Alternating Site ATPase Pathway of Rat Conventional Kinesin*

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The pathway of ATP hydrolysis by rat kinesin was established by pre-steady-state kinetic methods. A 406-residue long N-terminal fragment was shown by sedimentation equilibrium analysis to form a dimer with a $K_d$ of 46 nm. The pathway of ATP hydrolysis follows the Gilbert-Johnson pathway determined previously for a similar-sized N-terminal fragment of Drosophila conventional kinesin. However, the rates of ADP release were at least 3-fold faster, and ATP hydrolysis was ~5-fold faster. Paralleling our previous mechanistic data, these results support an alternating site ATPase pathway, including a captive head state as an intermediate in the kinesin ATPase cycle. The kinetic data presented in this report once again point to the importance of the captive head state and argue against a pathway that short-circuits this key intermediate. In addition, several unique aspects of the rat kinesin kinetics reveal new aspects of the ATPase-coupling mechanism. These studies provide a baseline set of kinetic parameters against which future studies of rat kinesin mutants may be evaluated and directly correlated with the structure of the dimeric kinesin.

Conventional kinesin is a plus-end-directed microtubule motor driving fast axonal transport of vesicles. Kinesin superfamily genes are represented in every eukaryotic genome examined, ranging in number from 6 genes in Saccharomyces cerevisiae to 45 in the human genome (1, 2). Members of this family are implicated in the transport of vesicles and organelles (3), as well as a variety of other microtubule-dependent processes, such as meiotic and mitotic chromosome movement (4), the maintenance and function of cilia and flagella (5), and the regulation of microtubule dynamics, as in the case of mitotic centromere-associated kinesins (6).

Kinesin is a dimer of mutually entwined $\alpha$-helices forming a coiled-coil structure, flanked on both ends with N- and C-terminal globular domains (7). The N terminus forms a globular motor domain of conserved sequence and structure, responsible for microtubule binding and ATP hydrolysis. Structures of the motor domain of rat conventional kinesin in both dimeric and monomeric forms containing ADP have been solved (8, 9), but subtle structural differences between the two heads suggest different conformational states that may be related to motility.

Conventional kinesin is a processive motor; for example, Drosophila kinesin dimers hydrolyze ~100 molecules of ATP before dissociating from the microtubule (10–12). Processivity is maintained by an alternating site ATPase pathway, in which one head of kinesin remains tightly associated with a microtubule while the other rapidly diffuses to the next binding site (13–15). Kinesins appear to achieve motility via a hand over hand mechanism, in which the two microtubule-binding heads of the protein alternate in relative position, advancing the molecule by 8 nm, the distance spanned by one $\alpha\beta$-tubulin dimer, with each step (16–19). This model is supported by structural studies of fixed kinesin/microtubule complexes, x-ray crystallography of monomeric and dimeric kinesin, spectroscopic analysis of kinesin dynamics using fluorescent and spin-label probes, physical measurements of kinesin force-generation using laser-trapping, and kinetic analysis of kinesin ATPase activity (14, 15, 20–23).

Despite extensive analysis by a variety of techniques, there remains some uncertainty concerning the enzymatic pathway by which processive motility is achieved, and the nature and order of the conformational changes. Taking advantage of rapid, transient-state kinetic analysis of Drosophila conventional kinesin a detailed model of kinesin ATPase emerged, based upon measurements of the rate and order of each step in the pathway (10, 11, 14, 15, 24–26). Similar studies using human kinesin (27–30) have produced a similar ATPase cycle with kinetic parameters that differ somewhat, especially with the extent to which ADP release may be partially rate-limiting. Data on each of the kinesin/microtubule ATPases provide evidence for the central role of a “captive head” state in the ATPase cycle where a nucleotide-free head is attached strongly to the microtubule while the other head retains ADP and interacts only weakly with the microtubule. Evidence for the alternating site ATPase pathway relies upon the observed effect of ATP binding to the open site stimulating the release of ADP from the second site. It can be argued that all of the data in support of an alternating site ATPase stem from experiments that are dependent upon the captive head state. In contrast, based upon purely structural and equilibrium data, Rice et al. (31) proposed a truncated model in which the captive head state is bypassed during processive movement.

One drawback of our kinetic studies using Drosophila kinesin is the absence of an x-ray crystal structure for this molecule. Given the potential utility of a crystal structure for structure-function relationship analysis, in which interactions between residues appearing in the crystal structure might be targeted for mutagenesis, it is unfortunate that one of the best-understood and most thoroughly characterized conventional kinesins has proved refractory to crystallization. Because Rattus norvegicus kinesin is the only source that has yielded the structure of the dimeric form of kinesin, we have turned our attention to rat kinesin for more detailed structure/function studies. In the present study, we examined the transient state kinetics of the ATPase hydrolysis cycle of a 406-residue N-terminal fragment of rat conventional kinesin. The results of this study establish the ATPase pathway for another kinesin whose structure is known and provide a standard against which the kinetics of rat kinesin mutants can be compared in the accompanying paper (32).

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli BLR(DE3) was obtained from Novagen Inc. (Madison, WI). E. coli XL1-Blue was from Stratagene (La Jolla, CA).

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QIAPrep Plasmid DNA extraction kits were purchased from Qiagen Inc. (Valencia, CA). QuikChange site-directed mutagenesis kits were purchased from Stratagene. Bio-Rex 70 resin (70- to 150-μm wet bead size) and Bio-Gel P-4 Gel (45- to 90-μm particle size) were from Bio-Rad Laboratories (Hercules, CA). DEAE-, Q-, and S-Sepharose Fast Flow chromatography resins were from Amersham Biosciences. Taxol (paclitaxel) was purchased from Sigma Co. Radiolabeled ATP (α-32P]ATP, >3000 Ci/mmol) was from PerkinElmer Life Sciences, and N-methylisothio cyanide and N-[2-(1-maleimidyl)ethyl]-7-diethyl- ylaminocoumarin-3-carboxamide (MDCC) were from Molecular Probes (Eugene, OR). Polyethyleneimine-cellulose F TLC and silica gel 60 F254 plates (EM Science) were purchased from VWR Scientific (West Chester, PA). Other chemicals were from Sigma or Fisher Scientific.

**Media and Buffers**—The following media and buffers were used for the experiments described: Buffer A (30 mM HEPES, pH 7.2, 4 mM MgCl₂, 0.1 mM EDTA, 20 μM ATP); Buffer B (50 mM Tris-HCl, pH 8.2, 4 mM MgCl₂, 0.1 mM EDTA, 20 μM ATP); ATPase Buffer (40 mM HEPES, pH 7.2 with KOH, 5 mM magnesium acetate, 50 mM potassium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol); and PM buffer (100 mM Na-PIPES, pH 6.6, 4 mM magnesium acetate, 1 mM EGTA). The pH of each buffer was adjusted at the temperature at which it was to be used.

**Data Analysis**—Thin-layer chromatography of radiolabeled nucleotide measured formation of ATPase hydrolysis product, which was quantified using a Storm 860 PhosphorImager and ImageQuaNT software (Amersham Biosciences). Linear and nonlinear regressions to kinetic data were performed using Excel (Microsoft Corp., Redmond, WA) and GraFit (Ehrithacus Software, Horley, Surrey, UK). Graphs were prepared using GraFit. Simulations of kinetic data were performed using the kinetic simulation software KinTekSim (33, 34) from KinTek Corp. (Austin, TX, www.kintek-corp.com).

**Construction of pKHC407A**—The plasmid used in the expression and purification of an N-terminal fragment of rat conventional kinesin heavy chain was derived from pKHC406 (35), a gift from Scott Brady (University of Texas Southwestern Medical Center, Dallas TX). The C-terminal polyhistidine tag encoded in the plasmid was removed by substitution of the non-fusion kinesin in the open reading frame of the plasmid with a nonsense codon, using site-directed mutagenesis (36). Kinesin mutants were likewise generated using PCR-mediated codon substitution. Oligonucleotides for site-directed mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA). For removal of the polyhistidine tag, the complementary primers 5′-CACCTGTGTTGACTAGCTTGCGGCCGCAC-3′ and 5′-GTGGGCGCGCAAGCTAGTCAACCAGGTG-3′ were used. The resulting plasmid, pKHC407A, expresses an N-terminal fragment of rat conventional kinesin terminating at asp407. A QuikChange site-directed mutagenesis kit (Stratagene), was used with a GeneAmp PCR System 2400 (PerkinElmer Life Sciences) for polymerase chain reaction (37). Temperature cycles were as recommended by the kit manufacturer: 95 °C (30 s), 55 °C (1 min), and 68 °C (6 min) for 16 cycles.

**Expression of Kinesin**—The pKHC406 plasmid and its derivatives are based on the isopropyl 1-thio-D-galactopyranoside-inducible pET expression vector (38–40). Colonies of transformed E. coli BLR(DE3) were grown overnight with shaking at 37 °C in 2 liters of LB broth plus 1% dextrose, 50 μg/ml kanamycin, and 10 μg/ml tetracycline. Induced colonies were centrifuged and pellets were frozen at −80 °C.

**Purification of Kinesin**—Kinesin preparations were kept on ice or at 4 °C. Cells from 4-liter-induced culture were resuspended in 50 ml of Buffer A plus 95 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.15 μg/ml leupeptin, 0.25 μg/ml lysozyme. The suspension was disrupted using a Branson Sonifier Model 450 for 4 bursts on ice, power setting 4, 20 s/burst. The lysate was then cleared by centrifugation (Beckman JA-25.50 rotor, 23,000 × g, 30 min), and loaded onto a 34-ml BioRex70 column (2 cm × 17 cm) pre-equilibrated with Buffer A plus 95 mM NaCl. The column was washed with 3 column volumes of the equilibrium buffer. Elution of protein was performed using a 4-column volume gradient spanning 95–485 mM NaCl, and 4-ml fractions were collected. The fractions were evaluated by SDS-PAGE, and kinesin appeared as an abundant ~45.5-kDa protein. Fractions were pooled and dialyzed for 2 h against 1 liter of Buffer B plus 95 mM NaCl. The dialyzed kinesin was loaded onto a 10-ml Q-Sepharose column (2 cm × 5 cm) pre-equilibrated with the same buffer. After a 3-column volume wash, kinesin was eluted with a 6-column volume gradient from 95 to 475 mM NaCl, and fractions were collected. Those fractions in which a ~45.5-kDa protein was predominant were selected and pooled. Pooled fractions were dialyzed twice for 2 h against 1 liter of Buffer A plus 40 mM NaCl. After dialysis, the preparation was loaded onto a 3-ml Q-Sepharose column (0.8 cm × 3.75 cm), pre-equilibrated with the same buffer. Elution was done with a 3-column volume gradient from 40 to 240 mM NaCl. Those fractions containing the 45.5-kDa protein were dialyzed twice for 2 h against ATPase buffer plus 0.1 μM ATP. Aliquots of purified protein were snap-frozen in liquid N₂ and stored at −80 °C. Before use, thawed kinesin aliquots were centrifuged (Heraeus Biofuge, 15,000 × g, 10 min) to remove debris. Active kinesin concentration was determined by measuring the time-dependent dissociation of kinesin-bound ADP as described (41).

**Mammalian Brain Tubulin and Microtubule Preparation**—Tubulin was extracted from bovine brain tissue by exploiting the temperature-dependent and reversible polymerization of the protein into microtubules (42, 43), and microtubule-associated proteins were then removed by DEAE-chromatography (44, 45). Microtubules were stored at −80 °C as centrifuged pellets. On the day of each experiment, pellets were thawed and resuspended in PEM buffer plus 1 mM GTP to a concentration of 10–15 mg/ml and depolymerized on ice for 20 min. Taxol was added stepwise to concentrations of 0.2, 2, and 20 μM, with 10-min incubations at 34 °C subsequent to each addition. The solution was then diluted 10-fold with PEM buffer plus 10 μM Taxol to stabilize the microtubules, and further incubated at 34 °C for 10 min. After centrifugation (Beckman JA-25.50 rotor, 39,000 × g, 30 min, 4 °C), the microtubule pellet was resuspended in ATPase buffer plus 20 μM Taxol. Microtubule concentration was determined using the method of Bradford (46), and reported molar concentrations of microtubules refer to the concentrations of αβ-tubulin dimer (molecular mass of 110 kDa).

**Nucleotide Analog-N-Methylanthraniloyl (mant) derivatives of ATP and ADP** were synthesized as described previously (47, 48). Spectrophotometric properties of the products were evaluated and conform to published values. 2′(3′)′-mantADP and 2′(3′)-mantATP are reported to have an A365/A496 ratio of ~4.0, reflecting the optical densities of the N-methylanthraniloyl and adenine moieties (47). Previous work showed the 2′-mant-3′-dATP and 3′-mant-2′-dATP gave results similar to the mixture of isomers (2′-mant-ATP and 3′-mant-ATP) (14); therefore, we only used the mixture in these studies.

**Phosphate Sensor**—Phosphate release experiments measured the rate at which inorganic phosphate is released from kinesin, and relied on an engineered E. coli phosphate-binding protein (PBPA179C) covalently coupled to a fluorescent dye MDCC (49, 50). The expression system for the protein component, consisting of the E. coli strain ANCC75 containing a pBR322-derived plasmid into which the modified phoS gene
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directing the production of PBP-A197C has been cloned, was obtained from M. Webb (National Institute for Medical Research, London, UK). PBP-A179C was expressed and purified as described (49). Conjugation of the protein with MDCC, and subsequent purification of the phosphate sensor, was performed according to the method described by Brune et al. (49), with subsequent modifications (50). The concentration of MDCC-PBP-A197C was determined spectrophotometrically, assuming an extinction coefficient at 280 nm of 68,575 M\(^{-1}\) cm\(^{-1}\). MDCC-PBP-A197C was divided into small aliquots, snap-frozen in liquid N\(_2\), and stored at −80 °C.

Steady-state ATPase Assays—The hydrolysis of [α-\(^{32}\)P]ATP by kinesin-microtubule complex was monitored at 35 °C by mixing labeled nucleotide with the enzyme, quenching the reaction after a predetermined time, separating the products on a TLC plate, and measuring the hydrolysis of the labeled nucleotide by standard radiation monitoring techniques as previously described (41). Velocity data for a range of substrate concentrations were then plotted and fit by nonlinear regression to a hyperbolic model, \(k_{\text{obs}} = k_{\text{cat}}[\text{ATP}]/(K_{\text{m,ATP}} + [\text{ATP}]) + C\), to determine values of \(k_{\text{cat}}\) and \(K_{\text{m,ATP}}\).

Sedimentation Equilibrium Study—A Beckman-Coulter Optima XL-1 analytical ultracentrifuge was used; it was fitted with an AnTi60 rotor and absorbance optics. Using three 6-channel charcoal-filled Epon centerpieces, nine kinesin concentrations could be evaluated simultaneously. Rotor speed was 13,000 rpm and run temperature was 24 °C. Equilibrium data were collected at 230 nm at a spacing of 0.003 cm with five averages in a step scan mode. Data sets were collected at 2-h intervals between 16 and 22 h after run initiation, and equilibrium was verified by comparing successive scans. Optical data were edited in Excel to extract data from individual channels and analyzed by nonlinear least-squares fitting to a self-association scheme using NONLIN (51), obtained from the Center for Analytical Ultracentrifugation of Macromolecular Assemblies at University of Texas Health Science Center at San Antonio. An estimate of 0.7350 cm\(^2\) mg\(^{-1}\) for the partial specific volume of the kinesin monomer was made based on amino acid sequence contribution, taking into account a contribution of −0.0030 cm\(^2\) mg\(^{-1}\) made by the bound ADP. Solvent density was determined volumetrically to be 1.0056 g cm\(^{-3}\). The extinction coefficient \(\varepsilon\) at 230 nm, the wavelength monitored during the analytical ultracentrifugation experiment, of KHC407A, was determined at 31,260 M\(^{-1}\) cm\(^{-1}\), and this value was used to convert the apparent association constants determined by NONLIN from optical density units to those of molarity according to the relationship \(K_a(M^{-1}) = K_a(\text{abs}^{-1}) \times (1.2\varepsilon/2)\), where 1.2 is the optical path length in centimeters of the rotor centerpiece. NONLIN was used to fit equilibrium optical profiles of the sample channels to a simple monomer-dimer association reaction, with no assumed nonideality.

Rapid Quench Experiments—Transient-state kinetic analysis of kinesin ATPase in ATPase buffer was performed at 35 °C using a KinTek RQF-3 chemical quench flow instrument (KinTek Corp.). Reactions were quenched with 2 M HCl and neutralized with 2 M Tris-3 M NaOH as described (24). 1.5 μl of each quenched sample was examined by polyethyleneimine-cellulose thin layer chromatography, developed with 0.6 M KH\(_2\)PO\(_4\), pH 3.4.

Stopped-flow Experiments—A KinTek Stopped-Flow apparatus (Model SF-2001, KinTek Corp., Austin, TX) was used for stopped-flow experiments. Experiments were performed as previously described (14, 52) at 35 °C in ATPase buffer. Indicated reagent concentrations represent concentrations achieved after mixing.

Phosphate release kinetics were measured in the stopped-flow instrument, using the fluorescent phosphate reporter MDCC-PBP-A197C (11, 49) at a concentration of 4 μM and including the “phosphate mop,” consisting of 0.1 mM 7-methylguanosine and 0.01 units/ml purine nucleoside phosphorylase. Excitation was at 425 nm, and fluorescence was measured using a 450 nm cutoff long-wave filter. Post mixing concentrations were 50 nM kinesin, 75 nM microtubules, and 500 μM ATP. Phosphate concentration was computed from the fluorescence based upon a standard curve. The time dependence of phosphate production was fitted to the burst equation \([P]_t = A\exp(-k_{\text{obs}}t) + k_c t + C\), with \(k_{\text{obs}}\) representing the rate of phosphate release under the conditions tested.

The rate constants governing the binding of nucleotide to kinesin were estimated using mantADP and mantATP (15). Fluorescence data were fit to a single exponential function, \(F = A\exp(-k_{\text{obs}}t) + C\), where \(k_{\text{obs}}\) is the rate constant governing nucleotide binding and \(k_c\) accounts for a slow, linear phase. Values of \(k_{\text{obs}}\) were plotted against nucleotide concentration and fit to a hyperbolic model: \(k_{\text{obs}} = k_{\text{max}}[\text{mantADP}]/(K_d + [\text{mantADP}]) + k_{\text{off}}\). In experiments measuring rates of dissociation of mantADP, excitation of the fluorophore was direct, at 360 nm, and a decrease in fluorescence accompanied dissociation of mantADP from the active-site as described previously (14). Fluorescence traces were either fit to a double-exponential model of the form \(F = A_1\exp(-k_1 t) + A_2\exp(-k_2 t) + C\), or else fit by simulated kinetic data.

Results

ATPase Pathway—The results presented in this report define the alternating site ATPase pathway shown in Fig. 1, which will be referenced to clarify the relationship between the data and this minimal model.

Active-site Titration—Isolated kinesin contains Mg-ADP tightly bound to the enzyme active-site (41, 53). To determine the concentration of kinesin active-sites in each preparation, the protein was incubated with [α-\(^{32}\)P]ADP, and then the concentration of the bound radiolabeled nucleotide was measured by quantifying the amount that was inaccessible to a regenerating system that converted all free ADP to ATP (41). When phosphocreatine kinase, phosphocreatine, and cold ATP were added to a kinesin-[α-\(^{32}\)P]ADP complex, all free [α-\(^{32}\)P]ADP was rapidly converted to [α-\(^{32}\)P]ATP while the remaining [α-\(^{32}\)P]ADP was slowly lost from the kinesin active-site, observable by its slow conversion to [α-\(^{32}\)P]ATP. The rate of disappearance of [α-\(^{32}\)P]ADP equals the rate of dissociation of ADP from kinesin, and the amplitude extrapolated to \(t = 0\) provides an estimate of the concentration of bound [α-\(^{32}\)P]ADP at the start of the reaction. In Fig. 2, the results of an active-site determination experiment are shown. The concentration of the kinesin preparation was estimated by the absorbance at 280 nm as 56.5 μM. A 1:1 mixture of kinesin and [α-\(^{32}\)P]ATP at 68.2 μM was prepared and allowed to come to equilibrium. The fraction of radiolabeled nucleotide, [ADP]/([ADP] + [ATP]), was plotted and fitted to a single exponential curve, \(F = A\exp(-k_1 t) + C\), to yield a rate of 0.0062 ± 0.0003 s\(^{-1}\) and an amplitude of \(A = 0.286 ± 0.005\). To correct for the dilution of radiolabeled ATP by the ADP already bound to the enzyme, we used the relationship [kinesin] = [ATP added]/(1 − A) to calculate the active-site concentration of 27.3 ± 0.5 μM. This value was then used as the active-site concentration of the preparation in all subsequent experiments. The active-site concentration decreased by <10% after 6 months at −80 °C and was equally stable after 5 days at 4 °C.

The rate of ADP release from the kinesin dimer determined in this assay was 0.0062 ± 0.0003 s\(^{-1}\), a value comparable to that obtained by other methods. This measurement defines the rate-limiting step during the steady-state ATPase reaction when microtubules are absent.

Sedimentation Equilibrium Analysis of KHC407A—To determine whether KHC407A formed a dimer in solution, analytical ultracentri-
fugation using the sedimentation equilibrium protocol was used. Analysis of truncated Drosophila conventional kinesin constructs showed that a 366-residue N-terminal fragment of the protein does not self-associate in solution, whereas a 401-residue fragment forms a dimer with a dissociation constant of 36 nM. Thus, it has been shown that the domain necessary for self-association is between residues 367 and 401 in this protein, corresponding to the coil-coil domain seen in the crystal structure of rat kinesin (9). Based upon the structural and sequence similarities between Drosophila and rat conventional kinesins, we constructed a 407-residue N-terminal fragment of rat conventional kinesin expecting it to form a dimer. Nevertheless, it was necessary to examine the dimerization state of the purified protein.

The sedimentation of KHC407A at equilibrium was examined by measuring the optical profiles of solutions of the protein at nine concentrations. The concentrations tested were 44, 154, 264, 374, 484, 594, 704, 814, and 924 nM. After 20 h of centrifugation at 13,000 rpm at 24 °C, sedimentation was judged to have achieved equilibrium. Fig. 3A shows the optical profiles of the nine samples at equilibrium. Each data set was translated along the y-axis so as to set to zero the extrapolated absorbance at the meniscus of each sample. The superimposed curves represent the best-fit monomer-dimer association model, whose single equilibrium association constant was determined by global fitting of all nine data sets by nonlinear regression. A value for $K_d$ of $2.15 \times 10^7$ M$^{-1}$ with a 95% confidence interval ($\pm 2$ S.D.) between 9.25 $\times 10^6$ and 5.70 $\times 10^7$ M$^{-1}$ was determined by NONLIN. Attempts to fit to monomer-dimer-trimer or monomer-dimer-tetramer models failed to yield con-
The kinesin dimer has a dissociation equilibrium constant of 46 nM, similar to the value of 36.5 nM found for the 401-residue long N-terminal truncation of its Drosophila counterpart (54). Note that the confidence contour is asymmetric allowing 95% confidence values in the range from 18 to 108 nM.

Steady-state ATPase Activity—The value of $K_{\text{0.5,Mo}}$, the concentration of microtubules required for half-maximal ATPase activity by kinesin at near-saturating ATP concentration, was estimated to be $1 \mu\text{M}$ (data not shown) comparable to that observed for Drosophila kinesin (41). To measure $k_{\text{cat}}$ of KHC407A, the concentration of microtubules was determined at various ATP concentrations ranging from 1 to 200 $\mu\text{M}$ and plotted in Fig. 4. The best fit to a hyperbola, $k_{\text{obs}} = k_{\text{cat}}[\text{ATP}]/(K_{\text{m,ATP}} + [\text{ATP}]) + C$, gave values of $k_{\text{cat}} = 40 \pm 1 \text{s}^{-1}$ (per site) and a $K_{\text{m,ATP}} = 54 \pm 5 \mu\text{M}$. The value for $k_{\text{cat}}$ is extracted from the data with no assumptions made concerning mechanism. If ATP hydrolysis by the kinesin dimer occurs by an alternating site mechanism, then only one subunit of the dimer is actively releasing product at any given time, and the slowest step in the pathway is therefore $80 \pm 2 \text{s}^{-1}$. These values contrast somewhat with those found for the Drosophila dimeric 401-residue N-terminal truncation, which gave values for $k_{\text{cat}}$ at 20 $\text{s}^{-1}$ and $K_{\text{m,ATP}}$ at 62 $\mu\text{M}$. It appears from these data that KHC407A has a maximum hydrolysis rate that is approximately twice that of its Drosophila counterpart, but the temperature of the measurement (35 versus 25 °C, respectively) may account for the difference. Finally, the apparent second-order rate constant ($k_{\text{app}}$) for the fluorescent complex, and is $5.6 \pm 1.7 \mu\text{M}^{-1} \text{s}^{-1}$.

Binding of mantATP to KHC407A—To obtain an estimate of the rate constant governing the binding of ATP to KHC407A, a fluorescent ATP analog was used in a stopped-flow experiment, in which the kinesin-microtubule complex was mixed with mantATP. Fluorescence resonance energy transfer between optically excited tryptophan residues in the protein and the N-methylanthraniloyl moiety of mantATP provided a means by which the binding rate can be measured. Excitation was at 280 nm, and fluorescence was detected by a photomultiplier tube fitted with a 400 nm cutoff long-wave pass filter. Although the experiment measures the binding kinetics of a substrate analog rather than those of the substrate itself, the results are considered a close approximation of the behavior of the enzyme toward its natural substrate, because $k_{\text{cat}}$ and $K_{\text{m}}$ for the fluorescent analog are within a factor of two of the corresponding values for ATP (15). Concentrations after mixing were 2 $\mu\text{M}$ KHC407A, 10 $\mu\text{M}$ microtubules, and 50 $\mu\text{M}$ mantATP. The dashed line indicates a best-fit curve $F = A \exp(-k_{\text{obs}}t) + k_{\text{off}}t + C$. Similar curves were generated for mantATP concentrations of 5, 10, 20, 30, 50, 70, and 100 $\mu\text{M}$ mantATP. Values for $k_{\text{obs}}$, obtained from fluorescence traces at each mantATP concentration are plotted against the independent variable. The solid curve represents the best-fit hyperbola $k_{\text{obs}} = k_{\text{max}}[\text{mANTP}]/(K_{d} + [\text{mANTP}])$ determined by nonlinear regression. Convergence of parameters provide estimates of $K_{d}$ at 210 $\pm 25 \text{s}^{-1}$ and $K_{\text{obs}}$ at $38 \pm 10 \mu\text{M}$. The ratio $k_{\text{obs}}/K_{d}$ provides a lower limit to the apparent second-order rate constant for mantATP binding to the complex, and is $5.6 \pm 1.7 \mu\text{M}^{-1} \text{s}^{-1}$.

In Fig. 5B values of $k_{\text{obs}}$ determined for different concentrations of mantATP are plotted. A best-fit hyperbola of the form, $k_{\text{obs}} = k_{\text{max}}[\text{mANTP}]/(K_{d} + [\text{mANTP}]) + k_{\text{off}}$, was obtained by nonlinear regression. The rate constant for ATP dissociation ($k_{\text{off}}$) could not be accurately determined by this experiment and was set to zero. The maximum achievable binding rate, to which $k_{\text{obs}}$ converges as the concentration of mantATP increases ($k_{\text{max}}$), was evaluated at $210 \pm 25 \text{s}^{-1}$, and, the apparent $K_{d}$ was $38 \pm 10 \mu\text{M}$. We also considered an alternative interpretation of the data based upon a two step binding sequence where the first step was not a rapid equilibrium with $k_{1} = 5.6 \mu\text{M}^{-1} \text{s}^{-1}$ and $k_{2} = 210 \text{s}^{-1}$. However, this model would predict that a lag should be seen in the kinetics at moderate concentrations (10–20 $\mu\text{M}$), and the failure to...
see a lag argues in favor of a rapid equilibrium binding for the collision complex.

The convergence of \( k_{\text{obs}} \) to a maximum value suggests that the fluorescence change does not come about as a direct result of mantATP binding, but is instead a function of an isomerization step, occurring at a rate of 210 s\(^{-1}\), after mantATP binding. The data fit a simplified model in which enzyme and substrate combine to form a "collision complex," which subsequently undergoes a conformational change accompanied by an increase in fluorescence (Reaction 1).

\[
M \cdot K + \text{ATP} \rightleftharpoons M \cdot K \cdot \text{ATP} \rightleftharpoons M \cdot K \cdot \text{ATP}^* \\
\text{REACTION 1}
\]

The existence of such a complex, although not certain, is supported by evidence from kinetic and structural studies, which suggested the possibility of two distinguishable sequential kinesin-ATP complexes (11, 28, 55). The observed single exponential in the fluorescence traces at all mantATP concentrations supports the conclusion that the initial binding step is a rapid equilibrium, and so, the fit to a hyperbola defines a ground state dissociation constant of 1/218 \( \mu \text{M} \) KHC407A, 10 \( \mu \text{M} \) microtubules, and 100 \( \mu \text{M} \) \([\alpha-32\text{P}]\text{ATP}\) and ([\alpha-32\text{P}]ADP formation examined by TLC. A linear fit (dashed line) was generated from the data (circles), estimating the hydrolysis rate at 34.5 ± 1.7 s\(^{-1}\) and the burst amplitude at 0.85 ± 0.07. A KinTekSim simulation using the reaction depicted in Fig. 1 (solid line) suggests values for kinetic parameters \( k_2 \) (ATP hydrolysis) at 523 ± 58 s\(^{-1}\), and \( k_2 \) (ADP release) at 43.6 ± 2.9 s\(^{-1}\). The rate of ATP binding was set at 5.6 \( \mu \text{M} \cdot \text{s}^{-1}\) based on results from mantATP binding experiments.

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The rate constants \( k_2 \) and \( k_3 \), representing the rate constants governing ATP hydrolysis and ADP release, respectively, were estimated indirectly from the burst amplitude of the quench flow data and the value of \( k_{\text{cat}} \), determined directly by steady-state methods. The burst amplitude (0.85 ± 0.07), a dimensionless number, is defined by the \( y \)-intercept of the linear extrapolation of the data in Fig. 6 divided by the active-site enzyme concentration. The values of \( k_2 \) and \( k_3 \) can be calculated from the relationships, burst amplitude \( A = \left(\frac{k_2}{k_2 + k_3}\right)^2 \) and \( k_{\text{cat}} = k_2 k_3 / (k_2 + k_3) \), with known amplitude \( A \) and steady-state rate constant \( k_{\text{cat}} \) (56), assuming that \( k_{-3} \) is negligible. Simultaneous solution of these two equations yields values of \( k_2 = 520 ± 60 \text{ s}^{-1} \) and \( k_3 = 44 ± 3 \text{ s}^{-1} \). This analysis depends upon the assumption that hydrolysis is not readily reversible; however, the consequences of this assumption are minor, and in either case the apparent rate of the burst was \( k_2 + k_{-2} = 520 \text{ s}^{-1} \). For example, if \( k_2 = 4 \) as in the case of skeletal muscle myosin, then the data would be fit by the parameters \( k_2 = 416 \text{ s}^{-1} \) and \( k_3 = 104 \text{ s}^{-1} \), and \( k_3 = 56 \text{ s}^{-1} \). If the rate of the burst had been measurable, then the assumption that \( k_{-3} \) was negligible would not have been necessary and all rate constants could have been solved by simultaneous solution of three equations defining the burst rate, burst amplitude, and \( k_{\text{cat}} \).

To illustrate the expected burst kinetics of KHC407A, the reaction was simulated using KinTekSim software, programmed with a simple three-step reaction mechanism described above and using estimates of each rate constant in the pathway. The apparent second-order rate constant for ATP binding to kinesin-microtubule complex (\( k_i \)) was previously estimated to be 5.6 \( \mu \text{M} \cdot \text{s}^{-1}\), so this value was used in the simulation. Fig. 6 shows the data, the linear fit to the data (dashed line), and the simulated curve (solid line). It is important to note that values obtained for \( k_2 \) and \( k_3 \) describe the hydrolysis of ATP and release of ADP, respectively, according upon the three-step model described above. If kinesin hydrolyzes ATP using an alternating site mechanism as has been proposed (13–15), then the hydrolysis of [\alpha-32\text{P}]ATP by the active-site to which it has bound will not immediately follow the binding step but will be delayed until the prescribed conformational changes occur within the other subunit of the kinesin dimer. Similarly, the alternating site model suggests that the release of product from one site may occur only after prerequisite rearrangements occur at the other. For this reason, values of \( k_2 \) and \( k_3 \) describe what are likely to be composite reactions that incorporate more than one distinguishable step. None-
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In the presence of KCl, the time dependence of phosphate release fits a double exponential. The fast phase has a rate of 510 s\(^{-1}\) and amplitude of 0.8 per kinesin, whereas the slow phase occurs at a rate of 62 s\(^{-1}\) and amplitude of 1.2 per kinesin. Interestingly, the elevated salt concentration appears to accelerate the rate of release of the first phosphate after ATP hydrolysis. Because the rate of phosphate release is linked to dissociation of the trailing head from the microtubule, it appears as though salt weakens the interaction of the head with the microtubule to accelerate its release.

**Binding of KHC407A to Microtubules**—The binding of kinesin to the microtubule is the first step in microtubule-dependent kinesin motility. Two methods were employed to examine the kinetics of kinesin binding to microtubules. Using the first method, binding was measured directly by exploiting the change in turbidity that accompanies kinesin/microtubule association. In the second method, which will be described in the next section, the release of mantADP from kinesin upon binding to microtubules served as a reporter for the initial association reaction.

KHC407A (2 μM after mixing) was rapidly mixed with microtubules at concentrations between 5 and 15 μM (after mixing) in the stopped-flow apparatus in the absence of nucleotide, and the intensity of 340 nm light transmitted through the mixture was monitored, which was used to compute the turbidity (defined by the natural logarithm of the intensity change). Fig. 8A shows a trace for 2 μM KHC407A rapidly mixed with 5 μM microtubules. The dashed line represents a fitted double exponential curve of the form \(T = A_1 \exp(-k_{1}t) + A_2 \exp(-k_{2}t) + C\). A similar analysis was performed on each turbidity trace in the microtubule concentration dependence series. For each microtubule concentration, a fast and a slow phase were identified based on the relative magnitudes of \(k_1\) and \(k_2\), and these rates were plotted against microtubule concentration, as depicted in Fig. 8B.

The rate of the fast phase (circles) shows a linear increase with microtubule concentration, whereas the slow phase (squares) is nearly constant. The slope of a linear fit to the fast phase data (2.9 ± 0.2 μM\(^{-1}\) s\(^{-1}\)) provides an estimate of the apparent second-order binding rate constant for KHC407A microtubule association, whereas the y-intercept (19 ± 2.2 s\(^{-1}\)) estimates the rate constant governing dissociation. A dissociation equilibrium constant \(K_{d} = 6.7 ± 0.9 \mu M\) is indicated from these two measurements. The significance of the slow phase data is not immediately clear. If the measured rates of the slow phase are relevant to the kinetics of kinesin/microtubule interaction, their independence from microtubule concentration suggests that they describe a process that is most likely first-order with respect to kinesin, the reaction component that is held constant at 2 μM throughout the series of experiments. For this reason, the fast phase rates are considered as indicating a second-order binding process, whereas the slow phase rates indicate an unknown first-order process, perhaps reflecting an isomerization or aggregation of the microtubule/kinesin complex. The slow phase could represent a change in structure leading to tighter binding of the kinesin.

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**TABLE ONE**

**Rate parameters from phosphate release data**

Phosphate release data shown in Fig. 7 was fitted by nonlinear regression to the burst equation \([P_r] = A \exp(-k_{obs}t) + k_2t + C\) to obtain the parameters \(A\) (amplitude), \(k_{obs}\) (exponential rate), and \(k_2\) (linear drift) in ATPase buffer with and without 100 mM KCl. After the addition of 100 mM KCl, the data fit a double-exponential burst equation to give two rates and amplitudes as shown.

| Conditions                  | \(A\)          | \(k_{obs}\)  | \(k_2\)  |
|-----------------------------|----------------|--------------|----------|
| ATPase buffer plus 100 mM KCl | 0.8 ± 0.01     | 74 ± 4       | 0.74 ± 0.006 |
|                            | 0.8 ± 0.07     | 508 ± 74     |          |
|                            | 1.26 ± 0.06    | 62 ± 5       | 0.19 ± 0.01  |
Fast and slow phase rates are plotted in Fig. 9B, as well as a linear fit to the fast phase rates. As described below, the signal in this case arises from the sequential release of two ADP molecules and the binding of one ATP molecule. Therefore, complete analysis of the time course requires fitting the data to four step model where $k_{\text{ADP}}$ represents kinesin with ADP bound to each site (Reaction 3).

$$M + K_{\text{ADP}} \rightleftharpoons M\cdot K_{\text{ADP}} \rightleftharpoons M\cdot K_{\text{ADP}}^{-1}$$

(REACTION 3)

In this experiment, the high ATP concentration makes step 3 very much faster than step 2, and the microtubule concentration dependence of the rate of release of ADP therefore provides a measurement of the microtubule binding rate. The observed rate of a reversible binding reaction is the sum of the forward and reverse rates, $k_{\text{obs}} = k_3[\text{Mt}] + k_7$. Curve fitting by linear regression sets the forward and reverse rates at 4.6 ± 0.1 $\mu\text{M}^{-1}\text{s}^{-1}$ and 30 ± 3.3 $\text{s}^{-1}$, respectively. These values are in reasonable agreement with the corresponding rate constants obtained more directly by turbidity analysis (2.9 ± 0.2 $\mu\text{M}^{-1}\text{s}^{-1}$ for binding, 19.5 ± 2.2 $\text{s}^{-1}$ for release), and cover a larger range of microtubule concentrations. Interestingly, despite the differences between the results obtained using turbidity and mantADP release methods, the

to the microtubule complex; however, the measured rates are too slow to be part of the ATPase cycle.

**Microtubule Dependence of mantADP Release from KHC407A**—In the absence of microtubules, conventional kinesin contains one ADP bound in each active-site (53). Upon binding to microtubules, the kinesin dimer will release both ADP molecules in succession (13–15). The rate at which the fluorescent analog mantADP is released from KHC407A upon binding to microtubules in the presence of a near-saturating concentration of ATP was examined using the stopped-flow apparatus. A kinesin-mantADP complex was formed by mixing the two components at concentrations of 4 $\mu\text{M}$ KHC407A (active-site) and 8 $\mu\text{M}$ mantADP. The mixture was allowed to come to equilibrium for 20 min, during which time bound ADP was presumably released from the kinesin active-sites and replaced by mant-ADP, which was then hydrolyzed, yielding the kinesin-mantADP complex.

KHC407A-mantADP complex was rapidly mixed with microtubules at concentrations from 8 to 80 $\mu\text{M}$ (after mixing) in the stopped-flow instrument. Fig. 9A shows a representative trace of time-dependent fluorescent decay after mixing 2 $\mu\text{M}$ KHC407A-mantADP complex with 18 $\mu\text{M}$ microtubules and 1 mM ATP. Like the turbidity data described in the previous section, the fluorescence traces from this experiment were best fit to a double exponential rather than a single. Fluorescence data at the highest microtubule concentrations could only be fitted to single exponential models.

The rate at which the fluorescent analog mantADP is released requires fitting the data to four step model where $k_\text{KHC407A}$ represents KHC407A association, whereas a $k_\text{binding}$ of 3.3 $\text{s}^{-1}$ is the apparent rate constant for dissociation of the complex.
apparent equilibrium dissociation constants for the initial microtubule–kinesin collision complex suggested by each experiment are nearly identical, 6.5 ± 0.7 µM indicated by the mantADP dissociation reaction, and 6.6 ± 0.9 µM by the turbidity assay. However, this may not represent a true dissociation constant, because the observed release of ADP is so fast. Therefore, ADP release (step 2) may be reversible in order for the net microtubule dissociation rate to contribute to the concentration dependence of the observed ADP release rate such that the observed rate of 20–30 s⁻¹ may represent a composite of $k_{-2}$ and $k_{-3}$.

The observed linear dependence of rate on microtubule concentration implies that the rate of ADP release must be much larger than the maximum observed rate of 400 s⁻¹ measured at 80 µM microtubules. Because it is not feasible to work at higher microtubule concentrations, we are unable to press the limit further to achieve a more precise estimate of the maximum rate of ADP release following microtubule binding. Nonetheless, the measured rate is considerably greater than that reported previously for rat kinesin (57, 58) and slightly greater than the extrapolated maximum observed for *Drosophila* kinesin (11).

**ATP Dependence of mantADP Release from KHC407A**—In previous analysis of conventional kinesin motility, two ADP release events were observed after kinesin bound to microtubules (13–15), the second of which was dependent upon ATP concentration. To examine the ATP concentration dependence of mantADP release, a KHC407A–mantADP complex was rapidly mixed with microtubules (20 µM) plus ADP (10 µM), or ATP (5, 10, 25, and 50 µM), and fluorescence decay monitored. Concentrations are post-mixing. **Solid lines** represent normalized fluorescence data. **Dashed lines** are kinetic simulations of the reaction, based on a reaction depicted in Fig. 1, rate constants in TABLE TWO, and initial reagent concentrations used in the experiments.

![FIGURE 10. Pre-steady-state ATP dependence of mantADP release from KHC407A.](image)

The data from this experiment resulted in a family of curves displayed in Fig. 10. Each trace was normalized to its initial signal intensity and displaced along the x-axis to account for an instrument dead time of ~1.5 ms so that the curves can be superimposed and compared with the results of computer simulation. In the absence of ATP, release of mantADP is clearly biphasic with rates of 140 ± 2 s⁻¹ and 1.44 ± 0.02 s⁻¹ for the release of the first and second ADP, respectively. Observation of the second ADP release is dependent upon the addition of 10 µM ADP to prevent the rebinding of mantADP. Thus, the release of the second ADP reaches equilibrium at a point that favors rebinding of the second ADP, and interactions with the microtubule stimulate the ADP exchange.

Increasing the concentration of ATP increases the rate of release of the second mantADP, which results in an observed increase in the amplitude of the fast reaction phase. At intermediate ATP concentrations, the observed reaction is a function of at least three steps, and their rates are not sufficiently different to be resolved meaningfully by conventional data fitting to a sum of exponential functions. Rather, the reaction sequence summarized in Fig. 1 was used to globally fit the data by computer simulation. In Fig. 10, **dashed lines** represent simulated data based on rate constants summarized in TABLE TWO.

Estimates for the rate constants governing KHC407A–microtubule association and dissociation, obtained from experiments described in previous sections, were used as known parameters for the simulations described here, leaving the remainder of the rate constants to float in fitting the data. As successive fittings improved, these values were permitted to vary slightly as well, until a stable set of rate constants was obtained. Note that the rate constants for initial KHC407A–microtubule association ($k_{+1}$) and dissociation ($k_{-1}$) have been previously investigated directly by turbidity measurements, and indirectly by mantADP dissociation measurements. The value determined here, 7.8 µM⁻¹ s⁻¹, is comparable to that obtained from mantADP dissociation experiments and turbidity measurements. The estimate for the dissociation rate constant for this process, 9 s⁻¹, is one-half and one-third that of the corresponding values determined by turbidity and mantADP dissociation, respectively. The ATP binding rate constant determined here, 1.7 µM⁻¹ s⁻¹, is less than one-third that of the mantATP binding rate constant, 5.6 ± 1.7 µM⁻¹ s⁻¹, determined previously. Lastly, an off-rate constant for ATP ($k_{-2}$) was undeterminable using the mantATP binding assay, but is estimated at 18.4 s⁻¹ by global fitting however these two experiments measure ATP binding to different kinesin states.

The most striking results of this experiment are the predicted rate constants for mantADP dissociation. A value of greater than 1000 s⁻¹ for both $k_{+2}$ and $k_{+b}$ were required for the fit seen in Fig. 10. Under conditions of the experiment, the release of the first ADP is limited by the rate of kinesin binding to the microtubule and therefore is not well defined. This was not the case for the *Drosophila* K401 N-terminal truncation of conventional kinesin, where the rate of release of mantADP was well defined at a rate constant of ~300 s⁻¹ (14).

On the longer time scale, there is a regain in fluorescence due to the partial rebinding of mantADP after the completion of ATP hydrolysis. This behavior was not seen in similar studies performed using the *Drosophila* K401 dimeric N-terminal truncation, which generated a family of traces converging to a common minimum within 1–2 s after mixing (14). Thus, the observations made using KHC407A suggest a qualitative difference affinity of the two enzymes for mantADP. Rat kinesin in complex with microtubules binds one molecule of ADP more strongly than does *Drosophila* kinesin. The reaction pathway for KHC407A simulation required the inclusion of a mantADP release step in the absence of ATP (step 6), occurring with a rate constant $k_{-2}$ of ~1 s⁻¹, to account for the slow decay in the presence of 10 µM ADP (0 µM ATP).
ADP Dependence of mantADP Release from KHC407A—The ATP-independent release of mantADP observed in the experiment described in the previous section prompted an investigation of the ability of ADP to stimulate mantADP release. A KHC407A-mantADP complex was mixed in the stopped-flow instrument with microtubules plus ADP. Post mixing concentrations were 1 μM KHC407A (active-site), 2 μM mantADP, 20 μM microtubules, and ADP at concentrations of 5, 10, 20, 50, and 250 μM, or ATP at 250 μM (top curve to bottom curve). Curves were fit to double-exponential models (F = A1exp(−k1t) + A2exp(−k2t) + C) by nonlinear regression (fit curves not shown), generating a set of parameters A1, A2, k1, k2, and C for each. B, fast-phase rates are plotted against ADP concentration, with a best-fit hyperbola of the form k_{obs} = k_{max}[ADP]/(K_{d} - [ADP]) + k_{off}, whose parameters were determined by nonlinear regression. Although there is uncertainty in the magnitude of k_{max} because of the paucity of data points at concentrations greater than the apparent K_{d,ADP}, the initial gradient of the curve is equal to k_{max}/K_{d,ADP} and provides an estimate of the apparent second order rate constant governing ADP binding to the stalled KHC407A-microtubule complex. From the data, this rate constant is 0.04 ± 0.01 μM^{-1} s^{-1}. The rate constant for mantADP dissociation in the absence of added nucleotide, k_{off}, is 0.8 ± 0.1 s^{-1}. The slow phase of mantADP release from KHC407A bound to microtubules in the absence of ATP consists of two components: one that is nucleotide-independent, occurring with a rate constant of ~0.8 s^{-1}, and one that can be stimulated ~5-fold to 4 ± 0.3 s^{-1} (the value of k_{max}) by weak ADP binding (100 μM K_{d}).

Binding of mantADP to KHC407A—We previously showed in Fig. 10 that after the release of mantADP from KHC407A upon binding to microtubules plus ATP there is an apparent reversal in the fluorescence decay that accompanies the conversion of mantATP to mantADP on the 0.1- to 3-s time scale. The most straightforward explanation for this behavior maintains that mantADP rebinds to the active-site of the enzyme. To further examine the interaction between mantADP and KHC407A, stopped-flow experiments were performed.

KHC407A-microtubule complex was formed and rapidly mixed with mantADP at various concentrations in the stopped-flow instrument. Based upon other measurements, we presume that at the start of this experiment kinesin will be bound to the microtubule in the captive head state, with one head tightly associated with the microtubule while the other retains ADP in a weakly bound state. Post mixing the concentrations were 1 μM KHC407A, 10 μM microtubules, and mantADP at 5, 10, 15, 20, 25, 30, and 35 μM. As in mantATP binding experiments, optical excitation was at 280 nm, which excites tryptophan residues within the protein, and emission detected by a photomultiplier tube fitted with a 400 nm long pass filter. Fluorescence resonance energy transfer between optically excited tryptophans in the protein and the N-methyl-lanthaniloyl moiety of mantATP, and the detection of fluorescence by the stopped flow instrument provides a means by which the binding rate can be measured after mixing. Fig. 12A shows a representative trace, in which the mantADP concentration was 5 μM. This and each trace were fitted to a burst equation of the form F = A_{1}exp(−k_{off}t) + k_{off}t + C. This equation provided a consistently better fit than did a simple exponential, because the second rate k_{off} was necessary to account for a slower shift in the fluorescence signal that invariably followed the exponential phase. For the curve in Fig. 12A, k_{off} was 0.940 ± 0.006 s^{-1} and k_{off} was 0.0146 ± 0.0004 s^{-1}. Fig. 12B shows observed rates of the exponential phases plotted against mantADP concentration. The data were evaluated as being better fit by a hyperbolic than a linear model. The equation k_{obs} = k_{max}[mADP]/(K_{D,ADP} + [mADP]) + k_{off} was used to fit the data by nonlinear regression. From the fitting, k_{max} = 6.1 ± 0.9 s^{-1}, K_{D,ADP} = 81.1 ± 18.8 μM, and k_{off} = 0.58 ± 0.04 s^{-1}.

The value of 0.08 ± 0.02 μM^{-1} s^{-1} for the apparent second order rate constant governing binding of mantADP to KHC407A in the absence of added nucleotide is calculated from the initial slope of the concentration dependence of the rate as the ratio of k_{max}/K_{D,ADP}. Based on this value and that of the off rate k_{off}, the equilibrium dissociation constant can be estimated at 7.7 ± 2.1 μM. This analysis shows that mantADP can bind weakly to the kinesin nucleotide-binding pocket in of the captive head.

DISCUSSION

The kinetics of ATP turnover by dimeric rat conventional kinesin (N-terminal truncation KHC407A) have been investigated in the experiments described in this report. Sedimentation equilibrium analysis

FIGURE 11. ADP dependence of mantADP release from KHC407A. A, the set of fluorescence traces resulting from the rapid mixing of kinesin-mantADP complex with microtubules plus ADP, and ADP at concentrations of 5, 10, 20, 50, and 250 μM, or ATP at 250 μM (top curve to bottom curve). Curves were fit to double-exponential models (F = A1exp(−k1t) + A2exp(−k2t) + C) by nonlinear regression (fit curves not shown), generating a set of parameters A1, A2, k1, k2, and C for each. B, fast-phase rates are plotted against ADP concentration, with a best-fit hyperbola of the form k_{obs} = k_{max}[ADP]/(K_{d,ADP} - [ADP]) + k_{off}. The parameters obtained by nonlinear regression estimate of the apparent second order rate constant for ADP binding to the stalled KHC407A-microtubule complex.
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**FIGURE 12.** Binding of mantADP to KHC407A-microtubule complex. A, representative trace (solid line) of fluorescence change observed when kinesin-microtubule complex was rapidly mixed with mantADP (1 μM kinesin, 10 μM microtubules, and 15 μM mantADP, post-mixing). The trace is fit by a single-exponential model containing a linear term ($F = A \cdot \exp(-k_{\text{on}}1 \cdot t) + k_t \cdot t + C$) by nonlinear regression with $k_{\text{on}}1 = 1.6 \text{s}^{-1}$ (solid line). Similar traces were obtained at mantADP concentrations of 5, 10, 15, 20, 25, 30, and 35 μM. B, rates obtained by nonlinear regression of fluorescence trace data to an exponential model are plotted against mantADP concentration. The data are fit to the hyperbolic model $k_{\text{obs}} = k_{\text{max}} \cdot \text{mantADP}/(K_d + \text{mantADP}) + k_{\text{off}}$, by nonlinear regression. Curve fitting yielded values of $k_{\text{max}} = 6.1 \pm 0.9 \text{s}^{-1}$, $K_d = 81.1 \pm 18.8 \text{μM}$, and $k_{\text{off}} = 0.58 \pm 0.04 \text{s}^{-1}$.

showed that the protein exists primarily as a dimer at low concentration, formed with a dissociation equilibrium constant of 46 nM (Fig. 3). Therefore, for most of the experiments described in this section, in which kinesin is used at micromolar concentrations, the motor exists as a dimer. Furthermore, the KHC407A dimer is of comparable stability as its Drosophila counterpart of similar length (54).

The interactions between KHC407A (with ADP bound) and microtubules were investigated using three methods. A rapid mixing experiment was performed in which the two components were mixed in the absence of ATP and turbidity change was measured (Fig. 8). The results of this experiment define a rate constant of $2.9 \pm 0.2 \text{μM}^{-1} \text{s}^{-1}$ for KHC407A-microtubule binding ($k_{\text{on}}$), and $19 \pm 2.2 \text{s}^{-1}$ for dissociation ($k_{\text{off}}$). Using mantADP release as a reporter event for the initial KHC407A-microtubule association reaction, values of $4.6 \pm 0.1 \text{μM}^{-1} \text{s}^{-1}$ and $29.8 \pm 3.3 \text{s}^{-1}$ were obtained for binding and dissociation, respectively (Fig. 9). This experiment, unlike the turbidity measurement, was performed in the presence of a high concentration of ATP to displace mantADP. Finally, an experiment was performed in which the release of mantADP from KHC407A was monitored after rapid mixing of the enzyme-mantADP complex with microtubules plus varying amounts of ATP, and simulated curves yielding estimates for the rate constants were fit to the data (Fig. 10). This experiment estimated $k_{1} = 7.8 \text{μM}^{-1} \text{s}^{-1}$ and $k_{2} = 9.3 \text{s}^{-1}$. Taken together, the experiments can be considered to describe upper and lower limits for estimate for the rate constants governing association and dissociation of the kinesin microtubule collision complex. Most importantly, each measurement indicates that the initial association of a kinesin-ADP complex with microtubules is a weak reversible equilibrium with a dissociation constant of $\sim 1-6 \text{μM}$. Thus in the initial collision kinesin interacts only weakly with the microtubule. One might presume that the release of ADP is coupled to the formation of a tighter complex, but if this were the case then the dissociation of the kinesin from the microtubule would not contribute to the observed binding kinetics, because ADP release is so rapid. These data may imply that the binding of kinesin to the microtubule must be weak until the binding of ATP leads to a tighter complex.

Rat kinesin may differ from its Drosophila counterpart in its affinity with which it interacts with microtubules. The experiments described above suggest a binding rate constant for the rat motor-microtubule interaction at between $\sim 3$ and $8 \text{μM}^{-1} \text{s}^{-1}$, and a rate constant for motor-microtubule dissociation from $\sim 10$ to $30 \text{s}^{-1}$ in the initial collision complex. For the Drosophila motor, the binding rate constant was evaluated at $19.5 \text{μM}^{-1} \text{s}^{-1}$ by measuring turbidity change in a kinesin-microtubule binding experiment (11). No dissociation was observed in the absence of ATP, suggesting that the Drosophila motor binds more tightly and less reversibly to microtubules than does its rat counterpart. Only in the presence of Mg-ATP does the Drosophila kinesin/microtubule complex appear to dissociate, with a rate constant of $13.6 \text{s}^{-1}$.

Two estimates have been made for the rate constants for ATP binding. The first comes from stopped-flow measurements on mantATP binding to KHC407A-microtubule complex (Fig. 5B), whereas the second was obtained from the global fitting of simulated curves to mantADP release traces (Fig. 10). The data indicated a binding rate of $5.6 \pm 1.7 \text{μM}^{-1} \text{s}^{-1}$ and an off-rate too small to measure. The global fitting to mantADP dissociation data estimated $k_{1} = 1.7 \text{μM}^{-1} \text{s}^{-1}$ and $k_{2} = 18.4 \text{s}^{-1}$. These two experiments may reflect different reactions. Although the latter measures ATP binding to a transient intermediate formed after kinesin initially binds to microtubules, the former involves the addition of ATP to an equilibrium mixture of kinesin and microtubules where the second head has time to interact with the microtubule and possibly release its ADP. Accordingly, the faster, less reversible ATP binding to a preformed microtubule-kinesin complex may reflect an altered state of the kinesin that is not normally on the pathway for ATP turnover. Experiments performed at low ATP concentration may allow an additional pathway in which ADP release from the second head precedes binding of ATP to the first head.

The binding of mantATP to the kinesin/microtubule complex follows a single exponential, like we had previously described for Drosophila kinesin (15). This is in contrast to a more recent report by Rosenfeld (59) where the observation of biphasic mantATP binding kinetics was used to argue for strain between the heads. Rosenfeld’s experiments were performed with a cysteine-light mutant, and the results could be explained by suggesting that the mutations loosen the tight coupling that normally prevents the binding of a second ATP. Our data argue for an even tighter coupling between the two heads such that strain between the two sites prevents the binding of a second ATP, as indicated by the monophasic ATP binding and single turnover of ATP hydrolysis in the pre-steady-state phase. Strain between the heads to prevent the binding of ATP to the second site of the dimer while both heads are...
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bound to the microtubule was implicit in the original Gilbert-Johnson model.

The most striking finding from our new set of experiments is the speed of mantADP release. Analysis of the dependence of mantADP release on microtubule concentration in a stopped-flow mixing experiment (Fig. 9B) revealed that this step was not rate-limiting at the highest microtubule concentration tested, at which $k_{on}$ was over 400 s$^{-1}$. Varying ATP concentration in a stopped-flow mantADP release experiment (Fig. 10) produced fluorescence traces which, when globally fit by kinetic simulation, revealed values for two mantADP release rate constants in excess of 1000 s$^{-1}$. This was unexpected, because the corresponding rate constants for the Drosophila counterpart have been measured at only 300 s$^{-1}$ (15). It seems to be of little advantage for rat kinesin to have accelerated product release to this extent, given that $k_{on}$ differs between the two enzymes by only a factor of two (20 s$^{-1}$ for Drosophila, 40 s$^{-1}$ for rat). In any event, it is clear that ADP release is not rate-limiting. It should also be noted that the Drosophila kinesin experiments were performed at 25 °C, whereas those using rat kinesin were preformed at 35 °C.

The rate at which KHC407A hydrolyzes ATP was too rapid to measure by quench flow analysis (Fig. 6), because the burst phase of ADP production was too brief to be resolved. Analysis of the data by simulation suggested an apparent ATPase rate constant of 523 ± 58 s$^{-1}$, five times faster than the 100 s$^{-1}$ ATP hydrolysis rate constant measured for Drosophila conventional kinesin (24). It is only slightly less than the rate at which 100 μM ATP binds to kinesin, given the ATP binding rate constant of 5.6 μM$^{-1}$s$^{-1}$ used in the simulation, suggesting that, under the conditions used in the quench flow experiment, ATP binding was the rate-limiting step. It is impossible, therefore, to assign a value to the rate constant governing ATP hydrolysis, or than to concede that it is too large to measure with this technique.

The rate of phosphate release by KHC407A-microtubule complex can be predicted using parameters measured in the experiments discussed thus far. Given a pathway in which ATP binding is followed by ADP release and ATP hydrolysis (in that order on alternating sites), both of which occur rapidly, phosphate release is predicted to be the rate-limiting step, with a rate constant of ~80–100 s$^{-1}$. This step is most likely the rate-limiting step of the kinesin motility cycle, as it is in the case of the Drosophila counterpart, which releases inorganic phosphate with a rate constant of 50 s$^{-1}$ (54).

The interaction between the kinesin and ADP was investigated in two ways. The ability of ADP to stimulate mantADP release from the KHC407A-mantADP complex in the presence of microtubules was measured in a stopped-flow experiment, as well as the binding rate of mantADP to the KHC407A-microtubule complex. The interaction studied here is not believed to be important in the motility cycle of the motor, although it may be relevant to the unexpected reversal in the fluorescence decay observed at ~0.5 s in the release of mantADP from the KHC407A-microtubule complex (Fig. 10). The fluorescent nucleoside diphosphate binds weakly ($K_{d} = 7.7$ μM) and slowly ($k_{on} = 0.08$ μM$^{-1}$s$^{-1}$), and ADP interacts with similar kinetics.

Maintaining the reactions on each of the kinesin heads out of phase with each other head is central to the mechanisms underlying processive movement. The most direct evidence for an alternating site ATPase pathway is based upon the observation that ATP binding to one kinesin site stimulates product release from the partner site when the kinesin interacts with the microtubule (Fig. 10). This experiment unequivocally defines the sequence of reactions and rates for steps a, b, 1, and 2 of Fig. 1. The rate of hydrolysis, step 3, is too fast to measure, but the amplitude of the burst provides an important bit of information; namely, that only one ATP is hydrolyzed in steps leading up to the rate-limiting step as the reaction approaches the steady state. This observation eliminates models that would allow the binding and hydrolysis of a second ATP. Finally, the slower rate of phosphate release and coincident rate of release of kinesin from the microtubule under nonprocessive conditions establishes the placement of step 4 next in the sequence. The approximate correspondence between the steady-state turnover rate and the rate of phosphate release establishes step 4 as rate-limiting.

The cycle is completed by returning to the captive head state via step 4. A captive head state of the kinesin-microtubule complex is operationally defined as the state where one head has bound to the microtubule, resulting in the rapid displacement of one ADP. Evidence indicates that the second head retains ADP and remains tethered in close proximity to the microtubule. It is noteworthy that ADP release from the second head in the captive head state is stimulated ~100-fold by the microtubules in the absence of ATP and that the binding of ATP to the first head results in a further 100-fold stimulation of ADP release from the second head to achieve a rate sufficient for turnover. The captive head state is therefore central to the evidence underlying the Gilbert-Johnson alternating site model (14).

The rate of release of the second ADP in the absence of ATP is too slow to account for steady-state turnover. Moreover, the equilibrium constant for the second ADP release in the absence of ATP (not shown) favors re-binding of ADP at the 1 μM concentrations of kinesin used in our experiments, so that the release of the second mantADP is not observed unless a low concentration of ADP is added to the solution to prevent the re-binding of mantADP. This phenomenon, the weak and reversible equilibrium binding of ADP to the captive head, may underlie some of the confusion surround the captive head state. Single molecule experiments performed at very low concentrations of kinesin and low ATP may favor a pathway in which ADP release precedes ATP binding.

A second component of the Gilbert-Johnson alternating site ATPase pathway that was notable in our description of the pathway was the observation that only one ATP bound per dimer prior to reaching the steady state and that steady-state turnover was limited by the release of the kinesin head (and phosphate) from the microtubule after ATP hydrolysis. ADP release and ATP hydrolysis are both much faster than steady-state turnover, whereas the release of kinesin from the microtubule occurs at a rate equal to the rate of steady state turnover. Thus another essential element of the Gilbert-Johnson pathway is implicit in the conclusion that ATP does not bind to the kinesin site left open by the release of ADP until the other head has hydrolyzed ATP and released from the microtubule, as shown in Fig. 1. Our data and that collected earlier on Drosophila kinesin imply that interactions between the two kinesin heads inhibit the binding of ATP to the leading head with both heads bound to the microtubule. Preventing the formation of a kinesin dimer with ATP bound to both sites contributes to maintaining the reactions on the two heads out of phase.

These two central features of the kinesin ATPase pathway have been overlooked by models that bypass the captive head state. Structural and equilibrium measurements have shown that the neck linker, a segment of β sheet structure along the kinesin surface, is docked in the presence of ATP and undocked in its absence (31). This structural model provides satisfying proposals for the means by which the interaction between the two heads might distinguish leading and trailing heads and thereby attenuate nucleotide binding affinity to achieve an alternating site ATPase pathway. However, the Rice et al. (31) pathway short-circuits the Gilbert-Johnson pathway by suggesting that ATP binds directly to the state with both heads attached to the microtubule, thereby bypassing the captive head state. There is no evidence for this short-circuited
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pathway and significant evidence against it. It should be noted that at very low ATP concentrations, the second head will bind to the microtubule and release ADP reaching an equilibrium dependent upon the concentration of ADP in solution. This may explain why single molecule experiments at low ATP concentration (17) suggest that ATP binds to a state with both heads bound to the microtubule, perhaps representing a nucleotide-free species that does not accumulate at moderate to high ATP concentrations but does form at sub-micromolar ATP and ADP concentrations.

The studies reported here reaffirm the Gilbert-Johnson ATPase pathway and lay the foundation for more detailed structure/function studies to address fundamental questions regarding the kinetic and thermodynamic basis for force production by kinesin. The accompanying report describes the beginning of this effort (32).

REFERENCES

1. Miki, H., Setou, M., Kaneshiro, K., and Hirokawa, N. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7004–7011
2. Vale, R. D. (2003) Cell 112, 467–480
3. Hirokawa, N. (1998) Science 279, 519–526
4. Endow, S. A. (1999) Eur. J. Biochem. 262, 12–18
5. Scholey, J. M. (2000) Annu. Rev. Cell Dev. Biol. 16, 487–508
6. Hunter, A. W., and Wordeman, L. (2000) Annu. Rev. Phys. Chem. 51, 985–994
7. Hackney, D. D. (1994) J. Biol. Chem. 269, 16155–16165
8. Kozielki, F., Sack, S., Marx, A., Thormählen, M., Schonbrunn, E., Hirose, K., and Mandelkow, E. M. (2004) Biochemistry 43, 3670–3680
9. Moyer, M. L., Gilbert, S. P., and Johnson, K. A. (1998) Biochemistry 37, 800–813
10. Schief, W. R., Clark, R. H., Crevenna, A. H., and Howard, J. (2003) Proc. Natl. Acad. Sci. U. S. A. 101, 1183–1188
11. Yildiz, A., Tomishige, M., Vale, R. D., and Selvin, P. R. (2004) Science 303, 676–678
12. Ashby, C. L., Fehr, A. N., and Block, S. M. (2003) Science 302, 2130–2134
13. Kaseda, K., Higuchi, H., and Hirose, K. (2003) Nat. Cell Biol. 5, 1079–1082
14. Mandelkow, E., and Hoenger, A. (1999) Curr. Opin. Cell Biol. 11, 34–44
15. Naber, N., Rice, S., Matuska, M., Vale, R. D., Cooke, R., and Pate, E. (2003) Biophys. J. 84, 3190–3196
16. Peterman, E. J., Sosa, H., Goldstein, L. S., and Moerner, W. E. (2001) Biophys. J. 81, 2851–2863
17. Peterman, E. J., Sosa, H., and Moerner, W. E. (2004) Annu. Rev. Phys. Chem. 55, 79–96
18. Moyer, M. L., Gilbert, S. P., and Johnson, K. A. (1996) Biochemistry 35, 6321–6329