Expression, Purification, and Characterization of the *Drosophila* Kinesin Motor Domain Produced in *Escherichia coli*†

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Received November 4, 1992; Revised Manuscript Received February 3, 1993

ABSTRACT: The *Drosophila* kinesin heavy-chain gene was truncated to obtain the N-terminal 401 amino acid motor domain (designated K401) containing both the microtubule and ATP binding sites. The plasmid construct with the truncated kinesin gene was used to transform *Escherichia coli*. After induction, K401 was expressed as soluble kinesin protein at high levels and purified to homogeneity in milligram quantities. The purified protein was active and behaved as native kinesin with respect to its steady-state kinetic properties: K401 demonstrated a very low ATPase activity (k_{cat} = 0.01 s^{-1}) which was stimulated ~1000-fold by the addition of microtubules (k_{cat} = 10 s^{-1}; $K_{0.5,MT}$ = 0.9 μM tubulin; $K_{m,ATP}$ = 31 μM). Like native kinesin, K401 when purified contained ADP tightly bound at its active site, and the release of ADP from the active site occurred at a rate equal to the steady-state ATPase k_{cat}. Active-site measurements using [α-^{32}P]ATP demonstrated a stoichiometry of one ATPase site per K401 molecule. Like native kinesin, K401 can also hydrolyze MgGTP, and in the presence of microtubules, the rate of hydrolysis was increased dramatically from 0.03 to 16 s^{-1} ($K_{0.5,MT}$ = 2 μM tubulin; $K_{m,GTP}$ = 3.5 mM). These results establish that an active kinesin motor domain can be bacterially expressed and that this domain, the N-terminal 401 amino acids of the *Drosophila* kinesin heavy chain without light chains or additional eukaryotic factors, has full catalytic activity with microtubules. Furthermore, we found that truncation of the kinesin heavy chain to approximately 400 amino acids was necessary to purify the milligram amounts of active motor domain necessary for further mechanistic and structural studies to establish the basis for force production.

Kinesin is a microtubule-activated ATPase that drives the translocation of cellular components to the plus-ends of microtubules at ~0.5 $\mu m \cdot s^{-1}$. The protein was originally discovered in neuronal tissue (Vale et al., 1985; Brady, 1985) and sea urchin eggs (Scholey et al., 1985). Since its discovery in 1985, kinesin has been studied intensively [reviewed in Vale (1987), Warner and McIntosh (1989), and Goldstein (1991)] and shown to be present in all eukaryotes examined to date. It is a tetrameric protein ($\alpha_2\beta_2$) with two identical heavy chains (α) of 110–140 kDa and two identical light chains (β) of 60–80 kDa, the molecular mass dependent upon species (Bloom et al., 1988; Kuznetsov et al., 1988).

Results from immunological and electron microscopic studies have revealed the structural organization of the native protein (Hirokawa et al., 1989; Scholey et al., 1989). In addition, the kinesin heavy-chain gene was cloned initially from *Drosophila melanogaster* (Yang et al., 1988, 1989), followed by the cloning of the analogous gene in squid (Kosik et al., 1990), sea urchin (Wright et al., 1991), and human (Navone et al., 1992). Furthermore, genetic studies of the *Drosophila* kinesin heavy chain in vivo have shown that the protein is essential and important in neuromuscular function (Saxton et al., 1991). Analysis of the deduced amino acid sequences of these kinesin heavy-chain genes predicted an amino-terminal globular domain, an extended α-helical domain, and a small, globular carboxyl-terminal domain. From structural predictions as well as studies with truncated kinesin polypeptides, it was revealed that the amino-terminal head domain contained both the ATP and microtubule binding sites and that this domain was sufficient for plus-end-directed motility (Yang et al., 1989, 1990). De Cuevas et al. (1992) demonstrated that kinesin was able to dimerize through the α-helical domains of each heavy chain to form the α-helical coiled coil of the stalk. The small globular domain at the carboxyl terminus was predicted to associate with the light chains and to be the domain that interacted with the cellular organelle to be transported.

Although much has been learned about the structural organization of the protein, its cellular localization, and its characteristics as a molecular motor, these studies have not provided the information necessary to understand the coupling of ATP hydrolysis to force production. Hackney et al. (1988, 1989) as well as Sadhu and Taylor (1992) have initiated mechanistic studies using native bovine brain kinesin. Their results show that in the absence of microtubules, kinesin hydrolyzes ATP at a very low rate ($\leq$0.003 s^{-1}). The initial steps, ATP binding and hydrolysis, were shown to be fast, and it was the release of ADP rather than phosphate release from the active site that was rate-limiting during steady-state turnover. Furthermore, microtubules were shown to increase steady-state ATPase activity dramatically ($\geq$1000-fold) to ~3 s^{-1}. However, mechanistic studies have been limited by the complexity of the native kinesin preparation. Both groups have reported heterogeneity in their preparations in part due to a mixture of kinesin dimers of two heavy chains (α_2) and kinesin tetramers consisting of heavy and light chains ($\alpha_2\beta_2$). Although Hackney et al. (1991) performed a very careful series of experiments characterizing the ATPase activity of both the dimer (α_2) and tetramer ($\alpha_2\beta_2$), there remained within each preparation a population of kinesin molecules that did not show the microtubule activation.

Mechanistic studies to understand the basis for force production would benefit greatly by a homogeneous preparation of kinesin motor domains that can be purified in

† This research was supported by grants to K.A.J. (GM26726 from the National Institutes of Health and S.P.G. (postdoctoral fellowship from the Muscular Dystrophy Association).

0006-2960/93/0432-4677$04.00/0

milligram amounts. Our approach has been to express the motor domain of the *Drosophila* kinesin heavy chain in bacteria. We have produced a truncated kinesin gene which encodes the amino-terminal 401 amino acids of the heavy chain, containing both the ATP and microtubule binding sites. These 401 amino acids represent a single motor domain believed to be incapable of dimerization because the α-helical domain is not present (Yang et al., 1990; De Cuevas et al., 1992). In this paper, we present the initial biochemical and kinetic characterization of the truncated kinesin polypeptide, K401. The results show that K401 can be purified in milligram amounts, is a homogeneous and active preparation, and shows properties expected of native kinesin in the experiments reported here.

EXPERIMENTAL PROCEDURES

Materials. Clone pET-K447 was the generous gift of Drs. Margaret de Cuevas and Lawrence S. B. Goldstein, Harvard University (Yang et al., 1990). The *Escherichia coli* cell lines BL21, BL21(DE3), HMS174, and HMS174(DE3) were kindly provided by Dr. Alan Rosenberg, Brookhaven National Laboratory, and taxol was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. [α-^{32}P]ATP (>3000 Ci/mmol) and [α-^{32}P]GTP (>3000 Ci/mmol) were purchased from ICN Biomedicals, Inc., restriction enzymes were from New England Biolabs, Inc., poly(ethylenimine) (PEI)[1] was from Aldrich Chemical Co., and PEI–cellulose F TLC plates (EM Separations of E. Merck, 20 × 20 cm, plastic backed) were from Curtin Matheson Scientific, Inc.

pET5b-K401 Construction. The plasmid described here derived its name from the translation vector pET5b (Rosenberg et al., 1987; Studier et al., 1990) and from the truncated kinesin gene which encodes the N-terminal 401 amino acids of the *Drosophila* kinesin heavy chain (Yang et al., 1989, 1990). The polymerase chain reaction (Saiki et al., 1988) was used to construct pET5b-K401. The oligonucleotides were chemically synthesized using an Applied Biosystems 380A synthesizer (Pennsylvania State University Biotechnology Institute, University Park, PA). The 5′-PCR oligonucleotide (5′-GCTAGGATCCTACATATGGCTAGCCGG-3′) consisted of a *Bam*HI site followed by *Nde*I and *Nhe*I sites and corresponds to the first four amino-terminal amino acids of the parent clone pET5-K447 (Yang et al., 1990). The 3′-PCR oligonucleotide (3′-TGCGGGTTGGACATCCCTAGGTCC-5′) consisted of 12 bases corresponding to amino acids 398–401 of the parent clone pET5-K447, followed by a stop codon and *Bam*HI site. Extra bases were added to each primer at the restriction sites to enhance binding by *Bam*HI. The template DNA was isolated from plasmid pET5-K447 which contains a truncated kinesin gene encoding the N-terminal 447 amino acids of the *Drosophila* kinesin heavy chain (Yang et al., 1990). The PCR conditions were those suggested by Perkin-Elmer Cetus (Norwalk, CT) with the following temperature cycle during amplification: 94 °C (2 min), 45 °C (1 min), 72 °C (3 min) for 25 cycles. The amplification products were subcloned into pET5b using *Bam*HI and *Nhe*I restriction sites, and the plasmid containing the truncated kinesin gene K401 was transformed into *E. coli* strain HMS174(DE3). In this expression system, the direct expression of K401 was under the control of the T7 promoter with the T7 RNA polymerase induced by IPTG (Studier & Moffatt, 1986; Rosenberg et al., 1987; Studier et al., 1990). The resulting K401 protein begins at the N-terminus of the kinesin heavy chain (Met-Ala-Ser-Arg...) as in the K447 protein, and K401 terminates at Leu401. Its molecular mass, 45 079 Da, was calculated from the amino acid sequence.

Media and Buffers. The following media and buffers were used for the experiments described: Miller LB broth (10 g of bacto tryptone, 5 g of bacto yeast extract, and 10 g of NaCl per liter); lysis buffer (50 mM Tris·HCl, pH 7.8, 2.5 mM EDTA, 1 mM DTT, 100 mM NaCl, and 2 mM PMSF); HEPES dialysis buffer (20 mM HEPES, pH 7.2, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1 mM EGTA, 50 mM KCl, and 0.5 mM PMSF); ATPase buffer (20 mM HEPES, pH 7.2, 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM potassium acetate, and 1 mM DTT); buffer A (50 mM Tris·HCl, pH 7.4, 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl, and 2 mM $MgCl_2$); buffer B (50 mM Tris·HCl, pH 7.8, 0.1 mM EDTA, 1 mM DTT, 20 mM NaCl, and 2 mM $MgCl_2$); PM buffer (100 mM PIPES, pH 6.7, 5 mM magnesium acetate, and 1 mM EGTA). The pH of the buffers was determined at the temperature at which they were used.

Expression of K401. Starter cultures were prepared by selecting single colonies of HMS174(DE3)/pET5b-K401 for growth in LB supplemented with ampicillin (0.1 mg/mL) at 37 °C to an A_{600} of 0.4–0.6. Five 4-L flasks, each containing 2 L of LB plus ampicillin, were each inoculated with 20 mL of the starter culture and shaken at 37 °C for 2 h. The cultures were then temperature-shifted to 22 °C and induced with IPTG (final concentration 0.1 mM). The cultures were shaken at 22 °C for 16 h, and the cells were harvested by centrifugation (Sorvall GSA rotor, 10 000 rpm, 5 min, 4 °C). Typically, 7 g of *E. coli* cells was obtained by this procedure from which ~0.5 mg of K401 can be purified to homogeneity as soluble protein (see below).

Alternatively, large-scale cultures (BioService 300-L fermentor; BioService, Inc., Bethlehem, PA) were grown to obtain ~120 g of *E. coli* from 230 L of LB. Single colonies were selected and grown at 37 °C to an A_{600} of 0.4–0.6 in LB supplemented with ampicillin. This initial culture was used to inoculate (at 1:100) a 2-L culture of LB plus ampicillin and shaken at 37 °C until the A_{600} was 0.4–0.6. This 2-L culture was used to inoculate 230 L of LB plus ampicillin and incubated at 37 °C for 2 h. The temperature was shifted to 20 °C and induced with IPTG (final concentration 0.1 mM). The fermentation was continued at 20 °C for 12 h and then shifted to 4 °C. The cells were harvested by centrifugation onto a mylar sheet initially (Sharples AS-16 supercentrifuge rotor, 16 800 rpm, feed-rate 1 L/min; Sharples Division, Alfa-Laval Separation, Inc., Warminster, PA) followed by centrifugation in a Sorvall SS34 rotor (10 000 rpm, 15 min, 4 °C). The cells were resuspended in lysis buffer (3 mL/g) and frozen as aliquots in liquid N_2 for storage at –80 °C.

Protein Purification. The procedures for the purification of K401 and tubulin were performed at 4 °C or on ice unless otherwise indicated.

Purification of K401 by Microtubule Affinity. Fourteen grams of cells was diluted to a final volume of 140 mL (1 g/10 mL) in lysis buffer plus lysozyme at 0.2 mg/mL and incubated on ice for 60 min with stirring. The cells were lysed by three cycles of freezing (liquid N_2) and thawing (37 °C). The cell

[1] Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; BSA, bovine serum albumin; DTT, dithiothreitol; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PEI, poly(ethylenimine); PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

extract was centrifuged (Sorvall SS34 rotor, 18 000 rpm, 30 min), and the viscous supernatant was removed and stored on ice. The pellet was resuspended in 47 mL of lysis buffer (one-third of the original volume) and homogenized using a Wheaton glass tissue grinder to extract additional soluble K401 that had become trapped in the pellet. The extract was centrifuged (Sorvall SS34 rotor, 18 000 rpm, 30 min) to obtain a second supernatant. The first and second supernatants containing soluble K401 protein were combined (total volume 180 mL). The DNA and proteins in the supernatant were precipitated with PEI. The PEI stock (5% in 50 mM Tris·HCl, pH 8) was added dropwise to the supernatant with vigorous stirring using a magnetic stirrer (final concentration of PEI, 0.3%). After the addition of PEI, the mixture was stirred more slowly for 30 min followed by centrifugation (SS34 rotor, 10 000 rpm, 5 min). The supernatants were discarded, and the pellets were resuspended in 45 mL of lysis buffer (one-fourth of the combined supernatant volume of 180 mL). The resuspended pellets were adjusted to 0.3 M NaCl (final concentration) and incubated for 15 min. The preparation was centrifuged (SS34 rotor, 15 000 rpm, 10 min) to obtain a supernatant enriched in K401 protein. This supernatant was clarified (SS34 rotor, 18 000 rpm, 30 min) and diluted 4-fold with HEPES dialysis buffer (45 mL plus 135 mL of buffer). The preparation was dialyzed in HEPES dialysis buffer overnight to reduce the NaCl concentration and to bring K401 to more physiological conditions for microtubule binding. On day 2, the dialysate was clarified by centrifugation (Beckman 60 TI rotor, 40 000 rpm, 60 min). The supernatant that resulted was adjusted to 2.5 mM tripolyphosphate, 0.1 mM MgGTP, 20 μM taxol, and microtubules to 0.05 mg/mL. The preparation was incubated for 20 min at room temperature (25–26 °C) followed by incubation at 34 °C for 10 min to promote K401 binding to the microtubules. The microtubules with K401 bound were collected by centrifugation (SS34 rotor, 18 000 rpm, 30 min) and resuspended in 5 mL of HEPES dialysis buffer plus 0.1 mM tripolyphosphate and 20 μM taxol. To the supernatant that resulted were again added 2.5 mM tripolyphosphate, 0.1 mM MgGTP, 20 μM taxol, and microtubules (0.05 mg of tubulin/mL). After incubation and centrifugation, the microtubule pellets were resuspended in 5 mL of HEPES dialysis buffer plus 0.1 mM tripolyphosphate and 20 μM taxol. The two aliquots of microtubules with associated K401 were then centrifuged, and the microtubule–K401 pellets were resuspended in 6 mL of HEPES buffer plus 5 mM ATP, 5 mM magnesium acetate, 50 mM KCl, and 20 μM taxol. The preparation was incubated 30 min at 34 °C followed by centrifugation (Beckman TY65 rotor, 40 000 rpm, 30 min). The supernatant that resulted was dialyzed against ATPase buffer until the estimated concentration of ATP was <1 pM. On day 3, the dialysate was clarified by centrifugation (Beckman TY65 rotor, 40 000 rpm, 60 min), and the supernatant that resulted containing purified K401 was concentrated to a protein concentration of 1–2 mg/mL using Centricon 30 microconcentrators (Amicon, Danvers, MA); the microconcentrators were precoated with bovine serum albumin (BSA). K401 was frozen as aliquots in liquid N_2 for storage at –80 °C. Typically, 1 mg of K401 was purified per 14 g of *E. coli* at >99% purity as determined by SDS–PAGE. This procedure has also been used starting with as little as 3–4 g of cells, yielding approximately 0.2–0.3 mg of K401 at >99% purity.

Large-Scale Purification of K401. Forty-five grams of cells was diluted to a final volume of 270 mL (1 g/6 mL) in lysis buffer plus lysozyme at 0.2 mg/mL and incubated on ice for 60 min with stirring. The cells were lysed by three cycles of freezing (liquid N_2) and thawing (37 °C). The cell extract was centrifuged (Sorvall SS34 rotor, 18 000 rpm, 30 min), and the viscous supernatant was removed and stored on ice. The pellet was resuspended in 90 mL of lysis buffer (one-third of the original volume) and homogenized in aliquots using a Wheaton glass tissue grinder. This resuspended pellet was again cycled 3 times, freezing and thawing to release additional soluble proteins. The cell extract was centrifuged (SS34 rotor, 18 000 rpm, 30 min) to obtain a second supernatant. The first and second supernatants were combined (350-mL total volume), treated with 5% stock PEI to a final PEI concentration of 0.35%, and stirred for 30 min. The extract was then adjusted to 0.4 M NaCl (final concentration) and stirred an additional 20 min. The soluble proteins were obtained by centrifugation (Sorvall GSA rotor, 10 000 rpm, 10 min), and the PEI pellet was discarded. The supernatant was adjusted to 2 mM $MgCl_2$ and clarified by centrifugation (SS34 rotor, 18 000 rpm, 30 min). The clarified supernatant was dialyzed against 2 × 6 L of buffer A. On day 2, the dialysate was clarified (Beckman 60Ti rotor, 40 000 rpm, 60 min) to yield a supernatant of ~370 mL. The clarified supernatant was loaded onto a 350-mL Bio-Rex 70 column (Bio-Rad Laboratories, Richmond, CA) that was equilibrated in buffer A. The column was washed with buffer A until the A_{280} returned to the base line. A 2000-mL linear NaCl gradient (150–600 mM NaCl) was run to elute K401 from the resin. On day 3, the fractions enriched in K401 were determined by A_{280} and SDS–PAGE. Most of the *E. coli* proteins elute in the flow-through with K401 being one of the few proteins in the preparation that binds to the Bio-Rex 70 resin under these buffer conditions; K401 eluted at ~325–375 mM NaCl. The fractions enriched in K401 were pooled (~320 mL) and dialyzed against buffer B (2 × 6 L). On day 4, the dialysate was clarified and loaded onto a 40-mL DEAE-Sephacel (Sigma Chemical Co., St. Louis, MO) column equilibrated in buffer B. A 900-mL linear NaCl gradient (50–400 mM) in buffer B was run, and K401 eluted at ~150–190 mM NaCl. On day 5, the fractions with pure K401 were determined by SDS–PAGE, pooled, and concentrated by Amicon ultrafiltration (Model 8050 stirred cell, Danvers, MA) to 2–8 mL. The K401 was dialyzed against ATPase buffer and on day 6 clarified by centrifugation (Beckman TY65 rotor, 40 000 rpm, 60 min). For some experiments, K401 was concentrated further by Centricon 30 microconcentrators. K401 was frozen as aliquots in liquid N_2 for storage at –80 °C. The large-scale procedure yields 10–12 mg of K401 per 40–45 g of *E. coli* at >99% purity as determined by SDS–PAGE.

Mammalian Brain Tubulin and Microtubules. Bovine brain microtubules were prepared by two cycles of temperature-dependent polymerization and depolymerization (Shelanski et al., 1973; Sloboda et al., 1976), and tubulin was separated from microtubule-associated proteins by the method of Borisy et al. (1974) as modified by Omoto and Johnson (1986). On the morning of each experiment, an aliquot of tubulin was thawed, diluted with PM buffer to 10–15 mg/mL protein, adjusted to 1 mM GTP, and cold-depolymerized for 30 min on ice. The tubulin was then centrifuged (microfuge, 14 000 rpm, 15 min, 4 °C) to sediment aggregates of tubulin. The supernatant with soluble tubulin was incubated at 34 °C for 20 min to polymerize the microtubules followed by dilution in PM buffer plus 20 μM taxol to dilute the GTP concentration to 0.1 mM and stabilize the microtubules. The preparation was incubated for an additional 15 min at 34 °C followed by centrifugation (microfuge, 14 000 rpm, 15 min, 4 °C). The

microtubule pellet was resuspended in ATPase buffer plus 20 μM taxol, and the protein concentration was determined by the Schacterle and Pollack (1973) modification of Lowry et al. (1951).

Determination of Protein Concentration and Extinction Coefficient. The protein concentration for K401 was determined spectroscopically using its extinction coefficient (ε) that was calculated on the basis of the amino acid sequence of K401 (Edelhoch, 1967). The ϵ_{K401} was calculated to be 26 740 M^{-1} cm^{-1} on the basis of 2 tryptophans (ϵ_{Trp} = 5690 M^{-1} cm^{-1}) and 12 tyrosines (ϵ_{Tyr} = 1280 M^{-1} cm^{-1}). A correction was made for one ADP bound per active site because K401 (see Results, Figure 3) like native kinesin (Hackney et al., 1989) when purified contains a stoichiometric amount of ADP. Although the absorption maximum for ADP is at 259 nm, ADP does absorb somewhat at 280 nm. The ε for ADP at A_{280} was determined experimentally to be 2500 M^{-1} cm^{-1}; therefore, the extinction coefficient used to calculate the protein concentration for K401 was 26 740 + 2500 = 29 240 M^{-1} cm^{-1}. With 1 mol of ADP bound per mole of K401, the contribution to the absorbance at 280 nm by K401 is 91%.

Nitrocellulose Binding Assay. A modification of the filter binding assay was used to determine the concentration of K401 active sites as well as the rate of ADP release from the active site. K401 (0–4 μM) in the absence of microtubules was incubated with [α-^{32}P]ATP (30-μL volume, 8 μM [α-^{32}P]-ATP in ATPase buffer plus 0.1 mg/mL BSA) for 60 min at 25 °C. This incubation would permit all ADP tightly bound at the active site to be exchanged with [α-^{32}P]ATP (see Results, Figure 3B). The samples (10 μL in duplicate for each K401 concentration) were applied to nitrocellulose in a dot-blot apparatus (see pretreatment below) and aspirated. Free nucleotides pass through the nitrocellulose, yet nucleotide tightly bound to K401 protein is trapped on the nitrocellulose. Two microliters (in duplicate) of the reaction mixture was spotted on the nitrocellulose without aspiration to determine the total counts in the reaction mix. The nitrocellulose membrane was air-dried and imaged by direct β-emission detection using a Betascope 603 blot analyzer (Betagen, Waltham, MA) to quantitate the radiolabeled nucleotides adsorbed to the nitrocellulose at each K401 concentration.

The binding of ATP is fast (Hackney, 1989) and dependent upon the release of ADP from the active site; therefore, the nitrocellulose binding assay can be used to make a direct measurement of the rate of ADP release. The reaction mixture (100-μL total volume; 3.2 μM K401 and 8 μM [α-^{32}P]ATP in ATPase buffer plus 0.1 mg/mL BSA) was incubated at 25 °C for varying times (eight time points, 0–60 min). At each time point, 10 μL of the reaction mixture was applied directly to the nitrocellulose in one well of the dot-blot apparatus and aspirated. A 2-μL aliquot (in triplicate) of the reaction mixture was applied to the nitrocellulose without aspiration to determine the total counts. The radioactivity for each time point was quantitated using the Betascope. The amount of radiolabeled ATP/ADP adsorbed onto the nitrocellulose represents nucleotide tightly bound at the active site of K401 and is a measure of K401 active sites. The data were fit to the single-exponential equation: $y = [K401][1 - \exp(-kt)] + b$, to determine the rate (k) of ADP release.

Pretreatment of Nitrocellulose. Nitrocellulose (0.2-μm pore size; Schleicher & Schuell, Inc., Keene, NH) was treated as follows to decrease nonspecific binding: The nitrocellulose (NC) was cut to fit the dot-blot apparatus (Bio-Rad Laboratories) and then added to ddH_2O at 100 °C for 3 min followed by a cold water wash. The NC was then treated with 0.5 M KOH for 5 min followed by extensive washing in ddH_2O. Lastly, the NC was equilibrated in ATPase buffer plus 0.1 mg/mL BSA. At the time of the assay, the dot-blot apparatus was assembled with the nitrocellulose. The thumbscrews of the apparatus were tightened again after aspiration and prior to application of the sample to prevent spreading of the sample on the nitrocellulose. In addition, each well to be used was checked with buffer to ensure an even and rapid flow rate. Directly prior to application of the radioactive sample, 30 μL of buffer was added to the well and aspirated.

NTPase Assays. The ATPase and GTPase measurements were made by following the hydrolysis of [α-^{32}P]ATP (or [α-^{32}P]GTP) to form products [α-^{32}P]ADP (or [α-^{32}P]GDP) during the initial linear phase of the reaction. These experiments were performed by preparing two tubes, each with 50 μL for a total reaction volume of 100 μL. The enzyme tube (A) contained K401, microtubules, ATPase buffer, and 20 μM taxol. The substrate tube (B) contained 1 μL of [α-^{32}P]-ATP (or [α-^{32}P]GTP), cold MgATP (or MgGTP), and ATPase buffer. The reactions were initiated by the addition of 5 μL of tube A to 5 μL of tube B and incubated at 25 °C for various times (eight time points per 100 μL). The 10-μL reaction for each time point was terminated by addition of 10 μL of 2 N HCl, followed by addition of 20 μL of chloroform, and lastly neutralization by the addition of 6 μL of 1 M Tris–3 M NaOH. The zero time points were determined by denaturing K401 (5 μL of tube A) with HCl and chloroform prior to the addition of radiolabeled substrate and neutralization with Tris–NaOH. An aliquot (1.5 μL) of the quenched reaction mixture for each time point was spotted onto a PEI–cellulose TLC plate, and the TLC plate was developed in 0.6 M potassium phosphate buffer, pH 3.4. Radiolabeled NTP and NDP were quantitated using the Betascope. Radiolabeled ATP and GTP as purchased contained a small amount of NDP (2% ADP; 4% GDP) as determined by TLC. Furthermore, these background levels of [α-^{32}P]NDP were equal to the zero time points for ATP (or GTP) hydrolysis. The concentration of product obtained at each time point was therefore corrected for the contaminating NDP determined in each reaction. For the experiments with microtubules, the microtubules and K401 were preincubated for 10 min to form the microtubule–K401 complex and to allow time for ADP to be released from the active site. Reactions were also performed with microtubules and radiolabeled substrate but in the absence of K401 to determine the rate of NTP hydrolysis attributed to the preparation of microtubules.

The addition of 1 μL of [α-^{32}P]ATP (or [α-^{32}P]GTP) resulted in a total of ~40 000 cpm per 1.5 μL spotted onto the nitrocellulose. The concentration of the product [α-^{32}P]-ADP (or [α-^{32}P]GDP) was plotted as a function of time. The data were fit to a straight line, and the rate of NTP hydrolysis (micromolar substrate converted to product per micromolar enzyme per second) was determined from the slope of the line and the K401 concentration used in the reaction. For the ATPase experiments in the absence of microtubules, the reaction times sampled were from 0 to 45 min, and for the microtubule-activated ATPase measurements, the times were from 0 to 4 min dependent upon the concentrations of NTP and tubulin (microtubules) used. For the GTPase experiments, the times for sampling were from 0 to 90 min, dependent upon the concentrations used. The rates of hydrolysis determined from these individual reactions were plotted in Figures 4 and 5 as a function of tubulin concentration and NTP concentration. The concentrations of the enzyme and substrates reported in the figure legends are the final concentrations. The data were fit to a hyperbola by nonlinear regression using

RS1 software (Bolt, Beranek and Newman, Inc., Cambridge, MA) to determine the steady-state kinetic constants K_m and k_{cat}.

Preparation of Antibodies, Immunoblotting, and SDS–PAGE. Antiserum against the kinesin heavy chain isolated from squid optic lobe was produced in New Zealand white rabbits. The proteins from partially purified preparations of squid kinesin were separated by SDS–PAGE; the band corresponding to the kinesin heavy chain (~116 kDa) was excised from the gel and equilibrated in PBS until the pH of the solution stabilized at pH 7. A quantity of the gel (~100 μg of protein) was diluted with a small volume of PBS and homogenized in an equal volume of Freund's complete adjuvant for subcutaneous injection into the rabbit. A booster injection with incomplete Freund's adjuvant was given after 4 weeks. Two weeks after the booster, the serum tested positive for kinesin heavy-chain antibodies. The rabbit was boosted after 2 weeks and bled 2 weeks after the injection. This boosting and bleeding schedule was continued. The IgG fractions from the preimmune and immune sera were obtained by precipitation with ammonium sulfate at 50% saturation, followed by resuspension of the pellet in and dialysis against PBS.

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose (0.2-μm pore size) was performed according to the method of Towbin et al. (1979). The kinesin antibodies were used at a dilution of 1:10 000 and the preimmune antibodies at 1:1000. The rabbit IgG–kinesin immune complexes were detected by the use of horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories) with enhanced chemiluminescence (ECL) reagents (Amersham Corp., Arlington Heights, IL) and autoradiography.

SDS–PAGE was performed using 8% acrylamide/2 M urea slab gels with 3% stacking gels according to the buffer formulations of Laemmli (1970) and stained with Coomassie brilliant blue R-250 (Fairbanks et al., 1971). The molecular mass standards used were rabbit muscle phosphorylase *b* (97 kDa), BSA (66 kDa), hen egg white ovalbumin (43 kDa), bovine carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21 kDa) (Bio-Rad Laboratories).

RESULTS AND DISCUSSION

Small-Scale K401 Expression and Purification by Microtubule Affinity. We have developed two different procedures to express and purify K401 protein, each procedure optimized for obtaining either a small amount (≤1 mg) or a much larger amount (>5 mg) of pure K401. Figure 1 shows representative samples taken from all the steps in the expression and purification of K401 using flasks for *E. coli* growth and purification by microtubule affinity. Lane d (18K supernatant) compared to lane e (the resulting pellet) as well as lane f (supernatant) compared to lane g (resulting pellet) shows that approximately 50% of K401 was expressed as soluble protein. The PEI precipitation steps (lanes h–j) were important for several reasons: At low salt concentrations, PEI precipitates nucleic acids and most of the proteins, thereby allowing protein to be precipitated out of a relatively large volume (180 mL). The resulting pellet was then extracted at increasing salt concentrations to obtain a fraction (lane i) enriched in K401 and free of most DNA and RNA. Lastly, the reduction in volume was important for the microtubule binding steps to keep the amount of taxol and microtubules used at a minimum. Lanes m–p, the microtubule supernatants and pellets, show that K401 and a low molecular weight *E. coli* protein were the only proteins that bind to the microtubules

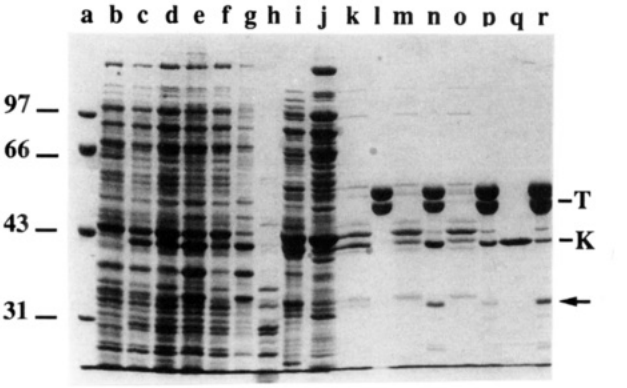

FIGURE 1: Small-scale K401 expression and purification by microtubule affinity. Coomassie blue stained 8% acrylamide/2 M urea gel showing all the steps in the purification of K401 protein as described under Experimental Procedures: lane a, molecular mass markers in kilodaltons; lane b, preinduced cell lysate; lane c, induced cell lysate; lanes d and e, first 18K supernatant and pellet, respectively; lanes f and g, second 18K supernatant and pellet, respectively, which show that ~50% of the K401 was expressed as soluble protein. The proteins and nucleic acids were precipitated with PEI. Lane h, supernatant showing those proteins soluble after PEI precipitation; lane i, 0.3 M NaCl extract of PEI pellets with K401 released to supernatant; lane j, 1.1 M NaCl extract of PEI pellets which compares the relative enrichment of soluble K401 in the 0.3 M NaCl extract relative to remaining K401 and *E. coli* proteins. The 0.3 M NaCl extract enriched in K401 (lane i) was diluted, dialyzed, and clarified. Lane k shows the dilute supernatant that resulted, that was used for microtubule binding. Microtubules (lane l), tripolyphosphate, and taxol were added to the supernatant (lane k). Lanes m and n show the supernatant and pellet, respectively, that resulted with K401 sedimenting with the microtubules (lane n). In addition, a low molecular weight *E. coli* protein (arrow) also sedimented with the microtubules. The microtubule binding was repeated, adding microtubules, tripolyphosphate, and taxol to the supernatant (lane m). Lanes o and p show the supernatant and pellet, respectively, that resulted; additional K401 sedimented with the microtubules. Both microtubule pellets with associated K401 (lanes n and p) were incubated with 5 mM MgATP plus 50 mM KCl; lanes q and r show the supernatant and pellet, respectively, that resulted. K401 was the only protein released to the supernatant (lane q) using these experimental procedures. T, tubulin; K, K401.

in the presence of tripolyphosphate. Only K401, however, dissociated from the microtubules in the presence of MgATP; the *E. coli* protein did not show a nucleotide-dependent interaction with the microtubules. AMP–PNP was as effective as tripolyphosphate in promoting K401 association with the microtubules (data not shown). There appeared to be no detectable difference in the yield of K401 or the steady-state ATPase activity using either analog for the K401 purification. However, we routinely used tripolyphosphate because it was less expensive than AMP-PNP. K401 migrated in our gel system (Figures 1 and 2) at ~42 kDa, which is in close agreement to the molecular mass (45 079 Da) calculated from the amino acid sequence.

The microtubule affinity procedure for purification of K401 resulted in 1 mg of K401 (>99% pure) per 14 g of cells. The procedure was used for successfully scaling the volumes as described under Experimental Procedures without compromising the purity or the yield of K401. For example, preparations starting with as little as 3 g of cells resulted in a yield of 0.2 mg of pure K401.

Fermentation and Large-Scale Purification of K401. The large-scale procedure was developed to purify amounts of K401 greater than a few milligrams at a time. Figure 2 shows representative samples from the purification as presented under Experimental Procedures. Comparison of lane h (18K soluble proteins) with lane i (proteins that sediment at 18K) shows

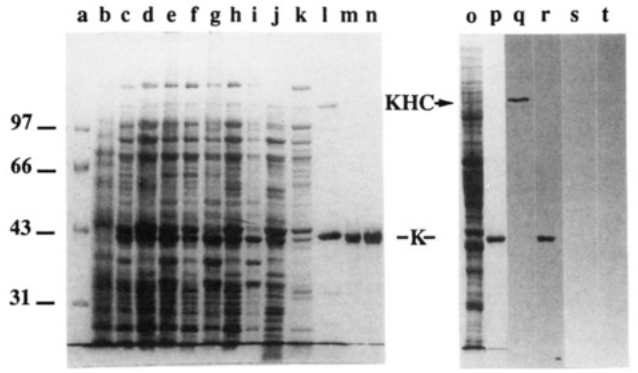

FIGURE 2: K401 expression by fermentation and large-scale purification. An 8% acrylamide/2 M urea gel stained with Coomassie R-250 showing all the steps in the purification: lane a, standards; lane b, preinduced cell lysate; lane c, induced cell lysate; lanes d and e, first 18K supernatant and pellet, respectively; lanes f and g, second 18K supernatant and pellet, respectively; lanes h and i, combined first and second supernatants and the resulting pellet, respectively. Each supernatant and pellet pair was loaded in equal volume to determine the relative concentrations of K401 as soluble and insoluble protein; ~50% of K401 was expressed as soluble protein. Lane j, 0.4 M NaCl supernatant that results after PEI precipitation; lane k, 1.1 M NaCl extact of the PEI pellet that remained; lane l, K401-enriched factions from the Bio-Rex column; lane m, purified K401 (4 μg) from the DEAE-Sephacel column; lane n, K401 (6 μg). Gel (lanes o and p), immunoblot probed with antibodies to the squid kinesin heavy chain (lanes q and r), and immunoblot probed with preimmune antibodies (lanes s and t): lanes o, q, and s, soluble bovine brain proteins with native kinesin; lanes p, r, and t, purified K401. KHC, native kinesin heavy chain; K, K401.

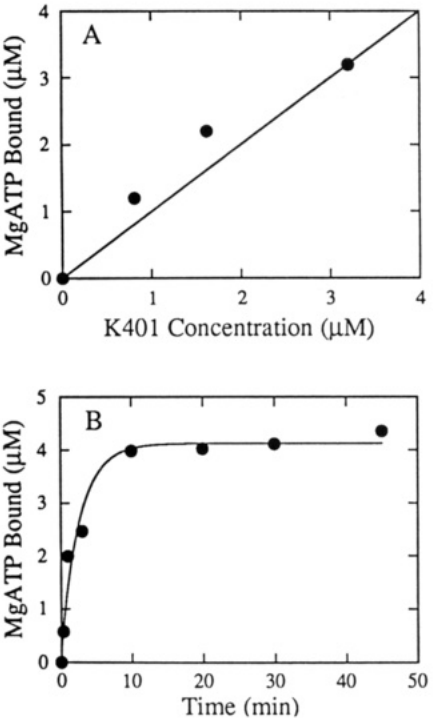

FIGURE 3: Nitrocellulose binding assay. (A) Active-site titration of K401: K401 (0, 0.8, 1.6, and 3.2 μM) was incubated for 60 min at 25 °C with 8 μM [α-^{32}P]ATP in the absence of microtubules. The samples were transferred to nitrocellulose and aspirated. The filled circles (●) show the concentration of radiolabeled ATP or ADP tightly bound to K401. The line with a slope of 1 shows the theoretical binding of one ATP per K401 active site. (B) Direct measurement of the rate of ADP release from K401: K401 (3.2 μM) was incubated with 8 μM [α-^{32}P]ATP for various times prior to application to the nitrocellulose. The filled circles (●) show the concentration of radiolabeled ATP or ADP bound to K401 as a function of time. The line shows the fit of the data to a single-exponential equation with the rate of ADP release of 0.006 ± 0.002 s^{-1}.

that ~50% of K401 was expressed as soluble protein. PEI precipitation was used to reduce the volume, precipitate nucleic acids and proteins, and permit the salt extraction of a fraction enriched in K401 (lane j). The Bio-Rex column resulted in the most significant purification of K401 because K401 was one of the few proteins to bind the resin (lane l). Pure K401 was eluted from the DEAE–Sephacel column (lanes m and n). The yield from the large-scale protocol has routinely been 10–12 mg of K401 per 40–45 g of *E. coli*.

Figure 2, lanes o–t, shows the gel and corresponding immunoblots probed with antibodies to the squid kinesin heavy chain or preimmune antibodies. The kinesin antibodies recognize antigenic sites of the bovine kinesin heavy chain (lane q) and purified K401 (lane r). The preimmune antibodies (lanes s and t) did not recognize antigens in either preparation. These results demonstrate that the protein purified from the recombinant cells is a kinesin polypeptide and indicates the high level of homology of the kinesin heavy chain between different species.

Active-Site Determination. We began the kinetic experiments to evaluate K401 activity by the determination of the steady-state ATPase activity in the absence of microtubules. The k_{cat} was determined to be 0.01 ± 0.002 s^{-1}; therefore, K401 demonstrated the very low ATPase activity characteristic of native kinesin (Vale et al., 1985; Kuznetsov & Gelfand, 1986; Hackney et al., 1988, 1991). Hackney et al. (1988, 1989) also showed that phosphate release was fast and that ADP was tightly bound to kinesin such that ADP release was rate-limiting in the absence of microtubules. Furthermore, Hackney et al. (1989) also reported that bovine brain kinesin when purified contained ADP in a stoichiometric amount bound at the active site.

The very low ATPase activity of K401 suggested that ADP was tightly bound to the active site; therefore, we anticipated that K401 when purified would also contain a stoichiometric amount of ADP. We used a nitrocellulose binding assay to test this hypothesis. K401 in the absence of microtubules was preincubated with [α-^{32}P]ATP to exchange ADP with radiolabeled ATP. If ADP were tightly bound to the active site, the [α-^{32}P]ADP would be trapped on the nitrocellulose membrane with K401, yet free [α-^{32}P]ATP or [α-^{32}P]ADP would pass through the membrane. Alternatively, if ADP were not tightly bound, the [α-^{32}P]ADP would be released from the active site during the time required for aspiration in the dot-blot apparatus. The released nucleotide would then pass through the nitrocellulose. Figure 3A shows the data for three concentrations of K401; the protein concentration was determined spectroscopically as described under Experimental Procedures. The data are close to a straight line with the theoretical binding of one molecule of ATP per one K401 active site. These results indicate that ADP was tightly bound at the active site such that [α-^{32}P]ADP/[α-^{32}P]ATP became trapped on the nitrocellulose in a concentration-dependent manner. In addition, the results show that the preparation of K401 purified was fully active at the detection limits of the nitrocellulose binding assay and the assay represents a relatively easy method to determine active-site concentration.

Because K401 contained a stoichiometric amount of ADP bound at the active site, the binding of [α-^{32}P]ATP to K401 in the nitrocellulose binding assay was dependent upon the release of ADP already present at the active site. Thus, this assay could also be used to make a direct measurement of the rate of ADP release. Figure 3B shows increased binding of [α-^{32}P]ATP as a function of time when K401 was incubated with [α-^{32}P]ATP. The data were computer-fit to a single-

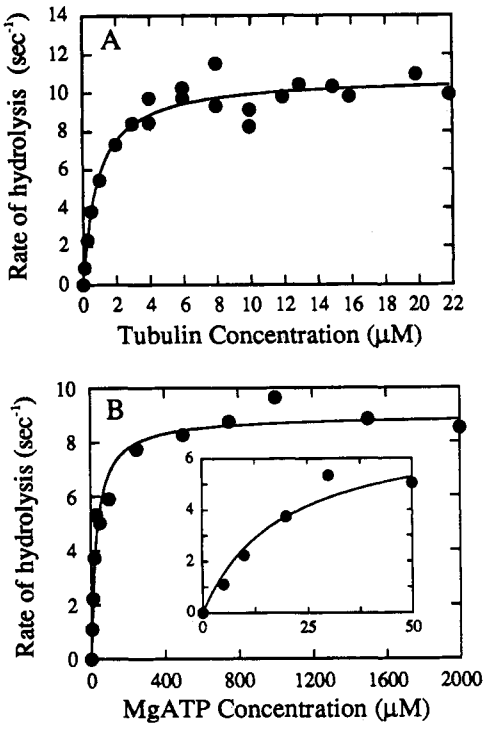

FIGURE 4: Steady-state ATPase activity of K401. (A) 0.5 μM K401 was incubated with 1 mM MgATP and microtubules (0–22 μM tubulin) in ATPase buffer at 25 °C. The data show the rate of [α-^{32}P]ATP hydrolysis as a function of tubulin concentration. In the absence of microtubules, the rate of ATP hydrolysis was 0.01 ± 0.002 s^{-1}. The line shows the computer fit of the data to a hyperbola with the k_{cat} = 10.9 ± 0.3 s^{-1} and $K_{0.5,MT}$ = 0.9 ± 0.15 μM tubulin. (B) K401 (0.1 μM) was incubated with microtubules (8 μM tubulin) and MgATP (0–2 mM) in ATPase buffer at 25 °C. The data show the rate of [α-^{32}P]ATP hydrolysis as a function of MgATP concentration, and the line is the fit of the data to a hyperbola with the k_{cat} = 9 ± 0.25 s^{-1} and $K_{m,ATP}$ = 31 ± 4 μM. The inset shows the initial part of the curve, 0–50 μM MgATP.

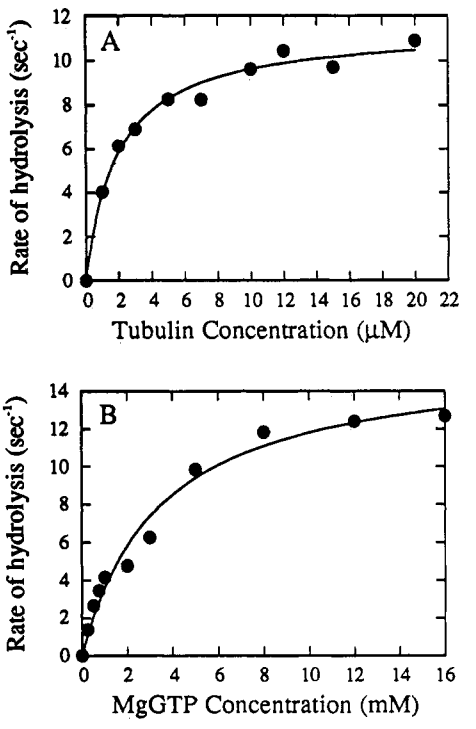

FIGURE 5: Steady-state GTPase activity of K401. (A) K401 (0.5 μM) was incubated with 10 mM MgGTP and microtubules (0–20 μM tubulin) in ATPase buffer at 25 °C. The data show the rate of [α-^{32}P]GTP hydrolysis as a function of tubulin concentration. In the absence of microtubules, the rate of GTP hydrolysis was 0.03 ± 0.002 s^{-1}. The line is the fit of the data to a hyperbola with k_{cat} = 11.5 ± 0.3 s^{-1} and $K_{0.5,MT}$ = 1.9 ± 0.2 μM. (B) K401 (0.5 μM) was incubated with microtubules (15 μM tubulin) and MgGTP (0–16 mM) in ATPase buffer at 25 °C. The data show the rate of [α-^{32}P]-GTP hydrolysis as a function of MgGTP concentration. The line shows the computer fit of the data to a hyperbola: k_{cat} = 16 ± 1 s^{-1} and $K_{m,GTP}$ = 3.5 ± 0.6 mM.

exponential equation with the ADP rate of release at 0.006 s^{-1}. This rate agrees with the rate constant determined for steady-state turnover and indicates that ADP release limits the steady-state kinetics of ATP hydrolysis. These results demonstrate that K401 shows the unique properties of native kinesin: the tight ADP binding, the very low ATPase activity, and ADP release as rate-limiting during steady-state turnover.

Microtubule-Activated ATPase and GTPase Activity. Like native kinesin, K401 showed a dramatic increase in the rate of hydrolysis of MgATP with microtubules. Figure 4A shows that as the tubulin concentration was increased, the rate increased from 0.01 s^{-1} in the absence of microtubules to a maximum rate of 10.9 s^{-1} with $K_{0.5,MT}$ = 0.9 μM tubulin. Figure 4B shows the microtubule-activated ATPase activity as a function of MgATP concentration with the microtubules at 8 μM tubulin. The k_{cat} for ATP hydrolysis was determined to be 9 s^{-1} with the $K_{m,ATP}$ at 31 μM. These results show 1000-fold activation of the ATPase activity by microtubules to ~10 s^{-1}. The ATPase activity of the microtubules (10 μM tubulin) in the absence of K401 was very low (≤0.001 s^{-1}); therefore, ATP hydrolysis by the microtubules was not responsible for the high rate (10 s^{-1}) observed.

A number of investigators have demonstrated that MgGTP will support kinesin motility (Porter et al., 1987; Cohn et al., 1989). In addition, the rate of motility with MgGTP was comparable to the rate of motility promoted by MgATP. Figure 5 presents the evaluation of MgGTP as a substrate for K401 in comparison to MgATP (Figure 4). In the absence of microtubules, the GTPase activity was 0.03 ± 0.002 s^{-1}. Figure 5A shows the activation of the GTPase activity by microtubules (0–22 μM) measured at 10 mM MgGTP. The fit of the data to a hyperbola defined a maximum rate of 12 s^{-1} with $K_{0.5,MT}$ = 2 μM tubulin. The rate of MgGTP hydrolysis by microtubules (10 μM tubulin) in the absence of K401 was not detectable (≤0.001 s^{-1}). Figure 5B shows the rate of GTP hydrolysis as a function of MgGTP concentration at 15 μM tubulin (as microtubules). The k_{cat} was determined to be 16 s^{-1} with the $K_{m,GTP}$ = 3.5 mM. Although the GTPase activity shows the microtubule activation to 16 s^{-1}, the higher k_{cat}/K_m ratio for ATP (0.3 μM^{-1} s^{-1}) as compared to that for GTP (0.005 μM^{-1} s^{-1}) indicates that MgATP is the preferred substrate in vivo.

Summary. The results presented here show that K401 demonstrates the kinetic properties of a native kinesin. K401, like native kinesin, exhibits a very low ATPase activity in the absence of microtubules, tight binding of ADP at the active site, and slow release of ADP that limits steady-state turnover. K401 also shows a remarkable level of microtubule activation (~1000-fold) of the ATPase activity that is characteristic of all native kinesins purified to date. Recent structural studies have established that K401 binds to microtubules with an axial repeat of 8 nm and a stoichiometry of one kinesin head per tubulin dimer (Harrison et al., 1993). In addition, K401 offers unique advantages as a kinesin preparation for mechanistic studies and for our understanding of kinesin as a microtubule-based motor. K401 can be purified in milligram amounts as a homogeneous preparation. As expected for a protein expressed in bacteria, K401 lacks eukaryotic post-

translational modifications[2] that may be important in regulation of activity or function in vivo. K401 represents a single motor domain. It is small (~45 kDa) and without associated light chains; therefore, it is less complex than native kinesin. Lastly, because K401 is expressed in *E. coli*, in vitro mutagenesis can be used to explore structure–function relationships.

ACKNOWLEDGMENT

We thank Drs. Maggie de Cuevas and Larry Goldstein (Harvard University) for the kinesin clone BL21(DE3)/pET-K447, Dr. Alan Rosenberg (Brookhaven National Laboratory) for cell lines and plasmids that we used for the subcloning, and N. R. Lomax (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute) for providing taxol. We recognize and acknowledge the contributions made by Susan Dencler, Mark W. Signs, and Kevin A. Smith (Bioprocessing Resource Center, Pennsylvania State University) that enabled the large-scale fermentation procedure to be developed. S.P.G. gratefully acknowledges Dr. Smita Patel for her guidance, valuable suggestions, and exciting discussions throughout the period of these experiments.

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[2] B. C. Harrison and K. A. Johnson, unpublished observations.