Pathway of the Microtubule-Kinesin ATPase

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ABSTRACT We have established pathway of the kinesin ATPase by direct measurement of each step in the pathway. Kinesin binds to microtubules with an 8-nm repeat and a stoichiometry of one kinesin monomer unit per tubulin dimer. Thus, the dimeric kinesin binds with both heads attached to the microtubule and on adjacent tubulin subunits. In the steady state, kinesin has a low ATPase activity that is limited by the rate of ADP release (<0.01 s^{-1}) in the absence of microtubules and is activated 2000-fold by the addition of microtubules to achieve a maximum rate of ~20 s^{-1}. Transient-state kinetic analysis has provided direct measurement of individual steps of the reaction to define the pathway of the microtubule-kinesin ATPase. These studies establish that the rate-limiting step in the ATPase pathway is the release of the kinesin-product complex (K·ADP·P) from the microtubule following ATP hydrolysis. After phosphate release, the rebinding of kinesin-ADP to the microtubule is fast, accounting for the high activation of the ATPase at low microtubule concentration. This ATPase cycle explains the phenomenological differences between myosin and kinesin observed in motility assays. Kinesin remains associated with a microtubule through multiple rounds of hydrolysis, because it spends only a small fraction of its duty cycle in the dissociated state. The discussion of this paper will focus on the new data, their interpretation, and significance for mechanisms of force production. The ATPase coupling mechanism will be compared with dynein and myosin.

INTRODUCTION

Our studies are based upon the direct measurement of each step in the microtubule-activated kinesin ATPase pathway; namely, ATP binding, ATP hydrolysis, phosphate release, ADP release, dissociation of the microtubule-kinesin complex, and rebinding of kinesin to the microtubule. For these studies, the *Drosophila* kinesin motor domain, consisting of the 401 N-terminal amino acids of the heavy chain, was expressed in bacteria and purified (Gilbert and Johnson, 1993). The protein forms a dimer in solution, retains a low ATPase activity (0.01 s^{-1}), and is fully activated by microtubules (2000-fold to a rate of 20 s^{-1}) (Gilbert and Johnson, 1994) and binds to microtubules with an 8-nm repeat (Harrison et al., 1993). Thus, the bacterially expressed head domain represents a biologically relevant and fully characterized protein preparation useful for mechanistic studies.

STRUCTURE OF THE MICROTUBULE-KINESIN COMPLEX

We first established the structure of the complex between kinesin (K401) and microtubules by electron microscopy of frozen, hydrated specimens (Harrison et al., 1993). Titrations based upon sedimentation of the microtubule-kinesin complex showed that the microtubule surface lattice was saturated at a ratio of one kinesin head domain (one 45-kDa unit) per tubulin dimer. Visual inspection of electron micrographs revealed an apparent 8-nm repeat of the kinesin along the microtubule lattice. However, this visual inspection alone does not rigorously establish the binding lattice. Rather, optical diffraction analysis of the micrographs revealed a strong 8-nm layer line in addition to the 4-nm tubulin layer line. This analysis and the absence of a 12-nm layer line established that the kinesin bound with a pure 8-nm repeat and not a mixture of 8- and 4- or 12-nm repeats that would occur with a staggered binding. Accordingly, the kinesin heads lie along the three-start microtubule lattice. Complex models for the interaction of kinesin with the microtubule surface lattice can be eliminated and the binding occurs simply by the interaction of each kinesin head with one tubulin subunit. Subsequent analysis has suggested that the kinesin binds only to the β-subunit (Song and Mandelkow, 1993), but those data do not exclude binding to the α-subunit, and binding to both subunits is possible.

KINESIN ATPASE PATHWAY

We have used transient-state kinetic analysis to measure each step in the pathway starting with the microtubule-kinesin complex. These studies lead to the pathway and set of rate constants as shown in Scheme 1, describing the most simple interpretation of all available data (Gilbert et al., 1995). The binding of ATP to the M·K complex is weak (K_d = 100 μM) due to the rapid dissociation of ATP from the kinesin while bound to the microtubule. ATP hydrolysis occurs at a rate of 100 s^{-1} while the cross-bridge is still attached to the microtubule. It is possible that an isomerization of the M·K·ATP complex precedes hydrolysis, but such a conformational change has not been detected in the present studies. Following ATP hydrolysis, release of kinesin from the microtubule and release of phosphate occur at the same rate (20 s^{-1}), which is equal to the maximum steady-state turnover rate. We interpret these observations in terms of a rate-limiting release of the K·ADP·P intermediate from the microtubule, followed by fast release of phosphate. The cycle

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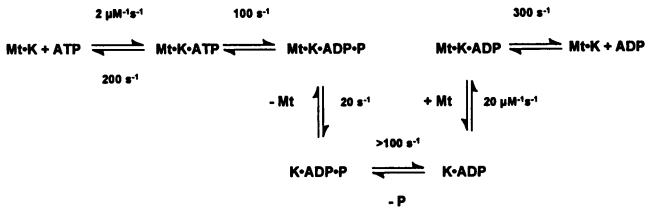
is then completed by the rebinding of microtubules to the K·ADP species and the fast release of ADP from M·K·ADP.



SCHEME 1 Pathway of the microtubule-kinesin ATPase. M represents microtubules, and K represents kinesin. The pathway and rate constants are listed for those steps that have been measured. The mechanism accounts quantitatively for the overall kinetic parameters of the ATPase.

It is important to note that the pathway accounts for the phenomenology observed in motility assays, in particular the apparent processivity of the kinesin in its tendency to continue for long distances along a single microtubule without dissociation. According to our model, the rate-limiting step is release of the K·ADP·P_i intermediate from the microtubule. The kinesin then releases phosphate and rapidly rebinds to the microtubule at a rate determined by the local microtubule concentration. It is reasonable to estimate a local microtubule concentration of ~1 mM near a kinesin head following its release. Accordingly, the kinesin would rebind to the microtubule at a rate of 20,000 s^{-1}, and the lifetime of the detached state would be ~50 μs. In motility assays the kinesin is attached to a glass surface or a polystyrene bead so that it cannot diffuse away from the microtubule on this short time scale. Therefore, we can account for the apparent processivity without the need to invoke a hand-over-hand mechanism of force production, as discussed further below.

It is important to consider whether the ATPase rates and observed motility rates can be reconciled with the estimated step size of 8 nm (Svoboda et al., 1993). Our truncated construct of kinesin, when attached to a flexible linker, moves beads at a rate of 400 nm/s. Accordingly, the 8-nm step size would require an ATPase rate of 25 s^{-1}/head to produce the observed rate of movement. Thus, the observed ATPase rate and the 8-nm step size are consistent with the observed motility.

Our model also reconciles the confusion surrounding the observations with the nonhydrolyzable ATP analog, AMPPNP. The initial observation that AMPPNP stimulates association of kinesin with the microtubule was taken to suggest that ATP binding might stimulate the formation of a M·K·ATP complex via a mechanism opposite that described for actomyosin. In previous studies on actomyosin and dynein, AMPPNP has not proven to be a good model for ATP in terms of the free energy of nucleotide binding. Thus, further work on AMPPNP will be required to obtain quantitative answers to the puzzles posed by its use in motility experiments. However, the current model seems to suggest that AMPPNP may simply lock the kinesin in a bound state, since ATP hydrolysis is required to precede microtubule release.

Force production in the pathway is most likely to occur in the attached state with the longest lifetime, namely, the steps associated with ATP hydrolysis prior to microtubule release. It also is possible that force production occurs with the release of ADP, but this is deemed less likely because of the short lifetime of the M·K·ADP state.

The microtubule-kinesin pathway differs from actomyosin and dynein in ways that are significant for the biology of these motor systems. Table 1 summarizes the differences in the rates of dissociation and reformation of the filament-cross-bridge complex for actomyosin and the microtubule-kinesin systems. The values for dynein (not shown) are similar to those reported for myosin (Johnson, 1985). Both myosin and dynein operate in ordered arrays. Thus, it is significant that in these systems the lifetime of the attached state after force production is quite short (~0.5 ms) compared with kinesin (50 ms). Under conditions of maximal actin activation, most of the myosin remains dissociated, an observation consistent with the concerted force production by multiple motors in an ordered array. In contrast, kinesin spends most of its duty cycle associated with the microtubule filament, consistent with its role as a lone motor pulling its cargo through the cytoplasm with only a few helpers. The apparent K_d for kinesin binding to microtubules, calculated from the ratio of the off rate divided by the on rate is 1 μM. In contrast, the apparent K_d for binding actomyosin at comparable salt concentration is 0.2 M. Thus, there is a 2 × 10^5-fold difference in the tendency of the kinesin to stay associated with the microtubule during force production. This accounts for the higher processivity seen in motility assays.

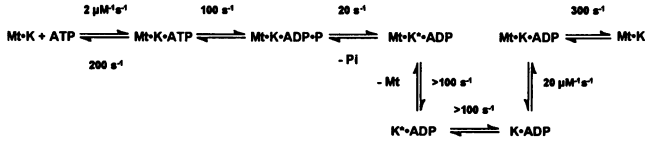
ALTERNATIVE MODEL

Although the pathway shown in Scheme 1 is the most simple to account for the available data, an alternative pathway is possible. Our results have shown that phosphate release and the dissociation of kinesin from the microtubule occur at the same rate and with no lag, implying that one step is fast following the other. In Scheme 2 below we consider the alternative hypothesis, which suggests that phosphate release precedes dissociation of the kinesin from the microtubule (see Scheme 1 for comparison). This model is attractive because it allows an additional step involving phosphate release as a potential site for the power stroke. However, this pathway is less desirable because it requires that there be a new K*·ADP state, for which there is no direct evidence. Moreover, phosphate release could still be thermodynamically

TABLE 1 Comparison of myosin and kinesin ATPase kinetics

Motor	Binding rate (M^{-1} s^{-1})	Dissociation rate (s^{-1})	Ratio: off/on (μM)
Kinesin	2 × 10^7	20	1
Myosin	1 × 10^4	2000	200,000
Kinesin is...	2000× faster	100× slower	200,000× bound

coupled to force production via Scheme 1. The first rule of kinetic analysis is to propose only those intermediate states for which there is direct evidence. Thus, for the model shown in Scheme 2 to be taken seriously, direct evidence for the postulated K*·ADP state must be obtained.



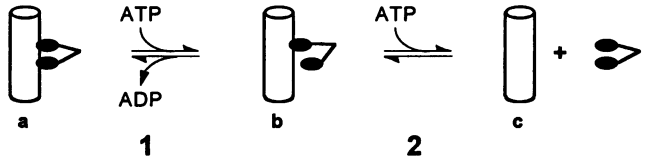
SCHEME 2 Alternative pathway of the microtubule-kinesin ATPase.

PROCESSIVITY OF THE KINESIN ATPASE

Processivity has been defined for DNA polymerases as the number of base pairs incorporated into growing DNA per encounter of the enzyme with the DNA primer-template (Johnson, 1993). Mechanistically, DNA polymerase processivity can be understood by the kinetic partitioning of the E·DNA complex and defined as the ratio of the rate of DNA polymerization divided by the rate of release of the enzyme from the DNA. Similarly, processivity of kinesin has been inferred in motility assays by the observation that kinesin tends to remain associated with the microtubule for long distances. However, the mechanistic basis for kinesin processivity may be somewhat more complex and will depend upon the ATPase pathway and the identity of the rate-limiting steps that govern continued ATP hydrolysis versus release of the kinesin from the microtubule.

Hackney (1994) has reported that only ~50% of the ADP is released from the kinesin following the binding of K·ADP to microtubules. Although this observation could simply be due to heterogeneity in the preparations or half-inactive protein, Hackney has argued that this observation implies that only one head of the kinesin can bind to the microtubule. To explain his results, he has postulated that the kinesin may walk along the microtubule in a hand-over-hand fashion. This large leap in interpretation from a single observation requires several additional hypothetical elements in the pathway that remain to be established. For example, to overcome the slow observed release of the ADP from the second head, one must postulate that ATP binding to the first head stimulates release of ADP from the second head. Moreover, his observations are contrary to our observed binding stoichiometry and our measurements of the ADP release kinetics.

As we have argued above, the majority of the observed processivity can be explained as being due to the relatively slow dissociation and fast formation of the microtubule-kinesin complex. Moreover, in motility assays, the kinesin is not free to diffuse away from the microtubule following its release from the surface lattice, and so it would tend to rebind and continue to interact with the microtubule, even if both heads of kinesin were to be released. A similar mechanism could operate in vitro and lead to processive vesicle movements. However, an additional mechanism could contribute to processive ATP hydrolysis due to the kinetic partitioning of the intermediate state (b) shown in Scheme 3 with one head bound to the microtubule. The kinetics of release of kinesin from the microtubule have indicated a sequential release of the kinesin heads as shown. Accordingly, processivity could be mechanistically defined as the probability of rebinding of the dissociated head relative to the rate of release of the bound head from the intermediate state (b).



SCHEME 3 Sequential kinesin head release and processive ATP hydrolysis.

If we defined k_1 as the rate of ATP binding and hydrolysis leading to the release of the first head and k_2 as the rate of release for the second head, then the rate of rebinding of the intermediate state, k_{-1} would govern the processivity. That is, the number of ATP molecules hydrolyzed per head per encounter of the kinesin with the microtubule would be defined by $k_{-1}/(k_{-1} + k_2)$. According to this model, processivity can then be understood in terms of the elementary steps of the pathway and quantified according to rate measurements.

One measure of processivity would be to determine the number of ATP molecules hydrolyzed per encounter of the kinesin with a microtubule in dilute solution. Under our conditions of moderate salt concentration (150 mM), it is important to note that the apparent second-order rate constant for microtubule activation of the steady-state turnover rate is identical to the rate constant measured directly for the binding of K·ADP to the microtubule (2×10^7 M^{-1} s^{-1}). This observation indicates that the K·ADP intermediate formed during net ATP turnover is the same as the K·ADP species purified with ADP bound at equilibrium. Moreover, under the conditions of our assays at moderate salt concentration, the binding of kinesin to the microtubule leads to the turnover of only one ATP per kinesin head in diluted solution. We have measured the kinetics of kinesin release and the rate and amplitude of phosphate release under these conditions and shown that the processivity is essentially unity, meaning that the rebinding of the intermediate state is slow relative to the rate of release of the second head. In fact, it was important that we perform our experiments under these conditions to measure the kinetics of release.

We have provided the first direct measurement of processive ATP hydrolysis, however, in experiments at lower salt concentration (50 mM). Under these conditions, the processivity is ~10 ATP/kinesin site, and the kinetics are consistent with a value of $k_{-1} = 200$ s^{-1}. Thus, these results open up the possibility that kinesin may proceed in a hand-over-hand fashion along the microtubule such that at least one head is nearly always contacting the microtubule surface.

MECHANISMS OF FORCE PRODUCTION

With an available crystal structure of myosin, we are coming closer to understanding the molecular details of the mechanism of force production. For myosin, it appears that a conformational change in the head is induced by ATP binding and then relaxed during ADP release to produce a force for movement, and a similar mechanism may operate for dynein. The kinesin ATPase may produce force by a similar mechanism, namely by coupling a change in protein structure to the interactions occurring at the nucleotide binding site. For example, in all three cases (myosin, dynein, and kinesin), the motors operate via a mechanism requiring a flexible linkage of the globular motor domain to its cargo. It has been argued that this flexible linkage is required to get unidirectional force from an ATPase cycle involving symmetrical conformational changes (Johnson, 1985). However, in the case of kinesin, the force is possibly produced at a different step of the cycle, following ATP hydrolysis and preceding the release of the kinesin from the microtubule as shown in Scheme 1. However, the slow release and fast rebinding open up the possibility that the fundamental mechanism of force production may involve an alternating activity of the two kinesin heads along the microtubule surface. It remains to be established how this cycle can be modulated to produce force in opposite directions.

Although we have established the elementary steps of the ATPase cycle and the pathway of coupling ATP hydrolysis to force production, the questions pertaining to the molecular details of the coupling pathway are by no means settled. The next few years promise to offer exciting new discoveries that will illuminate the mechanistic basis for kinesin-driven motility.

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DISCUSSION

Session Chairperson: Steven M. Block
Scribe: Kevin Hacker

YALE GOLDMAN: With the sequential release of two steps in series at 20/s, I think you would expect to see a lag in your turbidity signal when you mix in the ATP. Do you see that?

KENNETH JOHNSON: No, we don't. The answer to your question is a function of whether or not the intermediate species with one kinesin head dissociated contributes to the light scatter signal. If the kinesin molecule with both subunits dissociated was the only species contributing to the signal, then we would have seen a lag followed by a rate of 20/s. What we see is a rate which is a function of the sum of both species, which suggests that they both contribute equally. I think the interpretation of the turbidity signal is open to further investigation. The fact that the phosphate release kinetics, which is a signal proportional to the release of phosphate from each of these heads, gives a 13/s rate with no lag, I think really does substantiate our interpretation.

GOLDMAN: If one head is dissociated does phosphate release from that before the other head is dissociated?

JOHNSON: We don't know. I would guess that's quite likely to be correct. If the release is 100/s from the intermediate kinesin species with one head bound to the microtubule, then that phosphate will release faster than the second head. But that may not matter. Once the kinesin is off, then the release of the phosphate can occur randomly–I would expect–and the only cooperativity I would expect we are seeing is when the two kinesin heads are next to each other on the microtubule.

EMIL RIESLER: Is the dissociation an obligatory step in the release of phosphate in the pathway or can phosphate dissociate from an attached head?

JOHNSON: Well, we would have to cross-link the kinesin to the microtubule to test that, and even then that would be a questionable experiment. The kinetics show that phosphate release and microtubule release are occurring at the same rate, and neither one with a lag. So you can take the interpretation from there. The alternative pathway with phosphate release preceding kinesin dissociation from the microtubule is possible, but it requires a new intermediate.

DAVID HACKNEY: As you know we have a model which is not like your model, in that we think the predominant form