Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 6314–6318, September 1988
Biochemistry

Kinesin ATPase: Rate-limiting ADP release

(enzyme mechanism/motility/^{18}O exchange/energy coupling/tubulin)

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Communicated by Paul D. Boyer, May 23, 1988

ABSTRACT **The ATPase rate of kinesin isolated from bovine brain by the method of S. A. Kuznetsov and V. I. Gelfand [(1986) *Proc. Natl. Acad. Sci. USA* 83, 8530–8534)] is stimulated 1000-fold by interaction with tubulin (turnover rate per 120-kDa peptide increases from ≈0.009 sec^{-1} to 9 sec^{-1}). The tubulin-stimulated reaction exhibits no extra incorporation of water-derived oxygens over a wide range of ATP and tubulin concentrations, indicating that P_i release is faster than the reversal of hydrolysis. ADP release, however, is slow for the basal reaction and its release is rate limiting as indicated by the very tight ADP binding (K_i < 5 nM), the retention of a stoichiometric level of bound ADP through ion-exchange chromatography and dialysis, and the reversible labeling of a bound ADP by [^{14}C]ATP at the steady-state ATPase rate as shown by centrifuge gel filtration and inaccessibility to pyruvate kinase. Tubulin accelerates the release of the bound ADP consistent with its activation of the net ATPase reaction. The detailed kinetics of ADP release in the presence of tubulin are biphasic indicating apparent heterogeneity with a fraction of the kinesin active sites being unaffected by tubulin.**

A striking feature of the myosin ATPase is the very slow rate of product release in step 3 of the minimal scheme of Eq. 1 [see Taylor (1)].

$$E + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}ATP \underset{k_{-2}}{\overset{H_2O,\ k_2}{\rightleftharpoons}} E{\cdot}ADP{\cdot}P_i \xrightarrow{k_3} E + ADP + P_i \quad [1]$$

One consequence of this slow product release is that resynthesis of bound ATP from bound ADP and P_i by the reverse of step 2 is faster than product release with multiple reversals of the hydrolysis step occurring during each net turnover. These extra hydrolysis cycles can be detected by oxygen isotopic exchange. Thus if $k_3 >> k_{-2}$, then no reversal of step 2 occurs and only one water-derived oxygen is incorporated into the released P_i; whereas if k_{-2} is greater than or comparable to k_3, then reversal of the hydrolysis step can occur and more than one water-derived oxygen can be incorporated into the P_i before release. Binding of actin to the myosin-product complex stimulates product release that both increases the steady-state rate of ATP hydrolysis and decreases the incorporation of water-derived oxygens into the P_i due to the decreased lifetime of the complex with bound products.

The only other motile system to be characterized at this level is the dynein ATPase [for a comparative review, see Johnson (2)]. It too shows rate-limiting product release, although the absolute rate is much faster than for myosin and is only partially rate limiting. It is thus of interest to determine the product-release kinetics of the kinesin ATPase, which is a cytoplasmic microtubule-based motor for movement of intracellular membrane vesicles (3, 4). This enzyme has been isolated from a number of sources and contains a large subunit of 110–135 kDa and one or more smaller peptides of 45–70 kDa. The large subunit contains a nucleotide-binding site, as determined by affinity labeling (5), which may be the active site for ATP hydrolysis. Kuznetsov and Gelfand (6) have described a preparation of this enzyme from bovine brain with a low basal rate of ATP hydrolysis that is greatly stimulated by polymerized tubulin. It is reported here that this preparation hydrolyzes ATP with virtually no extra incorporation of water-derived oxygens over a wide range of tubulin and ATP levels, in striking contrast to myosin. Further analysis indicates that this is not due to a complete lack of rate-limiting product release but rather is due to a marked difference between the P_i and ADP release rates. The release of P_i is rapid and occurs before significant reversal of hydrolysis, whereas the release of ADP is extremely slow and represents the rate-limiting step in the absence of tubulin.

EXPERIMENTAL METHODS

Bovine brain kinesin was prepared essentially as described by Kuznetsov and Gelfand (6) with batch purification on DEAE-cellulose and phosphocellulose prior to affinity purification by binding to tubulin in the presence of triphosphate in excess of Mg^{2+} and by releasing with MgATP. Modifications included the omission of the second DEAE-cellulose step, the inclusion of bovine serum albumin (0.33 mg/ml) in the ATP release buffer, and the replacement of the final gel-filtration step after ATP-induced release from tubulin with an additional phosphocellulose step that concentrates the enzyme and removes tubulin and excess ATP. In this final step, the supernatant after ATP-induced release from tubulin was loaded on a 1 × 3 cm phosphocellulose column (P-11) equilibrated with buffer A (50 mM imidazole/0.5 mM $MgCl_2$/0.1 mM EDTA/1 mM 2-mercaptoethanol, pH 6.7) of Kuznetsov and Gelfand (6). The column was washed with 5 ml of buffer A and then with 5 ml of buffer A supplemented with 100 mM KCl to remove tubulin, bovine serum albumin, free ATP, and triphosphate, and the kinesin was eluted with buffer A supplemented with 600 mM KCl. The peak fractions were pooled (typically 2–3 ml) and dialyzed overnight versus ATPase buffer with one change of buffer after 3 hr. Kinesin concentrations are expressed as the molar concentration of the large subunit [reported to be 135 kDa by Kuznetsov and Gelfand (6), but found to be 120 kDa in our PAGE system in agreement with Vale *et al.* (3)] estimated by two-dimensional integration of the Coomassie blue-stained 120-kDa peptide after NaDodSO$_4$/PAGE with bovine serum albumin as standard.

Tubulin was prepared by the method of Williams and Lee (7) with two cycles of polymerization, chromatography on phosphocellulose, and storage in small vials at −70°C after fast freezing in liquid nitrogen. For ATPase measurements, a vial was thawed on the day of use and equilibrated with ATPase buffer by passage by 4°C through a centrifuge

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gel-filtration column (8). The tubulin concentration was determined by absorbance at 275 nm in 6 M guanidine hydrochloride by using a molecular mass of 110 kDa and an extinction coefficient of 1.03 $ml \cdot mg^{-1} \cdot cm^{-1}$. Tubulin was polymerized by incubation at 35°C for 15 min after addition of taxol (2- to 3-molar excess).

ATPase and binding measurements were performed at 25°C in the ATPase buffer of Kuznetsov and Gelfand (6) (buffer A supplemented with 50 mM KCl and 2 mM EGTA). $[^{32}P]P_i$ release from $[\gamma\text{-}^{32}P]ATP$ was determined by extraction of the molybdate complex of P_i as described (9). For determination of pyruvate kinase-inaccessible $[^{14}C]ADP$, a small volume of reaction mixture was quenched with 0.5 ml of 1 M HCl containing 0.8 mM carrier ADP, diluted with 5 ml of 0.2 M Tris base, and chromatographed on a 0.5-ml bed volume column of AG MP-1 anion-exchange resin (Bio-Rad) (10). The column was washed first with water and then with five 0.5-ml vol of 15 mM HCl, and the ADP was eluted with three 0.6-ml vol of 40 mM HCl. The second and third fractions of 40 mM HCl were pooled, and the amount of ^{14}C and the absorbance at 258 nm, which was used to correct for incomplete recovery of carrier ADP (typically 70%), were measured.

RESULTS

Oxygen Exchange. The dependence of the ATPase rate of bovine brain kinesin on the concentration of ATP and tubulin in the presence of pyruvate kinase and phospho*enol*pyruvate to regenerate ATP is shown in Fig. 1. The observed maximal velocity for this preparation of 8.7 $sec^{-1} \cdot mol^{-1}$ of 120-kDa polypeptide and the K_m values for ATP and tubulin of 8.3 μM and 6.9 μM are in good agreement with the results of Kuznetsov and Gelfand (6), with allowance for the difference in temperature (25°C versus 37°C). These incubations were performed with ^{18}O-labeled phospho*enol*pyruvate and ADP and thus the γ-phosphoryl group of the ATP synthesized by pyruvate kinase *in situ* was ^{18}O-labeled at the same level as the phospho*enol*pyruvate. The P_i produced was analyzed for ^{18}O content to determine if ATP hydrolysis was reversible at the enzyme active site as discussed above. Since these reactions were performed in unlabeled (^{16}O) water, exchange would result in a decrease in the ^{18}O labeling of the P_i beyond that resulting from incorporation of the one water-derived oxygen required by the hydrolysis. The control level of ^{18}O in the phospho*enol*pyruvate was determined by synthesis of labeled ATP with pyruvate kinase and ADP and then by hydrolysis of the ATP with the Ca-ATPase of myosin, which hydrolyzes ATP with incorporation of only one water-derived oxygen (11). It was found that there was only a slight shift in the labeling pattern of the P_i produced by kinesin from that of the Ca-ATPase control. This is best illustrated by the ratio of the species with two versus three ^{18}O per P_i. Incorporation of extra unlabeled water-derived oxygens would initially tend to increase this ratio as the amount of the species with two ^{18}O (and thus two unlabeled oxygens) increases at the expense of the species with three ^{18}O oxygens (and one unlabeled oxygen). This ratio in the Ca-ATPase control was 0.691 and only increased to an average of 0.716 for all the samples of Fig. 1 with a standard deviation of 0.005 and no indication of systematic variation with ATP or tubulin level. This is at the level of detection of the method and represents a ratio of k_{-2}/k_3 in Eq. 1 of only 0.027 when analyzed on the basis of probability (12). Thus P_i release likely occurs before significant reversal of the hydrolysis step can take place.

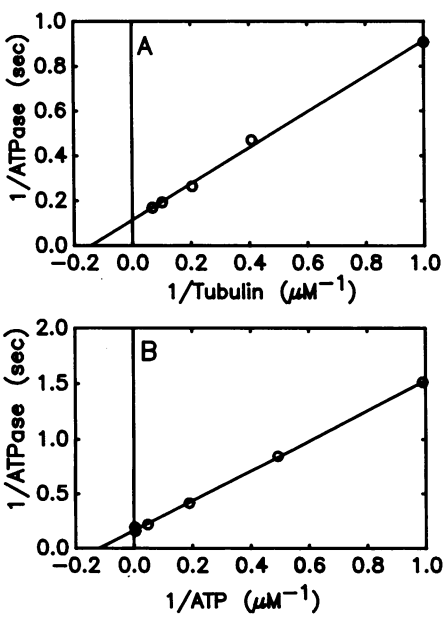

FIG. 1. Tubulin and ATP stimulation of kinesin ATPase. (*A*) Reaction conditions were 0.37–0.45 mM MgADP, 1.7 mM [^{18}O]-phospho*enol*pyruvate and pyruvate kinase (0.1 mg/ml) in ATPase buffer at various tubulin concentrations. (*B*) Reaction conditions were as in *A* except 9.6 μM tubulin was added with various ATP concentrations.

ATPase Kinetics. The ATPase kinetics of kinesin in the absence of tubulin and a 7.4-fold molar excess of $[^{32}P]ATP$ are shown in Fig. 2. The initial rates either with ATP alone or in the presence of pyruvate kinase/phospho*enol*pyruvate are similar and are equal to a turnover number of $\approx$0.009 sec^{-1} for each mol of 120-kDa peptide. Other experiments (data not shown) indicate that no substantial decrease in ATPase rate is observed, even down to 10 nM ATP and 1 nM 120-kDa peptide. Thus the K_m for ATP must be considerably less than 10 nM and the observed rate represents the V_{max} rate at saturating ATP. Thus the degree of activation of the ATPase rate by tubulin at saturating ATP is on the order of 1000-fold (0.009–8.7 sec^{-1}), indicating extremely tight coupling. The extent of activation observed by Kuznetsov and Gelfand (6) was less than this, mainly due to a higher basal rate in their case. There is an indication of what may be a small burst at $\approx$10% of the concentration of 120-kDa peptide,

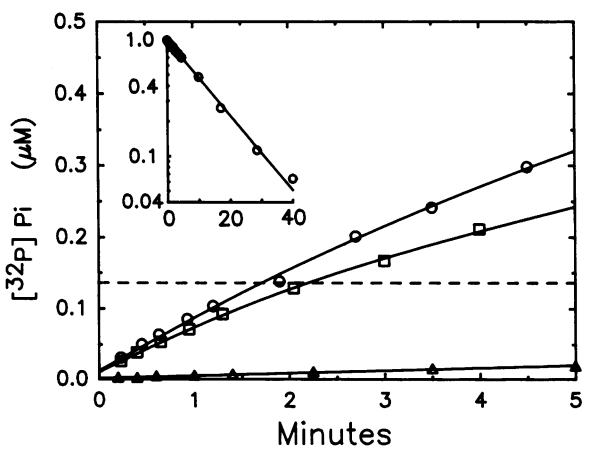

FIG. 2. Initial ATPase kinetics. The reaction was initiated by addition of $[^{32}P]ATP$ (final concentration, 1 μM) to 0.135 μM 120-kDa peptide in ATPase buffer supplemented with bovine serum albumin (0.26 mg/ml) alone (□), with pyruvate kinase (0.05 mg/ml) and 0.5 mM phospho*enol*pyruvate (○), or with 12.7 μM ADP (△). The molar concentration of 120-kDa peptide is indicated (dashed line). (*Inset*) Semilogarithmic plot of the final $[^{32}P]P_i$ (at 100 min) minus the observed $[^{32}P]P_i$ from 0 to 40 min of reaction time in presence of pyruvate kinase/phospho*enol*pyruvate.

but other preparations have often indicated smaller burst sizes and a larger burst has never been observed. Myosin exhibits an approximately stoichiometric burst due to its slow product release and the lack of a major burst with kinesin is again consistent with rapid P_i release.

For the reaction in the presence of pyruvate kinase phospho*enol*pyruvate, the rate of production of [^{32}P]P_i is expected to decrease with first-order kinetics due to the dilution of the specific activity of the [^{32}P]ATP pool as increasing amounts of ATP are derived from unlabeled phospho*enol*pyruvate. Fig. 2 (*Inset*) shows that this prediction is obeyed over a 40-min period with a first-order rate constant that corresponds to a flux rate of 0.0088 sec^{-1}. Thus the flux of total ATP hydrolysis remained constant during this whole period at the value initially observed in the first minute of the reaction. This is in contrast to the reaction in the absence of pyruvate kinase/phospho*enol*pyruvate that exhibited marked apparent product inhibition by ADP that became much more pronounced at longer times (data not shown). This product inhibition is confirmed by a 95% reduction in the initial rate when a 12.7-fold excess of ADP over ATP is included at zero time (Fig. 2). For competitive binding at saturating amounts of ATP, this represents a binding constant for ADP that is ≈2-fold tighter than the K_m for ATP or <5 nM. Addition of ADP also removes the small apparent burst.

Steady-State Titration. The extremely low K_m value for ATP indicated that it should be possible to observe ATP binding during steady-state hydrolysis by depletion of the free ATP levels as determined with myosin (9). Results of such a steady-state active site titration in the presence of pyruvate kinase phospho*enol*pyruvate are indicated in Fig. 3. The molar concentration of the 120-kDa peptide is 0.5 μM, which is at least 50-fold in excess of the K_m, and thus, for a simple kinetic scheme, most added ATP should be bound to the enzyme at steady state, as long as the molar concentration of the added ATP is less than the concentration of binding sites. This is not observed, however, and the concentration of free ATP at steady state is equal to the amount of added ATP even at low molar ratios of added ATP to 120-kDa peptide.

Bound ADP. This conflict between a very low K_m for ATP and lack of tight apparent nucleotide binding in a steady-state titration could be due to a number of reasons, including a rate-limiting conformational change of the enzyme in the absence of nucleotide, but the tight nature of ADP binding also observed above suggested that the enzyme as standardly isolated may already contain an ADP tightly bound at its active site. This was confirmed by direct nucleotide assay of a perchloric acid extract of the enzyme by the luciferase method. The neutralized extract contained no detectable ATP but did contain 1.0 mol of ADP per mol of 120-kDa peptide as determined by the increase in light production on addition of pyruvate kinase phospho*enol*pyruvate to phosphorylate the ADP to ATP.

The presence of a tightly bound nucleotide could also be demonstrated by rapid separation of free nucleotide from bound nucleotide by centrifuge gel-filtration chromatography (8) as shown in Table 1. Incubation of the enzyme with [^{14}C]ATP results in labeling of a stoichiometric level of bound nucleotide that is eluted from the column with the protein. The labeling is largely complete at 5 min and is totally reversible, with excess unlabeled ATP chasing the labeled ADP off the site with kinetics consistent with labeling at the steady-state ATPase rate. In contrast P_i release is rapid with no [^{32}P]P_i being eluted with the kinesin after incubation with [^{32}P]ATP.

To improve the time resolution of the labeling kinetics, the level of pyruvate kinase inaccessible ADP was determined. In this type of experiment [^{14}C]ATP is added to kinesin along with a large excess of pyruvate kinase and phospho*enol*pyruvate to rapidly recycle the free ADP produced by steady-state hydrolysis back to ATP. Thus the free ADP level will be negligible and any [^{14}C]ADP present at steady state must be inaccessible to pyruvate kinase and is likely to be tightly bound to kinesin. The results presented in Fig. 4 indicate that a stoichiometric amount of [^{14}C]ADP is in fact present at steady state and is generated from [^{14}C]ATP and chased out by excess unlabeled ATP with the kinetics expected for turnover of an active site at the steady-state ATPase rate. The kinetics of the initial labeling by [^{14}C]ATP are not strictly first order with a more rapid early phase. One factor is the dilution of the specific activity of the [^{14}C]ATP by the unlabeled ADP that is initially at the active site and is released during turnover (a smaller correction for this effect would also need to be applied to the results in Fig. 2). A second factor is the small burst observed in Fig. 2 that may represent a fraction of the sites without an initial ADP and that is labeled more rapidly. The kinetics of the chase of the [^{14}C]ADP by excess unlabeled ATP are not subject to these complications and are more closely, but not rigorously, first order. Fig. 4*B* shows the theoretical curve for a first-order reaction with a rate constant of 0.0082 sec^{-1}, which is essentially identical to the observed steady rate of ATP hydrolysis. The results of Figs. 2 and 4 have been observed with several independent preparations with the first-order rate constant for ADP

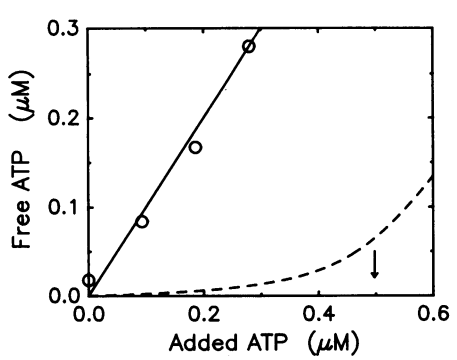

FIG. 3. Steady-state titration of free ATP. Mixture contained 0.5 μM 120-kDa peptide in ATPase buffer with pyruvate kinase (0.5 mg/ml), 1 mM phospho*enol*pyruvate, and luciferase/luciferin as described (9). Dashed line, theoretical values for 0.5 μM active sites (arrow) with a K_m of 0.01 μM; solid line, theoretical values for no tight binding sites.

Table 1. Centrifuge gel filtration

Reaction(s)	^{14}C or ^{32}P retained, mol per 120-kDa peptide
[^{14}C]ATP	
Pulse (5 min)	0.89
Pulse (20 min)	1.08
Pulse (5 min)/chase (0.5 min)	0.59
Pulse (5 min)/chase (20 min)	<0.01
[^{32}P]ATP	
Pulse (1 min)	<0.005
Pulse (5 min)	<0.005

[^{14}C]ATP or [^{32}P]ATP was added at the start of the pulse period to kinesin in buffer A to final concentrations of 0.05 μM 120-kDa peptide, 0.2 μM ATP, and bovine serum albumin (0.5 mg/ml). Carrier ATP was added to 1.0 mM at the start of the chase phase. At the indicated times the reaction mixture was applied to a precentrifuged column of Sephadex G-50-300 at 4°C and immediately centrifuged for 70 sec to elute the proteins. ^{14}C or ^{32}P was measured in samples and analyzed by $NaDodSO_4$/PAGE to correct for incomplete recovery of the 120-kDa peptide.

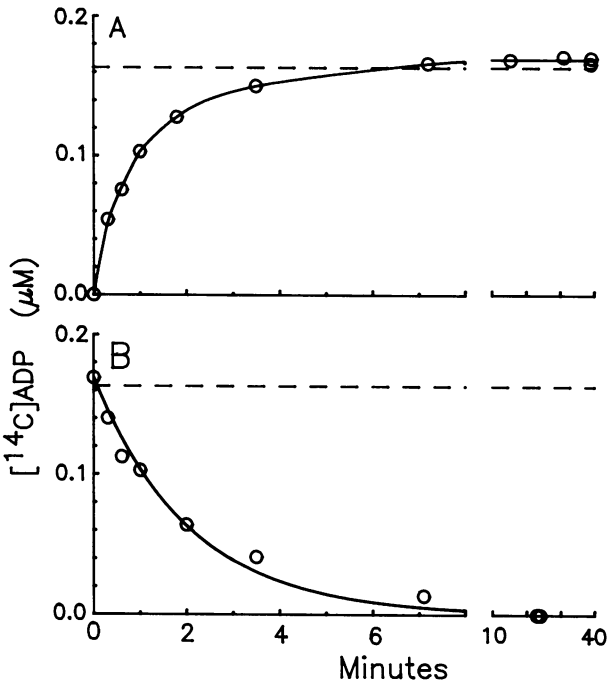

FIG. 4. (*A*) Pyruvate kinase-inaccessible [^{14}C]ADP. Kinesin at 0.163 μM 120-kDa peptide with pyruvate kinase (0.05 mg/ml) and 2 mM phospho*enol*pyruvate was mixed at zero time with [^{14}C]ATP to 1 μM (final concentration) and samples were removed for analysis of bound [^{14}C]ADP. (*B*) After equilibration, the bound [^{14}C]ADP was chased by addition of 100 mM MgATP to a final concentration of 200 μM.

release and the steady-state ATPase turnover rate being equal at 0.008–0.009 sec^{-1}.

Tubulin Stimulation. Since ADP release is the apparent rate-limiting step of the basal ATPase reaction of kinesin, it is expected that tubulin stimulation of the ATPase rate likely involves acceleration of this release rate; this was confirmed by the results presented in Fig. 5. In this experiment, the kinesin was preloaded with [^{14}C]ADP and the chase rate was determined with tubulin and excess unlabeled ATP included in the chase reaction mixture. The data are presented as a first-order semilog plot, and the results in the absence of tubulin are similar to those observed in Fig. 4. Addition of tubulin in the chase results in rapid loss of a significant fraction of the bound [^{14}C]ADP, indicating that tubulin has stimulated its rapid release. The release kinetics, however, are biphasic with the [^{14}C]ADP remaining after 0.25 min being lost approximately at the slower rate observed in the absence of tubulin. The principal effect of increasing the tubulin level appears to be to increase the fraction of the [^{14}C]ADP that is rapidly released with little effect on the kinetics of the slow phase. This indicates that the kinesin active sites are not kinetically equivalent in regard to their interaction with tubulin and that little exchange between classes of sites occurs on the time scale of Fig. 5.

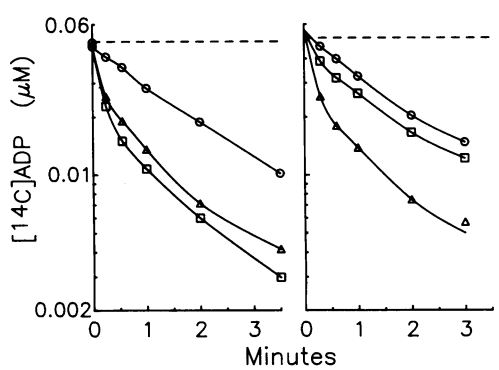

FIG. 5. Tubulin stimulation of [^{14}C]ADP release. Kinesin was equilibrated with a 4-fold molar excess of [^{14}C]ATP in the presence of pyruvate kinase (1 mg/ml) and 1 mM phospho*enol*pyruvate in ATPase buffer and the chase was initiated by addition of MgATP and tubulin to final concentrations of 0.9 mM ATP and 0 (○), 0.91 (△), and 2.7 (□) μM tubulin (*Left*) or 1.8 mM MgATP and 0 (○), 0.061 (□), and 6.1 (△) μM tubulin (*Right*). Dashed line, final concentration of 120-kDa peptide. Specific activity of [^{14}C]ATP was corrected for dilution with ADP bound to kinesin.

One factor that might slow ADP release in the presence of tubulin is that some or all of the active sites may not be able to directly interact with tubulin until after releasing their bound ADP by the unstimulated pathway or some other hysteretic process. This would predict a lag in the onset of steady-state ATP hydrolysis on addition of tubulin with a limited initial rate increasing over several minutes as the remaining kinesin molecules became activated. Fig. 6 indicates that a lag phase of this type is not observed on addition of tubulin. Even the short observed lag of ≈4 sec in the onset of coupled NADH oxidation is mainly due to the time required for mixing and closing the spectrophotometer lid and for the response times for the spectrometer and the coupled enzymes pyruvate kinase and lactate dehydrogenase. The observed initial ATPase rate is 4.9 sec^{-1} per 120-kDa peptide, and this rate was maintained unchanged during the remainder of the 14-min period before exhaustion of the NADH (data not shown). The lack of any further increase in steady-state rate over the time scale of turnover of the slow sites in Fig. 5 indicates that the population releasing ADP slowly in Fig. 5 is not subsequently recruited into the pool of sites stimulated by tubulin. This is consistent with tubulin being able to bind directly to one population of kinesin–ADP complexes to form ternary complexes that release ADP rapidly, while a second population of kinesin–ADP complexes is not stimulated by tubulin to rapidly release ADP.

DISCUSSION

The results presented here are consistent with ATP binding, hydrolysis, and P_i release being rapid and with the rate-limiting step being the subsequent slow release of ADP that can be accelerated by interaction with tubulin. The rapid

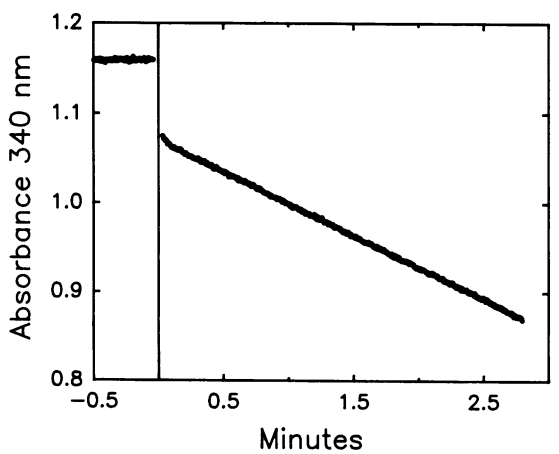

FIG. 6. Spectrophotometric ATPase rate. Reaction was initiated by addition of kinesin to a rapidly stirred cuvet containing tubulin to give final concentrations of 0.039 μM 120-kDa peptide, 3 μM tubulin, 1 mM MgATP, 1 mM phospho*enol*pyruvate, 0.2 mM NADH, pyruvate kinase (0.13 mg/ml), and lactate dehydrogenase (0.2 mg/ml). Kinesin was preincubated with 0.6 μM MgATP, 1 mM phospho*enol*pyruvate, and pyruvate kinase (0.05 mg/ml) before addition to the cuvet.

release of P_i is indicated for the basal reaction by the failure of the enzyme to retain P_i during passage through a centrifuge gel-filtration column and by the virtual lack of extra oxygen exchange during the tubulin-stimulated hydrolysis reaction. The tight ATP binding and rapid hydrolysis are indicated by the low K_m for ATP and by retention of bound ADP at the active site during the basal ATPase reaction as shown by both centrifuge gel filtration and analysis of pyruvate kinase-inaccessible ADP.

The lack of a pronounced burst or of a depletion of free ATP in a steady-state titration in spite of the low K_m for ATP and rate-limiting ADP release at first appears inconsistent with the above model but can be accounted for by the discovery that the enzyme as isolated already contains a tightly bound ADP at its active site. Thus no burst is observed because most of the active sites already contain a bound ADP and cannot bind a new ATP from the medium until this ADP is released at the steady-state rate. The K_i for ADP is <5 nM and this accounts for its retention through the final steps of the preparation, which were designed in part to ensure elimination of nucleotide present earlier in the procedure. This includes binding of the kinesin to a phosphocellulose column with a buffer wash to remove essentially all of the free nucleotide and then by an overnight dialysis with one change of buffer.

The mechanism of kinesin is thus broadly similar to that of myosin with the rate-limiting step, presumably a conformational change, occurring after hydrolysis. The principal difference is that with myosin this occurs before release of either product whereas with kinesin P_i release occurs before the rate-limiting step leading to ADP release. Even this difference is only qualitative since with myosin as well the release of P_i after the rate-limiting step is faster than the release of ADP, and ADP release can become partially rate-limiting at low temperature. The binding of ATP is tight with a K_m for the basal reaction of 10–30 nM for myosin (9) and an even lower value for kinesin. In both cases, interaction of the ATPase with its filament partner (actin or tubulin) stimulates the rate of product release resulting in an increase in ATPase rate and also a weakening of net ATP binding in an antagonistic manner with the K_m values for ATP of the stimulated reactions increasing into the micromolar range for both enzymes.

The reason for the biphasic kinetics of tubulin stimulation of ADP release observed in Fig. 5 is unclear. It may represent an intrinsic heterogeneity of the kinesin active sites, either as different populations of kinesin molecules or possibly as differences between the sites in each oligomer that interact in a negatively cooperative manner. In this regard, it is interesting to note that the fraction of the sites releasing ADP rapidly in the presence of 3–6 μM tubulin is $\approx$50%. Steric complications, perhaps in small aggregates of kinesin and possibly involving processive behavior, could also account for the heterogeneity if they prevented simultaneous productive access of all the kinesin active sites to microtubules. It is also possible that there are two active sites in the oligomer per 120-kDa peptide with one site having slow ADP release and being responsible for the majority of the ATPase flux of the basal reaction, while the other site has an even lower basal ATPase rate but is more highly tubulin stimulated and dominates the ATPase flux in the presence of tubulin. In particular, analysis at shorter times of the fast phase of ADP release is required to determine whether this release is rapid enough to account for the steady-state ATPase rate in the presence of tubulin. This heterogeneity and its possible variation with source and isolation procedure may be related to the failure to obtain high rates of tubulin-stimulated ATPAse with preparations obtained by affinity purification in the presence of adenosine 5′-[β,γ-imido]triphosphate. These slow ligand-release steps and heterogeneity may also be related to the slow recovery of movement observed on replacement of adenosine 5′-[β,γ-imido]triphosphate with MgATP (13). Regardless of these complications in the detailed nature of tubulin stimulation, however, it is clearly established here that the rate-limiting step of the unstimulated kinesin ATPase is ADP release and that tubulin accelerates the release of this ADP from a significant fraction of the sites.

The technical assistance of K. W. Wright, M. Carrozza, and L. A. Schick and the gift of taxol by Dr. M. Suffness are gratefully acknowledged. This research was supported by U.S. Public Health Service Grant AR25980 and an Established Investigatorship from the American Heart Association.

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