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Delayed start-up of kinesin-driven microtubule gliding following inhibition by adenosine 5′-[β,γ-imido]triphosphate

(fast axonal transport/motor proteins)

BRUCE J. SCHNAPP*†, BRUCE CRISE‡, MICHAEL P. SHEETZ§, THOMAS S. REESE¶, AND SHAHID KHAN‖

*Department of Physiology, Boston University Medical Campus, 80 East Concord Street, Boston, MA 02118; ‡Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510; §Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63310; ¶Laboratory of Neurobiology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892; and ‖Departments of Anatomy and Structural Biology/Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461

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ABSTRACT **Kinesin is a microtubule-activated ATPase that moves objects toward the plus end of microtubules and makes microtubules glide along a glass surface. Here we investigate a remarkable effect of the nonhydrolyzable analogue of ATP, adenosine 5′-[β,γ-imido]triphosphate (p[NH]ppA), on kinesin-driven microtubule gliding. Microtubule gliding that has been blocked by rapid replacement of ATP with p[NH]ppA requires 1–2 min of exposure to ATP before microtubule gliding resumes. This latency is not shortened by prolonged washing of p[NH]ppA-blocked microtubules in nucleotide-free buffer for up to 15 min, suggesting that ATP binding to a second nucleotide binding site on kinesin triggers the release of bound p[NH]ppA. To test this hypothesis, the release of [^{3}H]p[NH]ppA from kinesin–microtubule complexes was followed in parallel biochemical assays. In nucleotide-free buffer, the bound p[NH]ppA was released over several hours from the complexes. However, addition of ATP caused the release of p[NH]ppA from the kinesin–microtubule complexes within 2 min, which was similar to the latent period for start-up of microtubule gliding after p[NH]ppA inhibition. The stoichiometry of p[NH]ppA bound per kinesin heavy chain at saturation was estimated to be ≈1:2. These results suggest a model in which each molecule of kinesin has at least two nucleotide binding sites that alternately bind nucleotide.**

Kinesin is an ATP-driven soluble translocator (1–6) thought to be involved in microtubule-based transport of cytoplasmic organelles (7). The purification of kinesin was originally based on its tight binding to microtubules in the presence of the nonhydrolyzable ATP analogue adenosine 5′-[β,γ-imido]triphosphate (p[NH]ppA) (1, 8). Although this tight binding continues to be exploited as a means of purifying kinesin and its proteolytic fragments in a variety of systems (9, 10), the mechanism of p[NH]ppA-induced kinesin binding to microtubules is unclear. As noted (11), the tight binding of kinesin to microtubules is quite remarkable, given that ATP and its analogues typically dissociate other mechanochemical ATPases, such as myosin or dynein, from actin filaments or microtubules (12, 13). On the other hand, other properties of the kinesin ATPase are quite similar to those of myosin or dynein. For example, product release is the rate-limiting step in the kinesin ATPase cycle, and this step is accelerated by microtubule binding (14). Thus, the formation of a stable kinesin–microtubule complex induced by p[NH]ppA is the only known difference between kinesin and other mechanochemical enzymes. It is therefore of interest for understanding kinesin mechanochemistry to examine the actions of p[NH]ppA on these complexes.

Although it has been shown that the steady-state velocity of kinesin-driven microtubule gliding is inhibited by p[NH]ppA (5, 15), a mechanism for the p[NH]ppA-induced inhibition has not yet been proposed, and it is still not understood how p[NH]ppA acts to promote stable kinesin–microtubule complexes. Here we report that microtubule gliding that has been blocked by rapid replacement of ATP with p[NH]ppA requires 1–2 min of exposure to ATP before gliding resumes. This latency is unaffected by prolonged washing in buffer, and correlates with the time required for ATP-induced release of [^{3}H]p[NH]ppA from kinesin–microtubule pellets. The ATP-induced release of bound p[NH]ppA is explained by a model in which each kinesin has at least two sites that alternately bind nucleotide. Alternating nucleotide and microtubule binding could serve to minimize the fraction of time that kinesin is detached from the microtubule during the mechanochemical duty cycle, explaining how single kinesin molecules might drive the gliding of microtubules (16).

MATERIALS AND METHODS

Materials and Buffers. [^{3}H]p[NH]ppA (31.1 mCi·mg^{-1}; 1 Ci = 37 GBq; 94% purity) was prepared as a custom synthesis from Amersham. All other reagents were from Sigma. PEM buffer was 80 mM Pipes, pH 6.8/1 mM EGTA/1 mM $MgCl_2$. Motility buffer (half buffer X) was prepared as described (1). Taxol was a gift from M. Suffness (National Products Branch, National Cancer Institute).

Purification of Kinesin and Microtubules from Squid Optic Lobe. Kinesin was purified in motility buffer from squid optic lobe cytosol by p[NH]ppA-induced microtubule affinity (1), followed by velocity sedimentation on 5–20% (wt/wt) sucrose gradients (17), or by antibody affinity purification, followed by gel filtration chromatography (7). Salt-washed taxol-polymerized microtubules were purified from squid optic lobe cytosol (1).

Motility Experiments. A 75-μl flow cell (18) in which microtubule gliding could be directly observed in real time by video microscopy was preincubated with kinesin (0.1–1 μM). Taxol stabilized, salt-washed (0.5 M NaCl) squid brain microtubules (60 μg/ml) in PEM buffer containing 10 μM taxol were introduced into the flow cell and allowed to settle for 10 min, and the free microtubules and kinesin were washed out with PEM buffer containing 10 μM taxol and 1–2 mM ATP. Microtubule gliding was then blocked by washing in either PEM/taxol buffer without nucleotide or PEM/taxol buffer containing 10 μM to 1 mM p[NH]ppA. Solutions were exchanged with a peristaltic pump operating at a flow rate of

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Abbreviations: p[NH]ppA, adenosine 5′-[β,γ-imido]triphosphate; BSA, bovine serum albumin.

†To whom reprint requests should be addressed.

0.5 ml·min^{-1}, which was sufficient to wash out >95% of free nucleotide in 10 sec. The exchange properties of the flow cell have been measured (18). We confirmed that >95% of the fluid volume was replaced in 10 sec at the flow rate used by measuring the velocity of microtubule gliding on kinesin-coated glass before and after replacement of 1 mM ATP buffer with nucleotide-free buffer. In 1 mM ATP, microtubules slide at nearly maximum velocity, ≈0.5 μm·sec^{-1}, while after perfusion for 10 sec the velocity decreased to <0.18 μm·sec^{-1}, consistent with an ATP concentration of ≈50 μM (19).

Centrifugation Analysis of p[NH]ppA Release. Taxol-stabilized squid microtubules (final concentration, 300 μg/ml) were added to two 9-ml samples of 0.5 μM purified squid optic lobe kinesin in motility buffer and to one 9-ml sample of motility buffer alone. All three samples contained 10 μM ATP, 100 mM KCl, and 1 mM p[NH]ppA (0.95 mCi of [^{3}H]p[NH]ppA). Under these conditions, all of the kinesin binds to microtubules by the p[NH]ppA-dependent mechanism, since 10 μM ATP by itself completely dissociates kinesin from microtubules (data not shown). Samples were incubated for 1 hr, after which microtubules or microtubule–kinesin complexes were separated from the free p[NH]ppA by centrifugation (45 min at 45,000 × *g*). The pellets were washed repeatedly in PEM buffer containing 10 μM taxol and 10 mM $MgCl_2$, and then they were resuspended in 3.5 ml of PEM buffer. At this point, ATP (5 mM) was added to one of the resuspended kinesin samples releasing all of the bound p[NH]ppA within a few minutes (see Fig. 2*B*). Microtubules in all three samples were then sedimented by centrifugation, and aliquots of the supernatant were assayed for released p[NH]ppA (2 hr after initial separation of free p[NH]ppA). The three pellets were resuspended in buffer without ATP or in buffer containing 5 mM ATP. After 2 hr more, the samples were centrifuged and the supernatants were assayed for released p[NH]ppA. This process was repeated at 2-hr intervals for 10 hr, at which time all samples were resuspended in 5 mM ATP for the duration of the experiment. These experiments were done at room temperature.

Flow Dialysis. Salt-washed, taxol-stabilized microtubules (650 μg) from squid optic lobe were added to 0.2 μM purified kinesin and 7.5–50 μM [^{3}H]p[NH]ppA in 0.75 ml of PEM/taxol buffer for 1 hr at room temperature. Kinesin–microtubule complexes, recovered as a pellet after centrifugation, were washed by two cycles (≈1.5 hr) of resuspension in 10 ml of taxol/PEM buffer without nucleotide followed by centrifugation. The washed pellets were resuspended in 0.6 ml of fresh, nucleotide-free buffer, and divided into two samples of 0.4 and 0.2 ml. The 0.2-ml aliquot was used to determine the stoichiometry of p[NH]ppA bound to kinesin heavy chain (see below). The 0.4-ml aliquot was loaded into the upper cell of a flow dialysis apparatus (20), separated by dialysis membrane from the lower cell (0.4-ml vol), which was continuously perfused with buffer (2 ml·min^{-1}). The upper cell containing the complexes was continuously stirred while fractions were collected every 2 min from the lower cell and assayed to determine the rate of dialysis of p[NH]ppA across the membrane. Control experiments indicated that >90 min was required to reduce the concentration of p[NH]ppA in the upper cell by one-half. Net loss of p[NH]ppA is not significant within the time scale of this experiment, and the rate of dialysis is a reasonable measure of the concentration of free p[NH]ppA in the upper cell (for theory, see ref. 20). The concentration of free p[NH]ppA in the upper cell (plotted on the ordinate of Fig. 2*B*) was determined by calibration of the rate of dialysis with known concentrations of p[NH]ppA in the absence of protein. The relationship was linear above 30 nM p[NH]ppA. Below 30 nM p[NH]ppA, the counts in the fractions (taken at 2-min intervals) exponentially approached background, giving an overestimation of the concentration of p[NH]ppA in the upper cell. A new, steady-state rate of elution of p[NH]ppA was reached in 2–4 min after an instantaneous change in the concentration of p[NH]ppA in the upper cell.

Stoichiometry of p[NH]ppA Binding to Kinesin. A 0.2-ml aliquot of the washed and resuspended kinesin–microtubule ([^{3}H]p[NH]ppA) pellet, identical to those prepared for flow dialysis experiments, was divided into two parts. One-half was counted for radioactivity to determine the concentration of p[NH]ppA, and the other half was run at several dilutions on a polyacrylamide gel under denaturing and reducing conditions. A series of five bovine serum albumin (BSA) samples of known mass (0.1–1 μg per lane) were run on the same gel as standards. The gel was stained with Coomassie blue and the optical density of the kinesin heavy-chain bands and the BSA bands were measured at 595 nm with a Newvicon video camera and image processing system (Image-1, Universal Imaging, Media, PA). The BSA standards typically gave a linear standard curve with a correlation coefficient of 0.95–0.99. By comparing the optical density of a Coomassie-stained gel band of squid optic lobe kinesin heavy chain (purified by electroelution from SDS/polyacrylamide gels) of known mass (from amino acid analyses performed by the Washington University protein facility) with a series of BSA bands on the same gel, the actual concentration of kinesin heavy-chain bands was determined to be 0.87 times the concentration determined from the BSA standard curves; this correction was applied to determine the concentration of the kinesin bands on the gels. Stoichiometry was measured in four separate experiments; in each, the concentration of the kinesin heavy chain was determined by averaging the values calculated from three to five bands run at different concentrations on the same gel.

RESULTS

A 75-μl flow cell, designed for laminar flow to facilitate rapid exchange (18), was used to investigate the kinetics of p[NH]ppA inhibition and subsequent ATP reactivation of microtubule gliding on kinesin-coated glass. In 1 mM ATP, microtubules glide at almost 0.5 μm·sec^{-1}, while after perfusion of nucleotide-free PEM buffer for 10 sec the velocity decreased to <0.18 μm·sec^{-1}, consistent with an ATP concentration of ≈50 μM (19). Four consecutive 10-sec washes with nucleotide-free buffer completely stopped microtubule gliding.

Microtubule gliding also stopped when ATP-containing buffer was replaced with 1–2 mM p[NH]ppA. Surprisingly, when the p[NH]ppA buffer was subsequently replaced by 2 mM ATP, a latent period of ≈1 min preceded the start-up of microtubule gliding (Fig. 1). By contrast, when microtubule gliding was completely blocked by perfusion of nucleotide-free buffer, microtubules resumed gliding immediately upon application of 2 mM ATP (Fig. 1). The latent period associated with p[NH]ppA was not simply due to passive release of the bound p[NH]ppA, because it was unaffected by previous perfusion of buffer at 0.5 ml·min^{-1} for up to 15 min. An additional experiment showed that the latent period is unlikely to be caused by trace amounts of p[NH]ppA remaining in the flow cell after the 15-min wash with nucleotide-free buffer. When microtubule gliding was blocked with 50 μM p[NH]ppA perfused into the flow cell, a latency was consistently observed following replacement of the p[NH]ppA with ATP. However, when gliding was blocked with <10 μM p[NH]ppA, no latency was observed upon addition of ATP, presumably because microtubule gliding was inhibited solely by the absence of ATP at this low concentration of p[NH]ppA.

We considered two plausible explanations to account for the latent period for the start-up of microtubule gliding after p[NH]ppA inhibition. One possibility is that p[NH]ppA binds to kinesin, changes its conformation, and then dissociates, leaving behind a kinesin that binds ATP more slowly. Alter-

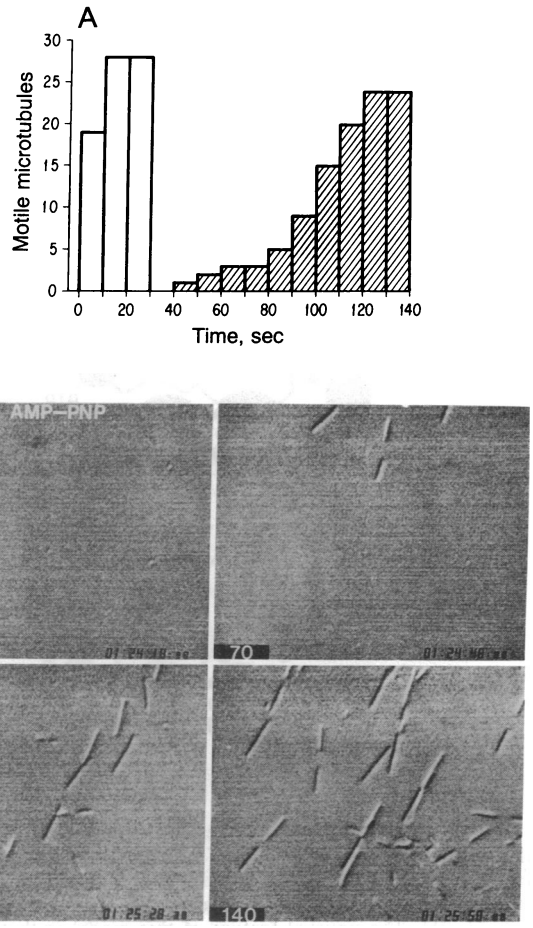

FIG. 1. Return of microtubule gliding after p[NH]ppA block. (*A*) Microtubule gliding was blocked by nucleotide-free buffer (open bars) or 1 mM p[NH]ppA (hatched bars). To initiate recovery, ATP (2 mM) was perfused for 10 sec preceding time 0, at which time exchange was >95% complete (ref. 18; see below). Data are from one experiment using the same region of the same coverslip for consecutive cycles of block and recovery. Microtubule gliding blocked by nucleotide-free buffer recovered immediately upon addition of ATP, whereas following inhibition by p[NH]ppA microtubules do not resume gliding until 40 sec after application of ATP. (*B*) Image processing technique used to determine the numbers of moving microtubules during consecutive 10-sec intervals. Video frames were stored in a frame memory every 10 sec immediately following introduction of 2 mM ATP into a flow cell containing p[NH]ppA (AMP-PNP)-blocked microtubules. Successive frames were subtracted, providing a difference image that captured only those microtubules that moved during the 10-sec intervals between successively stored frames. Difference images are shown 40, 70, 110, and 140 sec after washing p[NH]ppA-blocked microtubules with ATP. For instance, three microtubules started to move in the interval between 40 and 70 sec (the image processing yields two images of each moving microtubule—one from the present frame and one from the previous frame—but here two of the paired images are cut off by the edge of the picture). The number of moving microtubules increased exponentially with time. (×4900.)

natively, p[NH]ppA might bind one of the two nucleotide binding sites on each kinesin molecule, leading to formation of a stable p[NH]ppA–kinesin–microtubule complex; ATP binding to the second nucleotide binding site triggers a conformational change that leads to dissociation of the p[NH]ppA.

To distinguish between these two possibilities, it was necessary to follow the release of p[NH]ppA from kinesin–microtubule pellets. We therefore prepared p[NH]ppA–kinesin–microtubule complexes in the presence of [^{3}H]p[NH]ppA. To measure release of p[NH]ppA in nucleotide-free buffer (Fig. 2*A*), the complexes were separated from free p[NH]ppA by centrifugation and resuspended in nucleotide-free PEM/taxol buffer. At 2-hr intervals, the resuspended complexes were separated from the released p[NH]ppA by centrifugation, the supernatant was counted, and the microtubule pellets were resuspended in fresh, nucleotide-free buffer. In nucleotide-free buffer half of the p[NH]ppA was released in 6–8 hr (Fig. 2*A*). In parallel samples exposed to 10 mM ATP immediately after separating the kinesin–microtubule complexes from free p[NH]ppA, at least 85% of the total bound p[NH]ppA was released within the 45 min required to sediment the microtubules (Fig. 2*A*). Relative to the kinesin-containing samples, a control sample of microtubules alone bound ≈1/3rd as much p[NH]ppA. This microtubule-bound p[NH]ppA was released exponentially with time and the release did not increase significantly in response to the addition of ATP. As noted (1), release of kinesin was not detected in the absence of ATP. However, in the presence of ATP, from 50% to 95% of the kinesin was released within the 45 min required to pellet the microtubules (1). There was essentially no further release at subsequent time points; for reasons that are unclear, the fraction of kinesin that was not released by ATP was variable.

Flow dialysis measures changes in free ligand (20) more rapidly than the centrifugation method detailed above, and it was used to measure release of [^{3}H]p[NH]ppA from resuspended kinesin–microtubule complexes (Fig. 2*B*) with a time resolution that allowed better comparison to the recovery of microtubule gliding. Upon addition of ATP, half of the [^{3}H]p[NH]ppA was released within 2 min (n = 4), which is indistinguishable from the response time of the flow cell to an instantaneous change in concentration of free ligand (data not shown). Thus, the latency for release of p[NH]ppA is less than or equal to the latency for recovery of microtubule gliding.

Both the centrifugation analysis of [^{3}H]p[NH]ppA release and the flow dialysis experiments indicate that p[NH]ppA release in the absence of ATP is too slow to account for the 1-min latent period required for microtubule gliding to recover. These results argue against the possibility that p[NH]ppA could have bound and altered kinesin conformation and then have been released to leave behind an altered kinesin that would bind ATP more slowly. The results are more consistent with a second site model, in which p[NH]ppA release is accelerated by at least 2 orders of magnitude when ATP binds at the other, unoccupied nucleotide binding site (Figs. 2 and 3).

Available evidence indicates that each molecule of bovine and sea urchin kinesin has two heavy chains (110–130 kDa; refs. 6, and 21–24). Squid optic lobe kinesin shadowed on mica has essentially the same structure (data not shown). The amino-terminal domain of each heavy chain is a globular "head" containing microtubule and ATP binding sites (21–24). The structure of kinesin is thus consistent with each molecule containing two nucleotide and two microtubule binding sites, one of each per head (Fig. 3). A further test of a second site model, therefore, would be measurement of the molar ratio of bound p[NH]ppA to kinesin heavy chains.

Determination of the number of p[NH]ppA binding sites per heavy chain by equilibrium binding methods was not feasible because of a limited amount of kinesin. Furthermore, the results of such an approach would be difficult to interpret because the interactions of p[NH]ppA with kinesin and microtubules are complex. An estimate of the molar ratio of bound p[NH]ppA to the kinesin heavy chain, however, was made by washing p[NH]ppA–kinesin–microtubule pellets (prepared by incubation of kinesin and microtubules in 7.5–50 μM [^{3}H]p[NH]ppA) for 1–2 hr in nucleotide-free PEM/taxol buffer and then counting the radioactivity to determine the p[NH]ppA concentration. Gels were stained with Coomassie blue to measure the concentration of kinesin

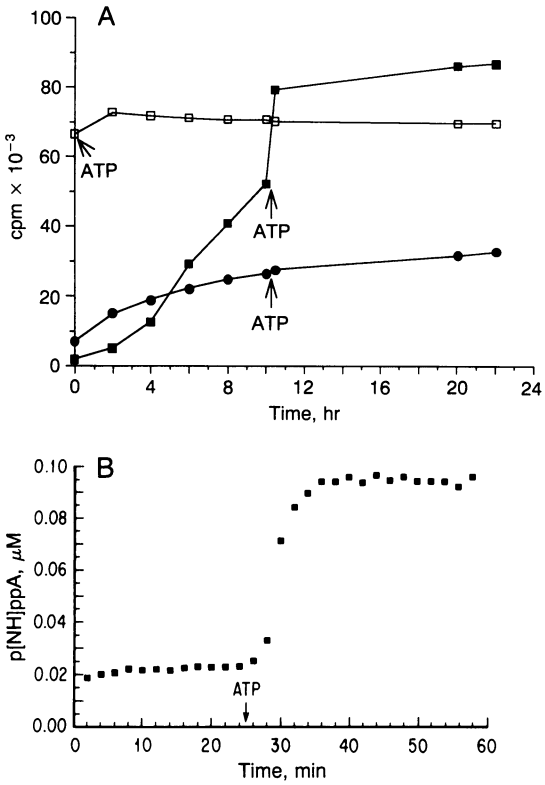

FIG. 2. Release of p[NH]ppA from kinesin–microtubule complexes loaded with [^{3}H]p[NH]ppA. (*A*) Time course of release of [^{3}H]p[NH]ppA from kinesin–microtubule complexes (squares) or microtubules alone (circles). Taxol-stabilized squid microtubules (final concentration, 300 μg/ml) were added to 9 ml of either 0.5 μM microtubule-purified squid optic lobe kinesin (ref. 1; squares) in motility buffer (half buffer X) or 9 ml of motility buffer alone (circles). Each of the three samples contained 10 μM ATP, 100 mM KCl, and 1 mM labeled p[NH]ppA (0.95 mCi of [^{3}H]p[NH]ppA). Under these conditions, all of the kinesin binding to microtubules must depend on p[NH]ppA because 10 μM ATP by itself completely dissociates kinesin from microtubules. Samples were incubated for 1 hr, after which microtubules or microtubule–kinesin complexes were separated from the free p[NH]ppA by centrifugation. The pellets were washed repeatedly in PEM buffer containing 10 μM taxol and 10 mM $MgCl_2$ and resuspended in 3.5 ml of buffer. ATP (5 mM) was added to one of the resuspended kinesin samples (open squares) releasing all of the bound p[NH]ppA within a few minutes (see *B*). Microtubules in all three samples were then sedimented, and aliquots of the supernatant were assayed for released [^{3}H]p[NH]ppA at time 0, which was actually 2 hr from initial separation of free p[NH]ppA. The three pellets were resuspended in buffer without ATP (solid symbols) or in buffer containing 5 mM ATP (open squares). After 2 hr, the samples were centrifuged and the p[NH]ppA supernatants were assayed for released [^{3}H]p[NH]ppA. This process was repeated at 2-hr intervals for 10 hr, at which time all samples were resuspended in 5 mM ATP for the duration of the experiment. Note that the ordinate denotes the cumulative counts over the course of the experiment. For each time point on this graph, the release of [^{3}H]p[NH]ppA from microtubules alone (circles) was subtracted from the release of [^{3}H]p[NH]ppA from the kinesin–microtubule complexes (squares) to obtain the rate of release from kinesin. (*B*) Time course of p[NH]ppA release measured by flow dialysis (20). Kinesin–microtubule complexes resuspended in nucleotide-free PEM buffer were exposed to ATP (final concentration, 10 mM) after 25 min. The rate of the ATP-induced release of p[NH]ppA could not be distinguished from the response of the flow dialysis system to an instantaneous change in free p[NH]ppA concentration; hence, approximately one-half of the p[NH]ppA was released in 4 min. The apparent 20% free [^{3}H]p[NH]ppA before addition of ATP is likely to be an overestimate because the counts are close to background. The free p[NH]ppA in the flow cell prior to addition of ATP is presumably due to residual free p[NH]ppA, to release from the microtubules, and to passive release from the kinesin (see *A*).

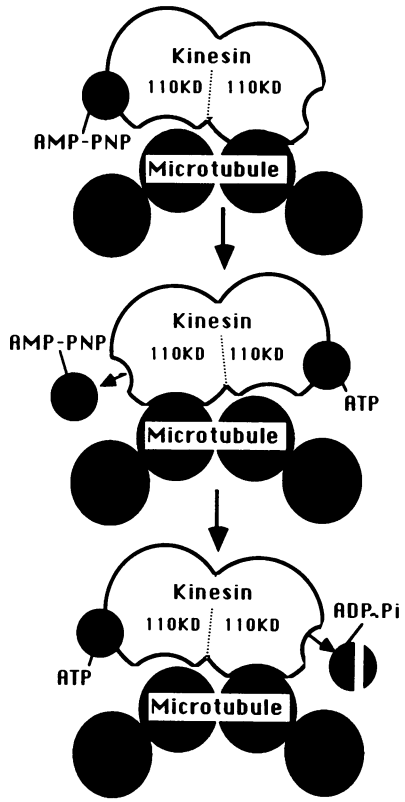

FIG. 3. Model for mechanochemical cycling of kinesin. Each of the two 110-kDa heavy chains of kinesin includes nucleotide and microtubule binding sites (21–24). Binding of nucleotide and microtubules to each heavy chain is antagonistic, as in other ATP motors (12, 13, 25). The unusual feature of this model is that nucleotide binding to a heavy chain triggers conformational changes that cause the adjacent heavy chain to bind microtubule protofilaments and release nucleotide. This interaction between heavy chains results in kinesin binding to microtubules in the presence of p[NH]ppA (AMP-PNP). p[NH]ppA is released very slowly (half-time, >6 hr) in the absence of ATP but much more rapidly when ATP is added. The 1- to 2-min latency to start up microtubule gliding after addition of ATP depends on the displacement of p[NH]ppA by ATP. Although the subunit with bound p[NH]ppA is drawn as if completely dissociated from the microtubule, this feature of the model is hypothetical. The binding of nucleotide alternately at the two sites, however, is implied by the experimental results, which suggest a model for normal cycling (*Bottom*). Microtubule binding by one head is associated with product release at that head while the other head would bind ATP and release the microtubule. p[NH]ppA would be released more slowly than ADP and P_i if it bound more strongly. The kinesin then tends to remain bound to the microtubule as it moves along it, with the attachment alternating between the two heavy chains. It should be noted that this model does not explain why kinesin moves in only one direction along microtubules.

heavy chain; a sample of purified heavy chain subjected to amino acid analysis was used as the standard. Calculated in this way, the molar ratio of p[NH]ppA/kinesin heavy chain was 1:2.4 (SE = 0.2; n = 4). The accuracy of this estimate is limited by the fact that microtubules alone bind ≈1/3rd of the p[NH]ppA and ≈15% of the kinesin-bound p[NH]ppA would have been released during the 2-hr wash period (see Fig. 2). This estimate, however, is unlikely to be very far off, since these complications would affect the calculation in nearly equal and opposite ways.

DISCUSSION

We observed that microtubule gliding previously blocked by rapid replacement of ATP with p[NH]ppA required 1–2 min

of exposure to ATP before gliding resumed. Furthermore, the latent period for resumption of microtubule gliding was unaffected by prolonged washing in nucleotide-free buffer, suggesting that p[NH]ppA release is accelerated by the binding of ATP to a second site. Analysis of the release of [^{3}H]p[NH]ppA from kinesin–microtubule pellets indicated that the release of p[NH]ppA is indeed accelerated by at least 2 orders of magnitude by binding of ATP. These observations have led us to propose a model in which there are two interactive nucleotide binding sites per kinesin, as explained in Fig. 3. This model is further supported by the observations that the rate of release of p[NH]ppA induced by ATP roughly correlates with the latent period for resumption of microtubule gliding after p[NH]ppA inhibition, and with the stoichiometry of p[NH]ppA to kinesin heavy chain of ≈1:2.

The latency to recover microtubule gliding after replacement of p[NH]ppA by ATP in the microscopy flow cell cannot be explained by simple exchange at a single binding site. The concentration of free p[NH]ppA in the flow cell at the time of ATP addition would be expected to be negligible because the washout rate from the flow cell (18) is 1 order of magnitude faster than the rates of p[NH]ppA release and rebinding. The rate of p[NH]ppA release from one kinesin binding site would be the time taken for release of p[NH]ppA upon addition of saturating concentrations of ATP, ≈2 min. Binding of p[NH]ppA to kinesin–microtubule complexes takes several minutes as measured by flow dialysis (data not shown), so that rebinding of released p[NH]ppA to sites vacated during the continuous perfusion of buffer would be unlikely. Thus, simple exchange at a single binding site would predict that p[NH]ppA bound to kinesin would be washed away during the 15 min of perfusion, so that microtubule gliding would start up immediately upon addition of ATP, which is contrary to the observed latency for start-up of microtubule gliding under these conditions.

While more complex schemes cannot be categorically ruled out on the basis of the existing data, p[NH]ppA release following a conformational change at an adjacent site is the simplest explanation and fits well with estimates of the stoichiometry of p[NH]ppA binding to kinesin heavy chain and to the known molecular structure of kinesin. Alternating nucleotide binding, coupled to rotation, has also been proposed for the energy transducing, protonmotive force ATPase (26).

The unusually tight p[NH]ppA-dependent kinesin binding to microtubules does not necessarily imply that ATP facilitates binding rather than dissociation of kinesin from microtubules, as has been suggested (11). In our model, the tight, p[NH]ppA-dependent kinesin binding to microtubules occurs because of interactions between the kinesin heads (Fig. 3). Individually, the force-generating kinesin head is postulated to behave similarly to other force-generating ATPases (e.g., myosin and dynein; refs. 12, 13, and 25) in that nucleotide binding is associated with release of the motor from the microtubule, and binding of the motor to the microtubule is associated with product release (25). Indeed, the ATPase kinetics of kinesin in solution have recently been shown to mimic myosin and dynein (14).

Recently, a single-headed kinesin fragment has been prepared by proteolysis of the intact protein (9, 10). This fragment is reported to bind to microtubules in the presence of p[NH]ppA, but this observation is not necessarily inconsistent with our model for p[NH]ppA-induced binding of kinesin to microtubules (Fig. 3) since the basic features of this model would not be changed if the head that binds p[NH]ppA remains attached to the microtubule, although with less affinity than the subunit with the vacant site. It is, of course, also possible that the head with the bound p[NH]ppA as well as the head with the vacant site both attach strongly to the microtubule, but it would then remain to be explained how nucleotide and microtubule binding to kinesin modulate each other to generate a mechanochemical cycle. Nevertheless, it would be of interest to determine whether single-headed kinesins attached to microtubules with p[NH]ppA are released by ATP, and, if so, how the kinetics of release differ in the presence and absence of ATP. It is also possible that the isolated heads would behave differently than the individual heads in the intact molecule.

Although the data presented here deal only with the effect of p[NH]ppA on the interaction of kinesin with microtubules, the results suggest that the two heavy chains could cycle out of phase with each other during the normal mechanochemical cycle (Fig. 3 *Bottom*). If the release of ADP were considerably faster than the release of p[NH]ppA then the cycling could be fast enough to enable the observed kinesin-driven transport along microtubules. The strategy of alternating binding of the paired kinesin heads to the microtubule could minimize the fraction of the duty cycle in which kinesin is detached from the microtubule. Such behavior might enable a single kinesin to move a microtubule, as recently observed (16), and could minimize the number of kinesin molecules needed to keep an organelle attached to a microtubule. Vesicle movement, like microtubule gliding, is presumably unloaded, so the efficiency of organelle transport would increase as the number of kinesin units decreases, while the speed of gliding remains the same.

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