with the B-subfiber is coupled to the binding and hydrolysis of ATP (Brokaw, 1975; Brokaw and Benedict, 1968; Brokaw and Gibbons, 1975; Gibbons and Gibbons, 1972). A rigor-type bond is formed in the absence of ATP and released by the addition of ATP (Gibbons and Gibbons, 1974; Gibbons, 1975; Zanetti et al., 1979). Therefore it is reasonable to propose a simple, four-state cross-bridge cycle analogous to models for muscle contraction (Huxley, 1969; Taylor, 1979). Without committing oneself to the precise conformational states of the dynein arm in each step of the cycle, this model provides a framework with which to examine the reactions involving the coupling of ATP binding and hydrolysis to the association and dissociation of dynein with the B-subfiber.

Since ATP causes the release of the rigor bond, the first question to address is whether ATP hydrolysis precedes or follows the ATP-induced dissociation. Our previous kinetic analysis showed that ATP induces an extremely rapid dissociation of dynein from the microtubule. Stopped flow light-scattering measurements established that the lifetime of the microtubule-dynein-nucleotide complex following ATP binding was much less that 1 ms. The apparent second order rate constant for ATP binding was $4.7 \times 10^8$ M$^{-1}$ s$^{-1}$.

To establish the pathway by which ATP hydrolysis is coupled to the microtubule-dynein cross-bridge cycle, it is necessary to measure the kinetics of ATP binding and hydrolysis on a time scale short enough to resolve the transients. Previous analysis of the dynein ATPase has indicated the existence of a presteady state phosphate burst, based upon extrapolation of steady state data at low temperature (Shimizu et al., 1979; Takahashi and Tonomura, 1979; Terashita et al., 1983) or relatively short times (Evans, 1982). These data suggest the presence of an enzyme-bound product intermediate with a long lifetime relative to its rate of formation and have been used to argue that dynein may share some kinetic properties with myosin.

In this report, I measure the kinetics of ATP binding and hydrolysis by chemical quench flow methods and show that hydrolysis is slower than the ATP binding-induced dissociation of the dynein from the microtubule. Preliminary reports of these data have been presented (Johnson and Porter, 1981, 1982a, 1982b).

**MATERIALS AND METHODS**

**Preparation of Proteins**—Dynein, tubulin, and the microtubule-dynein complex were prepared as described previously (Porter and Johnson, 1982a). Column-purified dynein, obtained by chromatography on DEAE-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ), was used without further purification. This was dictated by the quantities of material needed for the chemical quench flow experiments. The column-purified dynein consists of a mixture of 30 S dynein (80%) and 14 S dynein (15%). Since the chemical quench flow experiment provides a signal that is directly proportional to the molar concentration of enzyme sites, the contribution of the 14 S dynein to the observed signal should be less than or equal to 15%, based upon our preliminary estimate of the molecular weight of the 14 S dynein. We have failed to observe a phosphate burst in isolated 14 S dynein, but further work is currently underway using higher concentrations of protein and a newly constructed device able to use smaller sample volumes. The concentration of 30 S dynein was calculated as 80% of the total protein. The calculation of the molecular weight per ATP site (see Fig. 2) is therefore based upon the assumption that the 14 S dynein does not contribute to the observed signal.

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The concentration of dynein was determined by readings of absorbance at 280 nm using an extinction coefficient of 0.97 cm/mg based upon a calibration using dry weight of 30 S dynein. This was within 3% of the value obtained by the Lowry method. The concentration of tubulin was determined by the Lowry method, with a bovine serum albumin standard.

All of the experiments reported here were performed at 28 °C in a buffer consisting of 50 mM PIPES' and 4 mM MgCl₂ titrated to pH 7.0 with NaOH. The experiments were completed within 24 h of the preparation of the protein.

Stopped Flow Light-scattering Measurements—Light scattering at 90 ° to the incident beam was measured as described by Porter and Johnson (1983b) using a stopped flow spectrophotometer. Data were collected and stored digitally using a microcomputer (On-Line-Instruments Systems, Inc., Jefferson, GA). The data were fit to a single exponential by a modification of the Method of Moments (Dyson and Isenberg, 1971).

The microtubule-dynein complex was formed at ~1 mg/ml of tubulin and dynein, as described previously (Porter and Johnson, 1983a, 1983b), then diluted 5-fold with warm buffer and immediately loaded into one syringe of the stopped flow apparatus. The reaction was initiated by mixing the microtubule-dynein complex 1:1 with ATP in the same buffer. All of the ATP concentrations reported are the concentrations after mixing.

Chemical Quench Flow Measurements—The kinetics of ATP binding and hydrolysis was measured over the period of 5–100 ms using a quench flow apparatus constructed in this laboratory. The device consisted of three syringes, as diagrammed in Fig. 1A, which were simultaneously driven by an air cylinder to force the mixing of dynein first with substrate, [γ-32P]ATP, and then with a quench solution. The syringes were mixed using Berger ball-type mixers (Research Instruments and Mfg. Co., San Diego, CA). Each time point required 200 μl of enzyme, 200 μl of [γ-32P]ATP, and 400 μl of quench solution. Sample lines were flushed between consecutive runs as indicated in Fig. 1A. The time between mixing with substrate and the quench solution was varied from 5–100 ms by varying the length of the delay line between the two mixers and the rate at which the syringes were driven. The volume of solution between the mixers (delay volume) was measured using [32P]phosphate as a tracer by loading and flushing in the appropriate sequence to recover the solution that was contained between the two mixers. The progress of the pistons was monitored using a linear position transducer (Bourns, Riverside, CA) connected in series with a constant voltage supply, the output of which was fed into a storage oscilloscope. As shown in Fig. 1B, the movement of the pistons was linear with time. The progress of the pistons was timed (drive time = time from start to stop) and used to calculate the reaction time for each sample collected according to the equation: reaction time = drive time × (delay volume/sample volume), where the sample volume was the sum of the enzyme and substrate volumes (400 μl).

The time course of ATP hydrolysis (formation of acid-labile phosphate) was obtained by quenching with 1 N perchloric acid (final concentration) in the chemical quench flow apparatus and then measuring the concentration of phosphate released in each sample, as described below.

The time course of ATP binding was measured by quenching with a 200-fold excess of unlabeled ATP, allowing the reaction to continue for 1–2 s (10–20 half-lives of the enzyme turnover) and then stopping the reaction by manually adding 1 N perchloric acid. In this millisecond pulse-chase experiment, any tightly bound [γ-32P]ATP will be recovered as [32P]phosphate. The term tightly bound is operationally defined with the requirement that the rate of the forward reaction (hydrolysis and product release) must be 1 order of magnitude or more faster than the rate of substrate dissociation to get quantitative recovery of the bound ATP as phosphate. The observed increase in recovery of phosphate in the cold chase experiment provides evidence in support of a relatively slow substrate dissociation step. Preliminary evidence based upon measurement of the rate of synthesis of ATP from ADP and phosphate supports this assumption.

The steady state turnover rate was determined immediately following each quench flow experiment using the same dynein and ATP samples and under conditions identical with those used in the chemical quench flow apparatus. Dynein and [γ-32P]ATP were manually mixed and the reaction was stopped with 1 N perchloric acid to obtain reaction times from 3–30 s.

The concentration of [32P]phosphate was determined by applying the sample to a charcoal column to adsorb unreacted [γ-32P]ATP and measuring the radioactivity of the effluent relative to the total sample as described by Bagshaw and Trentham (1973) and Taylor (1977), with the following changes. For each sample a 2-ml column was prepared using activated coconut charcoal (50–200 mesh, Fisher Scientific Co., Pittsburgh, PA); this charcoal provided both a rapid flow rate and quantitative retention of [γ-32P]ATP while greater than 95% of the phosphate was recovered in the eluate. The charcoal was extensively washed before use with a solution consisting of 65 mM H₃PO₄ and 10 mM Na₃PO₄ (wash solution). The wash solution and columns were kept cold (0–4 °C). Immediately after stopping the reaction with acid, 0.5 ml of the wash solution was added to the sample and then a 0.8-ml aliquot of the mixture was placed onto a charcoal column. The column was then washed three times with 2.5 ml of the wash solution and the effluent was collected directly into a plastic scintillation vial (sample A). A 0.2-ml aliquot of the reaction mixture was added directly to another scintillation vial containing 8 ml of wash solution (sample B). The radioactivity of each was determined by the Cerenkov method using a Beckman LS 7000 liquid scintillation counter (Beckman Instruments, Inc., Irvine, CA) on the tritium channel. The concentration of phosphate was then calculated.

1 D. B. Clutter and K. A. Johnson, manuscript in preparation.
2 The abbreviations used are: PIPES, pipermazine-N,N'-bis(2-ethanesulfonic acid); STEM, scanning transmission electron microscopy.
3 K. A. Johnson, manuscript in preparation.
4 K. M. Arndt and K. A. Johnson, unpublished observations.
as the ATP concentration of the original sample multiplied by the activity of sample A divided by four times the activity of sample B. \([\gamma^{32}\text{P}]\text{ATP}\) was synthesized by the method of Schenkel and Wells (1973) and purified on a 2-ml DEAE-Sephadex A-25 column (Pharmacia Fine Chemicals, Piscataway, NJ) eluted with steps of 0.1, 0.2, and 0.4 M triethylammonium bicarbonate. The peak fractions of ATP in the 0.4 M step were evaporated to dryness, resuspended in water, and stored frozen until use. The \([\gamma^{32}\text{P}]\text{ATP}\) was used within a few days of synthesis to ensure a background of \([\gamma^{32}\text{P}]\text{phosphate}\) less than 1% relative to \([\gamma^{32}\text{P}]\text{ATP}\). In the two experiments shown in Fig. 2, A and B, backgrounds of 0.74 and 0.85% were recorded, corresponding to blanks of 0.24 and 0.37 \(\mu\)M, respectively. Although these blanks were relatively large, they were constant within 4-6% in replicate measurements. The blanks were subtracted from the data to obtain the concentration of phosphate produced during enzymatic hydrolysis. These data were then fitted as described in the Appendix to obtain a measurement of the number of phosphates produced per enzyme site. The concentration of phosphate was used to calculate the concentration of ATPase sites and thus the molecular weight per site.

The concentration of ATP in each experiment was determined by absorbance measurements using an extinction coefficient of 15.0 cm\(^2\)/
\(\mu\)mol. A trace of labeled ATP was then added to obtain an activity of approximately 500,000 cpm/ml of sample giving a specific activity of \(\sim 0.01\) Ci/mmol of ATP.

**RESULTS**

The kinetics of ATP binding and hydrolysis was examined in the millisecond time scale by chemical quench flow methods. Fig. 2, A and B, shows the results obtained at 30 and 50 \(\mu\)M ATP, respectively (concentration during the reaction period) for two separate experiments using two different dynein preparations. The data are plotted as the phosphate released per enzyme site, calculated by the fitting process described in the Appendix. The filled symbols show the kinetics of ATP hydrolysis (formation of acid-labile phosphate) and the open symbols show the kinetics of ATP binding, each of which is followed by steady-state turnover.

The data can be understood in terms of the following pathway with the minimum number of steps:

\[ E + \text{ATP} \xrightarrow{k_1} E-\text{ATP} \xrightarrow{k_2} E-\text{ADP-P} \xrightarrow{k_3} E + \text{ADP} + P, \]

where \(E\) represents a dynein enzymatic site. The data were fit to the complete solution of the above equation using all four rate constants as described in the Appendix. The smooth lines in Fig. 2 were computed according to Equations 9 and 10 (see Appendix) with the rate constants given in the legend. Table I summarizes the best estimate of the rate constants consistent with both sets of data.

The data shown were obtained using two separate enzyme preparations and are representative of six separate experiments. Identical results were obtained using a preformed microtubule-dynein complex in quench flow experiments at 30 \(\mu\)M ATP (data not shown). The ability of one set of constants to quantitatively account for data obtained at two ATP concentrations and using two separate enzyme preparations increases one's confidence in the accuracy of the estimates. A discussion of the fitting method and an estimate of the probable error is described in the Appendix. Approximate 90% confidence limits for the best estimates of the rate constants in fitting each data set are given in Fig. 2.

In each experiment, the rate of ATP binding was faster than the observed rate of ATP hydrolysis. The pseudo-first-order rate constant used to calculate the line for the ATP-binding transient \((k_1[\text{ATP}])\) was 120 \(s^{-1}\) at 30 \(\mu\)M ATP and

<table>
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<tr>
<th>Reaction</th>
<th>Parameter</th>
<th>Value</th>
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<tr>
<td>ATP binding</td>
<td>(k_1)</td>
<td>(4.7 \times 10^6 \text{M}^{-1}\text{s}^{-1})</td>
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<tr>
<td>ATP hydrolysis</td>
<td>(k_2)</td>
<td>(55 \text{s}^{-1})</td>
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<tr>
<td>ATP synthesis</td>
<td>(k_{-2})</td>
<td>(10 \text{s}^{-1})</td>
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<td>Burst amplitude</td>
<td>(k_3)</td>
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<td>Product release</td>
<td>(k_4)</td>
<td>(8 \text{s}^{-1})</td>
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<td>Michaelis constant</td>
<td>(K_m)</td>
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was 240 s⁻¹ at 50 μM ATP. These values are in agreement with the apparent second order rate constant of 4.7 × 10⁶ M⁻¹ s⁻¹ (Porter and Johnson, 1983b). Although the data at 50 μM ATP do not cover a short enough time interval to resolve the kinetics of ATP binding, they demonstrate that the rate was at least faster than 200 s⁻¹ and probably equal to the rate of 240 s⁻¹ obtained by stopped flow light-scattering measurements under the same conditions (see below).

The ATP hydrolysis transient was slower than the observed rate of ATP binding at each ATP concentration and was best fit by forward and back rate constants of \( k_1 = 55 \) s⁻¹ and \( k_2 = 12 \) s⁻¹, respectively. A lag phase is apparent in the fitted curve that is not necessarily demonstrated by the data obtained in the acid quench experiment. Rather, the fitting process includes a lag phase since the kinetics of binding is known and is slow enough to lead to a lag in the hydrolysis reaction according to the complete solution to the transient (see Appendix). Omission of the lag from the fitting process would lead to an underestimate in the rate of hydrolysis.

There is considerable margin for error in the estimate of the rate of the back reaction, \( k_2 \), since it is dependent only upon the relative amplitude of the ATP hydrolysis transient and a 2-fold variation in rate would lead to only a 20% variation in amplitude. Preliminary estimates of \( k_2 \) by analysis of medium \(^{32}\)P phosphate-H₂O exchange (Hackney et al., 1980), catalyzed by dynein in the presence of ADP, has given a value of 4 s⁻¹ for the rate of ATP synthesis.

The slope of the curves following the transient defines the rate constant for product release equal to 8 s⁻¹. This leads to a steady state turnover of 6 s⁻¹ since only ~70% of the enzyme is in the form of the enzyme-product intermediate, as defined by the values of the constants obtained for ATP binding and hydrolysis (see Appendix, term \( k_{on} \)).

The rate of ATP-induced dissociation of the microtubule-dynein complex was measured by stopped flow light-scattering methods, under conditions identical with those for the quench flow experiments. The results obtained at 30 and 50 μM ATP are shown in Fig. 3. The rate of the ATP-induced dissociation at 30 μM ATP (Fig. 3A) was 140 s⁻¹ and the rate at 50 μM ATP (Fig. 3B) was 240 s⁻¹. These results show that the rate of ATP-induced dissociation of the microtubule-dynein complex is equal to the observed rate of ATP binding, obtained by chemical quench flow methods (Fig. 2) and thereby indicate the following pathway:

\[
MD + ATP \overset{k_1}{\rightarrow} MD-ATP \\
\downarrow k_0 \\
D + ATP \overset{k_1}{\rightarrow} D-ATP \overset{k_2}{\rightarrow} D-ADP-P_i \overset{k_5}{\rightarrow} D + ADP + P_i
\]

where \( D \) represents dynein and \( MD \) represents the microtubule-dynein complex. The present results show that \( k_1 = k_1' \) and establish the values of the rate constants as given in Table I. Data from Porter and Johnson (1983b) put a lower limit on the rate of dissociation, \( k_0 \approx 1000 \) s⁻¹.

**DISCUSSION**

The observation and measurement of a presteady state phosphate burst provide several important bits of information. The existence of a burst implies that some step in the pathway following ATP hydrolysis is rate limiting during the steady state turnover. Presumably, the rate-limiting step is the release of products from the dynein-ADP-Pᵢ intermediate. Measurement of the amplitude of the ATP-binding transient provides an active site titration giving the apparent molecular weight per ATP-binding site. The observed site \( M_s = 750,000 \) gives 2.7 ATP-binding sites/2 million \( M_s \) particle (Johnson and Wall, 1983). Titration of the light-scattering amplitude for ATP-induced dissociation of the microtubule-dynein complex also gave an apparent molecular weight per ATP-binding site of 750,000 (Shimizu and Johnson, 1983b). Allowing for 10% inactive protein, the most reasonable interpretation of available data would suggest that there is one ATP-binding site on each of the three dynein heads (Johnson and Wall, 1983), but this remains to be firmly established.

It is interesting to note that burst measurements based upon extrapolation of steady state data for sea urchin dynein has given an amplitude of 1 ATP site/650,000 Da (Evans, 1982). Preliminary STEM analysis of sea urchin dynein-1 has indicated a two-headed particle with \( M_s = 1.3 \) million,⁵ again

⁵ K. A. Johnson, I. R. Gibbons, and J. S. Wall, unpublished observations.
Fig. 4. The pathway of ATP hydrolysis. A hypothetical cross-bridge cycle is illustrated coupled to the ATPase cycle established thus far, where M represents a microtubule and D represents dynein. It is suggested, but not yet established, that the $D$-$ADP$-$P$ intermediate (in parentheses) rebinds to the microtubule to complete the cycle. Each cycle results in the net upward movement of the adjacent microtubule and the hydrolysis of one ATP.

There has been no convincing demonstration of activation of the dynein ATPase in solution by microtubules. Following ATP-induced dissociation, the dynein reassociates with the microtubule after a lag period, the duration of which is directly proportional to the ATP concentration (Porter, 1982). However, in experiments to date, the rate of reassociation was not fast enough to distinguish whether dynein reassociated with the microtubule before or after the release of products. Our preliminary measurements of the rate of association of dynein with microtubules gave an apparent second order rate constant of $2 \times 10^7$ M$^{-1}$ s$^{-1}$ (Porter, 1982). At tubulin concentrations that are inaccessible in solution (up to 100 $\mu$M), the rate of dynein-microtubule association is too slow relative to the turnover rate of 6 s$^{-1}$ to have a noticeable effect on the rate of product release. However, the local concentration of tubulin in the axoneme would be sufficiently high to account for the more rapid turnover in vivo. In fact, Gibbons and Brokaw have observed a 4-fold increase in ATPase activity occurring with motility (Brokaw and Benedict, 1968; Gibbons and Gibbons, 1972). This is consistent with our measurements which might suggest an activation of $\sim 35:6$ from the ratio of the rate of turnover in the axoneme to the rate on the isolated dynein molecule.

It has been suggested that calmodulin activates the steady state dynein ATPase in a calcium-sensitive manner (Blum et al., 1980). However, it is likely that the release of products from the free dynein molecule, which determines the rate of steady state turnover, is not part of the cross-bridge cycle in vivo (see Fig. 4). Clearly, further work will be required to establish whether calmodulin serves to regulate the microtubule-dynein cross-bridge cycle and this cannot be addressed by studies on isolated dynein in the absence of microtubules.

There are several models in the literature proposing conformational changes that occur to produce the force for sliding (Goodeough and Heuser, 1982; Satir et al., 1981; Witman and Minervini, 1982; Tsukita et al., 1983). However, all of these models are based upon interpretations of electron microscopic images of dynein in terms of a rather rigid, bulky multisubunit arm. Our recent STEM analysis of dyneins from Tetrahymena (Johnson and Wall, 1983) and Chlamydomonas (Witman et al., 1983) has indicated that the ciliary outer arm dynein consists of a bouquet of three globular heads connected to the A-tubule by three independent stems (see also Witman and Minervini, 1982). Our data indicate that three molecules of ATP are required to dissociate each dynein molecule (Shimizu and Johnson, 1983b) indicating that the three globular heads interact with the B-subfiber to produce force for sliding. The simplest model would propose a rotation of the head on the microtubule surface to produce the sliding force analogous to current models for muscle contraction (Huxley, 1969; Taylor, 1979). The existing data can be interpreted in terms of this model.

suggesting that there may be one ATP binding site per head. The basis for the difference in the number of heads is currently being studied.

The rate of ATP binding equals the rate of ATP-induced dissociation of the microtubule-dynein complex. This observation serves to define the first two steps of the pathway by which ATP hydrolysis is coupled to the cross-bridge interaction as shown in Fig. 4. ATP binding causes a very rapid dissociation of dynein from the microtubule and hydrolysis occurs as a slower step following dissociation. It is significant that the lifetime of the microtubule-dynein-ATP ternary complex is much less than 1 ms (Porter and Johnson, 1983b). The predominant pathway is the kinetically favorable route and although ATP hydrolysis conceivably could occur without dissociation (Stein et al., 1981), the lifetime of the ternary complex is too short to allow time for the reaction to occur prior to dissociation. The rapid dissociation is also significant since the rigor complex formed at the end of the power stroke must have a short lifetime so that it will not impede the sliding forces generated by other cross-bridges. Models of flagellar motility developed by Brokaw (1975) require this rapid dissociation (>2,000 s$^{-1}$) at the end of the power stroke.

The Michaelis constant for the isolated dynein, calculated from our presteady state data (Table I), agrees with the observed $K_m$ of 1 $\mu$M based upon steady state measurements (Shimizu, 1981). In contrast, the ATP concentration dependence of the flagellar beat frequency gives an apparent Michaelis constant of $\sim 0.2$ mM (Brokaw and Benedict, 1968; Gibbons and Gibbons, 1972). This relatively high value is probably due to a more rapid rate of product release when the ATPase is coupled to movement, but may also be due to the requirement for a coordination of the interaction of many dynein arms to produce the sliding velocity needed for wave propagation.

Further work will be needed to establish the steps involved in recombination of the dynein with the microtubule. It should be noted that analysis of the mechanism of vanadate inhibition of dynein (Shimizu and Johnson, 1983a) suggests that phosphate release precedes ADP release and that the release of phosphate may occur with a large free energy change similar to the case for actomyosin (Taylor, 1979). In addition, the microtubule-dynein complex could be dissociated by ADP plus vanadate, this reaction may represent the reverse of the normal forward pathway involving recombination of the dynein-ADP-P intermediate with the microtubule. Attempts to directly establish this pathway have not been successful. However, measurements of ATP turnover in reactivated flagella (Brokaw, 1975; Brokaw and Benedict, 1968; Gibbons and Gibbons, 1972) have indicated that two molecules of ATP are hydrolyzed per dynein arm per beat of the flagellum. This would require that the slowest step in the pathway be no slower than the beat frequency of 35 s$^{-1}$. Product release from free dynein, which occurs at a rate of 8 s$^{-1}$, is too slow to be part of the cross-bridge cycle in vivo and one would predict that microtubules should activate the steady state ATPase.

It is suggested, but not yet established, that the D-ADP-Pi intermediate (in parentheses) rebinds to the microtubule to complete the cycle. Each cycle results in the net upward movement of the adjacent microtubule and the hydrolysis of one ATP.

Furthermore, in experiments to date, the rate of reassociation was not fast enough to distinguish whether dynein reassociated with the microtubule before or after the release of products. Our preliminary measurements of the rate of association of dynein with microtubules gave an apparent second order rate constant of $2 \times 10^7$ M$^{-1}$ s$^{-1}$ (Porter, 1982). At tubulin concentrations that are inaccessible in solution (up to 100 $\mu$M), the rate of dynein-microtubule association is too slow relative to the turnover rate of 6 s$^{-1}$ to have a noticeable effect on the rate of product release. However, the local concentration of tubulin in the axoneme would be sufficiently high to account for the more rapid turnover in vivo. In fact, Gibbons and Brokaw have observed a 4-fold increase in ATPase activity occurring with motility (Brokaw and Benedict, 1968; Gibbons and Gibbons, 1972). This is consistent with our measurements which might suggest an activation of $\sim 35:6$ from the ratio of the rate of turnover in the axoneme to the rate on the isolated dynein molecule.

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We have neglected effects due to multiple dynein heads. There is some evidence, at low ATP concentrations, for the kind of kinetic behavior expected for a three-headed dynein molecule (Shimizu and Johnson, 1983b), but further work will be required to establish the differences and functions of the three heads. The observation that the ATP-induced dissociation of the three-headed dynein molecule occurs as a single exponential at high ATP concentration might imply an inherent difference in the rate constants for the three heads or may be due to positive cooperativity in the ATP-binding step.

It is significant that the cross-bridge cycle for the microtubule-dynein interaction is analogous to that described for actomyosin (Lynn and Taylor, 1971; Taylor, 1979). In fact, the kinetic constants for ATP binding, hydrolysis, and ATP-induced dissociation of the cross-bridge are within a factor of 2–3 (Taylor, 1979; Johnson and Porter, 1982b). The major structural differences between dynein and myosin (Johnson and Wall, 1983; Taylor, 1979) suggest that the similarities in kinetic constants and pathway are more likely to be a function of the general principles of catalysis rather than due to any common ancestry. The ATP-induced dissociation of the cross-bridge is the critical step in establishing the pathway. Substrate-binding energy is presumably used to couple the ATP hydrolysis reaction to the vectorial process (Jencks, 1980).

The major difference in rates is in the product-releasing steps, where the rate observed for dynein exceeds that for myosin by a factor of 100. This accounts for a corresponding difference in the Michaelis constant. The slower turnover by myosin may be a function of the required control mechanisms where a muscle is in a relaxed state for long periods of time. In contrast, once a cilium or flagellum starts beating, it continues without interruption. As we learn more of the similarities and differences between dynein and myosin, the basic principles governing the conversion of chemical energy to mechanical force production and regulation may become more apparent.

**APPENDIX**

**The Pathway of ATP Hydrolysis by Dynein: Derivation of the Kinetics of ATP Binding and Hydrolysis**

Typically, presteady state transient chemical quench flow data are fitted as a single exponential followed by linear steady state. This approach is valid only when substrate binding and catalysis are well separated or cannot be distinguished kinetically. However, if one wishes to simultaneously resolve the kinetics of binding and catalysis over a single time interval, the experiments must be performed under conditions where the two rates differ by less than 5-fold. For example, experiments on the dynein ATPase (see main text) have provided measurements of the kinetics of ATP binding and hydrolysis where the two rates differed only by a factor of 3 or 5. Under these conditions, one expects a lag in the formation of product due to the kinetics of substrate binding. Although chemical quench flow data are rarely accurate enough to establish a lag, omission of the lag from the fitting process leads to significant underestimation of the rate of product formation. In this section, a derivation of the complete equations describing binding and hydrolysis of ATP and release of product is presented. The use of these equations in fitting chemical quench flow data, using a computer graphics subroutine, is then described.

The derivation was begun with the following model:

\[ E + S \overset{k_1}{\rightarrow} ES \overset{k_3}{\rightarrow} EP \overset{k_4}{\rightarrow} E + P \]  

where \( E \), \( S \), and \( P \) represent enzyme, substrate (ATP), and product (ADP and Pi), respectively. Substrate binding was assumed to be irreversible on the time scale of the quench experiment in the sense that catalysis and product release were much faster than substrate release. This assumption is reasonable and is, in fact, operationally defined by the ATP quench/chase experiment because one only observes ATP that is converted to products during the chase period. Preliminary evidence, based upon measurements of the rate of the reverse reaction, the synthesis of ATP from ADP and phosphate, supports this assumption.

Scheme 1 leads to the following set of differential equations:

\[ \frac{d[E]}{dt} = -k_3[E][S] + k_4[EP] \]

\[ \frac{d[ES]}{dt} = k_3[E][S] - k_4[ES] + k_4[EP] \]  


\[ \frac{d[P]}{dt} = k_4[EP] \]

Simultaneous solution of the equations gives the time dependence of each intermediate during the transient where \( \frac{[E_0]}{[E]} = \frac{[E_0]}{[ES]} + [EP] \).

\[ \frac{[E]}{[E_0]} = a_1 \left( \frac{\lambda_1 + \eta - k_4[S]}{\lambda_1 - \lambda_2} \right) e^{-\eta t} \]

\[ + \left( \frac{\lambda_1 + \eta - k_4[S]}{\lambda_1 - \lambda_2} \right) e^{-\eta t} \]

\[ \frac{[EP]}{[E_0]} = a_0 \left( \frac{\lambda_2}{\lambda_1 - \lambda_2} \right) e^{-\eta t} \]

\[ \frac{[P]}{[E_0]} = a_0 \frac{\lambda_2}{\lambda_1 - \lambda_2} e^{-\eta t} \]

The release of phosphate with time is obtained by integration of the equation,\[ \frac{[P]}{[E_0]} = \frac{[dP]}{[E_0]} = k_4[EP]/[E_0] dt \]

substituting the time dependence of the EP intermediate from Equation 4 and integrating to yield:

\[ \frac{[P]}{[E_0]} = k_4 a_0 + k_4 a_0 \left( \frac{\lambda_2}{\lambda_1 - \lambda_2} \right)^2 \]

The amplitude terms are defined as follows:

\[ a_0 = k_1 k_4[S]/[k_5 k_4[S] + k_2 + k_3] + k_2 k_3 \]

\[ a_1 = k_1 k_4[S]/[k_5 k_4[S] + k_2 + k_3] + k_2 k_3 \]

The values of \( a_0 \) and \( a_1 \) determine the fractions of enzyme as EP and E, respectively, during the steady state. Accordingly, the steady state turnover rate is given by \( V_{\text{st}} = k_4 a_0 [E_0] \), such that \( k_5 = k_2/k_3 \), and \( V_{\text{max}} = k_4 a_0 [E_0] \).

The exponential rate constants are the roots of the quadratic equation:

\[ \lambda_{1,2} = [(k_5 [S] + k_2 + k_3 + k_4) \pm \sqrt{(k_5 [S] + k_2 + k_3 + k_4)^2 - 4(k_5 [S] + k_2 + k_3)^2}] / 2 \]

The kinetics of ATP binding was measured by quenching the reaction with an excess of unlabeled ATP on a millisecond time scale and then with acid after a period of time sufficient to convert all tightly bound ATP to products. The kinetics of ATP hydrolysis (more precisely, the kinetics of the formation of acid-labile phosphate) was measured by quenching the reaction with acid on a millisecond time scale (Johnson, 1983). The ATP and Acid quench experiments measure the time dependence of the following sums in units of product per site:

\[ \text{Acid quench} = \frac{[EP]}{[E_0]} + \frac{[P]}{[E_0]} \]

\[ \text{ATP quench} = \frac{[ES]}{[E_0]} + \frac{[EP]}{[E_0]} + \frac{[P]}{[E_0]} = 1 - \frac{[E]}{[E_0]} - \frac{[P]}{[E_0]} \]

6 K. M. Arndt and K. A. Johnson, unpublished observations.
The time dependence of $[E^P]/[E^o]$, $[E]/[E^o]$, and $[P]/[E^o]$ are defined by Equations 3, 4, and 5. Since the above equations can most easily be solved in parts using a microcomputer, we have not made the substitutions of Equations 3–8 into Equations 9 and 10 for the final result.

The amplitudes of the Acid and ATP quench presteady state transients are obtained by extrapolation of the linear phase to $t = 0$. This can be defined algebraically by setting $t = 0$ for the linear terms and $t = \infty$ for the exponential terms in the above equations to get the following:

\[
\text{Acid quench amplitude } = a_0 (1 - k_2 \lambda_1 + \lambda_2)/\lambda_2 \lambda_3 \quad (11)
\]

\[
\text{ATP quench amplitude } = a_0 (k_2 + k_2 + k_3)
- k_2 (\lambda_1 + \lambda_3)/\lambda_2 \lambda_3 \quad (12)
\]

The complete solution was programmed into a computer graphics subroutine and the best fit to the data was evaluated visually and refined by an iterative nonlinear regression analysis (Draper and Smith, 1981) based upon trial and error. The steady state turnover rate was independently measured over a period of several seconds immediately following each experiment and was used in the fitting process to define the slope of the line following the transient. In addition, the data were normalized with respect to the presteady state ATP binding transient in order to calculate the number of phosphates per site from the measured value of phosphate concentration. This calculation originally assumed an amplitude of 1 phosphate/site and was subsequently corrected to an amplitude 0.85 phosphate/site according to Equation 12 using the best estimates for the rate constants. This normalization factor was then used to calculate the apparent molecular weight per ATP binding site from the measured protein concentration.

Visual evaluation of the calculated curve provides an essential criterion for judging the fit to the experimental data, especially during the initial stages of the fitting process. The major advantage of the visual evaluation is that one can readily distinguish those aspects of a curve that are particularly sensitive to a given parameter. For example, the rate of ATP binding ($k_1[ATP]$) primarily affects the shape of the presteady state ATP binding transient (ATP quench) and has only secondary effects on the fit to the linear phase following the transient or on the ATP hydrolysis curve. On the other hand, the rate of ATP hydrolysis ($k_2$) primarily affects the shape of the presteady state ATP hydrolysis transient (Acid quench) and has little effect on the fit to the linear phase; the rate of ATP synthesis ($k_4$) primarily affects the amplitude of the ATP hydrolysis transient. Only the rate of product release ($k_3$) has a marked effect on both the ATP binding and the ATP hydrolysis curves by affecting the slope of the linear phase following each transient.

The optimal method of obtaining the best fit to the data took advantage of the unique effects of each parameter on the two sets of experimental data. First, estimates for the rate of ATP binding ($k_1[ATP]$) and the rate of product release ($k_3$) were observed as the best fit to the presteady state and linear phases of the ATP-binding curve, respectively. At this stage of the fitting process, initial guesses for the constants $k_2$ and $k_4$ could be used because these have only secondary effects on the fit to the ATP-binding curve. Next, estimates of the values of $k_2$ and $k_4$ were obtained by fitting the net rate of ATP hydrolysis ($k_2 + k_4$) to the presteady state phase and fitting the rate of the ATP synthesis ($k_4$) to the amplitude of the ATP hydrolysis (Acid quench) transient, using the values of $k_1$ and $k_3$ obtained in fitting the ATP-binding curve. The best estimates of $k_2$ and $k_4$ were then used to refine the estimates of $k_1$ and $k_3$ in fitting the ATP-binding curve again.

The process was repeated and the final solution was reached when each constant gave a minimum in the sum square error, when the other constants were held fixed at their best estimate. The sum square error was calculated as $\sum(Y_{obs} - Y_{calc})^2$ for each data set.

Confidence limits for each constant were evaluated by constructing a confidence contour for each parameter (Draper and Smith, 1981). The sum square error was calculated while varying one parameter and holding the other three variables fixed at their best estimate. The approximate 90% confidence limits of each rate constant were determined as the variation in rate that led to a 40–50% increase in the sum square error (Draper and Smith, 1981).

The best fit provides a unique solution. Since one must simultaneously fit two data sets (ATP and Acid quench) and since each constant defines a particular aspect of the shape of the curves, one cannot obtain two similar curves by compensating for a change in one constant by altering another. Moreover, there is only one global minimum in the sum square error term for the data presented.

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