THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 11, Issue of March 18, pp. 8610–8615, 1994
Printed in U.S.A.

Microtubule Movement by a Biotinated Kinesin Bound to a Streptavidin-coated Surface*

(Received for publication, September 30, 1993, and in revised form, December 6, 1993)

Elise Berliner‡, Hansraj K. Mahtani§, Sher Karki§, Lawrence F. Chu§, John E. Cronan, Jr.¶, and Jeff Gelles§‖**

From the ‡Biophysics Program, the §Graduate Department of Biochemistry, and the ‖Center for Complex Systems, Brandeis University, Waltham, Massachusetts 02254 and the ¶Departments of Microbiology and Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Kinesin, an ATP-dependent microtubule motor, can be studied *in vitro* in motility assays where the kinesin is nonspecifically adsorbed to a surface. However, adsorption can inactivate kinesin and may alter its reaction kinetics. We therefore prepared a biotinated kinesin derivative, K612-BIO, and characterized its activity in solution and when bound to streptavidin-coated surfaces. K612-BIO consists of the N-terminal 612 amino acids of the *Drosophila* kinesin α subunit linked to the 87-amino acid C-terminal domain of the biotin carboxyl carrier protein subunit of *Escherichia coli* acetyl-CoA carboxylase. The C-terminal domain directs the efficient post-translational biotination of the protein. We expressed K612-BIO at high levels using the baculovirus expression vector system and purified it to near-homogeneity. The expressed protein is completely soluble, and >90% is bound by streptavidin. K612-BIO steady-state ATPase kinetics ($K_{M,ATP}$ = 24 μM, $K_{0.5,microtubule}$ = 0.61 mg ml^{-1}, V_{max} = ~25 s^{-1} head^{-1}, 25 °C) are similar to those reported for intact kinesin. ATPase kinetics are not affected by the addition of streptavidin. Enzyme bound to a surface coated with streptavidin drove microtubule gliding in the presence of 2 mM ATP at 750 ± 130 nm s^{-1} (26 °C). Activity was abolished by pretreatment of the surface with biotin, indicating that the microtubule movements are due to specifically bound enzyme. Motility assays based on specific attachment of biotinated enzyme to streptavidin-coated surfaces will be useful for quantitative analysis of kinesin motility and may provide a way to detect activity in kinesin derivatives or kinesin-like proteins that have not yet been shown to move microtubules.

Kinesin is a motor enzyme that hydrolyzes ATP and uses the released free energy to move subcellular organelles along microtubules (1). Understanding the mechanism of energy transduction by this enzyme requires knowledge of the mechanical processes that move a kinesin molecule along a microtubule and of the kinetic rules that couple these mechanical processes to steps in the hydrolysis of ATP.

Kinesin movement is studied *in vitro* by using light microscopy to detect the gliding of microtubules over a kinesin-coated surface or the movement of kinesin-coated microscopic particles along immobilized microtubules. The motility velocity and the force produced by single or multiple kinesin molecules can be measured in such assays (1–6). However, quantitative interpretation of data derived from such motility assays can be problematical. Existing assays are based on nonspecific adsorption of kinesin to a glass or polymer surface, and there is evidence that surface adsorption can completely or partially inactivate kinesin molecules or alter their reaction kinetics. A kinesin derivative can display normal motility activity when adsorbed to one type of surface and reduced or no activity when it is absorbed to another (2, 7, 8). Some fragments of the α-subunit of kinesin display normal ATPase kinetics but produce no movement in motility assays unless they are prepared as fusion proteins containing a large segment of an unrelated protein (8, 9). The unrelated protein segment may serve to promote the surface adsorption of the kinesin fragment in a functional orientation. To facilitate detailed analysis of single-molecule movement kinetics (10) and quantitative comparison of the motility activity of surface-bound kinesin with the ATPase activity of kinesin in solution, it may be useful to study microtubule movement driven by kinesin molecules attached to a non-adsorbing surface by a specific chemical linkage in a uniform, functional orientation.

Biotination of enzymes provides a convenient method for attachment of the enzymes to protein-coated surfaces by taking advantage of the high affinity of biotin for streptavidin or avidin (11, 12). Chemical biotination of large proteins (*e.g.* with *N*-hydroxysuccinimidyl biotin reagents) frequently produces inhomogeneous mixtures of biotinated protein products. Cronan (13) developed a general method to produce fusion proteins that are biotinated at a single lysine residue near the C terminus using a conserved biotin-acceptor domain. Recombinant proteins that contain at their C terminus the ~80 C-terminal amino acids of the biotin carboxyl carrier protein (BCCP)[1] subunit of *Escherichia coli* acetyl-CoA carboxylase are efficiently post-translationally biotinated *in vivo* when the proteins are expressed in *E. coli* (13).

Intact kinesin molecules contain two globular head domains, each of which consists of between 340 and 450 of the N-terminal amino acids of a kinesin α subunit (14–16). Goldstein and

* This work was supported by grants from the National Institutes of Health and the Whitaker Foundation, by scholar awards from the Lucille P. Markey Charitable Trust and the Searle Scholars Program (to J. G.), by a National Institutes of Health training grant, and by fellowships from Brandeis University and the Brandeis University Undergraduate Fellows Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Biochemistry, Brandeis University, 415 South St., Waltham, MA 02254. Tel.: 617-736-2377; Bitnet: GELLES@BRANDEIS; Internet: GELLES@BINAH.CC.BRANDEIS.EDU.

[1] The abbreviations used are: BCCP, biotin carboxyl carrier protein; K612-BIO, fusion protein containing 612 N-terminal amino acids of the *Drosophila* kinesin linked to the 87 C-terminal amino acids of *E. coli* BCCP; AMP-PNP, 5′-adenylyl imidophosphate; BSA, bovine serum albumin; PIPES, piperazine-*N*,*N*′-bis-2-ethanesulfonic acid; DTT, dithiothreitol; bp, base pair(s).

co-workers (8, 9) expressed in *E. coli* truncated proteins containing the head domain and demonstrated that this domain is sufficient to produce the motility and the microtubule-activated ATPase activity that are characteristic of intact kinesin. Gilbert and Johnson (17) purified to homogeneity a bacterially-expressed kinesin fragment containing only the N-terminal 401 amino acids of the *Drosophila* kinesin α chain. The steady-state ATPase kinetics of this protein are similar to those of intact kinesin. However, it is not yet known whether such kinesin α-chain fragments can display all functional properties of intact kinesin including the ability to generate piconewton forces (4, 6) and the ability of single molecules to move continuously along microtubules (2, 3). Such functions might require the presence in the recombinant protein of a substantial portion of the kinesin α-helical coiled-coil rod domain (18) in order to produce a two-headed structure analogous to that in intact kinesin molecules.

We have purified and characterized a specifically biotinated kinesin fusion protein, K612-BIO, and studied its function in a motility assay based on the high affinity attachment of the biotinated enzyme to a streptavidin-coated surface. To ensure that it contains the structural features of kinesin necessary for full chemomechanical function, K612-BIO contains 612 N-terminal amino acids of the *Drosophila* kinesin α chain, a segment that includes the entire kinesin head domain and >160 amino acids of the rod domain. The C terminus of the rod domain is fused to the BCCP biotin-acceptor domain, an arrangement that spatially separates the head domain from the surface attachment point. K612-BIO expressed in an insect cell line using the baculovirus expression vector system is fully soluble and binds to streptavidin. We have purified K612-BIO to near-homogeneity and characterized its microtubule-stimulated ATPase activity. We have also measured the velocity of microtubule movements driven by K612-BIO specifically bound to a streptavidin-coated glass surface. This work is the first characterization of the mechanochemical activity of a purified kinesin that is not nonspecifically adsorbed to a surface.

MATERIALS AND METHODS

Plasmids—We prepared plasmids for expressing K612-BIO in *E. coli* and in the baculovirus expression vector system by sub-cloning a fragment of the *Drosophila* kinesin α subunit cDNA (clone 1, described by Yang *et al.* (Ref. 19), gift of L. S. B. Goldstein, Harvard University) and a fragment of the *E. coli* BCCP sequence from plasmid pCY142. The DNA sequence in the pCY142 fragment is derived from the *E. coli* chromosomal *KpnI/PstI* fragment encoding BCCP residues 70–133 (20) and a synthetic oligonucleotide encoding amino acids 134–156. To construct the bacterial expression plasmid pSK4, we ligated the 1830-bp *KspI/NcoI* fragment from clone 1, the synthetic oligonucleotide 5′-AGCTTGCGGC-CGCATATGTCCGC-3′/3′-ACGCCGGCGTATACAGG-5′, and the 2370-bp *HindIII/NcoI* fragment from pCY142 to produce an intermediate plasmid, pSK2. The 2123-bp *NdeI/SalI* fragment of this intermediate was subcloned into the polylinker of the pT7-7 expression vector (Ref. 21, gift of S. Tabor, Harvard Medical School) to give pSK4. To construct the baculovirus expression plasmid pSK3, we ligated the 1830-bp *KspI/NcoI* fragment from clone 1, the synthetic oligonucleotide 5′-AGCTTGCGGCC-GCCATGTCCGC-3′/3′-ACGCCGGCGGTACAGG-5′, and the 2370-bp *HindIII/NcoI* fragment of pCY142 to produce an intermediate plasmid, pSK1. The 2123-bp *NotI/BamHI* fragment of the intermediate was isolated and ligated to the 9602-bp *NotI/BamHI* fragment of pVL1392 (Invitrogen) to give pSK3. The validity of all plasmid constructs was tested by restriction mapping using the overlapping restriction sites in the synthetic oligonucleotides.

Bacterial Expression—To express K612-BIO in *E. coli*, we transformed strain BL21(DE3)pLysS (22) with pSK4. Mid-log cultures in TB medium (23) supplemented with 24 mg/liter biotin were induced with 1 mM isopropylthio-β-D-galactoside and incubated for 5 h at 37 °C. Harvested cell pellets were lysed by resuspending in 50 mM Tris-Cl⁻, pH 7.5, 10% sucrose, 1 mM EDTA, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} pepstatin A, 10 μg ml^{-1} N^{α}-*p*-tosyl-L-arginine methyl ester, 10 μg ml^{-1} *N*-tosyl-L-phenylalanine chloromethyl ketone, 1 μg ml^{-1} leupeptin, 10 μg ml^{-1} soybean trypsin inhibitor at 3 ml g^{-1} pellet and incubating for ~12 h at 0 °C. Lysis was verified by light microscopy.

Insect Cell Culture Expression—K612-BIO was expressed using the baculovirus expression vector system as described by Summers and co-workers (24, 25), with the following modifications. Multiple strains of recombinant virus were isolated from cells co-transfected with wild-type baculovirus DNA and pSK3 DNA. Control strains were prepared by substituting pVL1392 DNA for pSK3 DNA. Expression cultures were grown in 250-ml spinner flasks (Bellco) containing 135 ml of Sf9 cells in TNM-FH media, modified by Summers *et al.* (24) from Hink (26), supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin G sodium, 50 μg/ml streptomycin sulfate, 0.1 μg/ml fungizone (Life Technologies, Inc.), 200 mg/liter *d*-biotin and 2 mg/liter 20-hydroxyecdysone (27). Cultures were seeded at 1.5 × 10^6 cells ml^{-1} with cells preinfected with recombinant baculovirus at 10 plaque-forming units cell^{-1} and harvested after 72 h of growth at 26 °C and 60 rpm.

Protein Purification—Sf9 expression cultures were pelleted, and the cells were lysed by resuspension in 0.8 ml/135 ml culture of 50 mM imidazole, pH 6.7, 50 mM KCl, 4 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 10 μg/ml N^{α}-*p*-tosyl-L-arginine methyl ester, 10 μg ml^{-1} *N*-tosyl-L-phenylalanine chloromethyl ketone, 1 μg ml^{-1} leupeptin, 10 μg ml^{-1} soybean trypsin inhibitor. Lysates were spun at 10,900 × *g* for 15 min and 200,000 × *g* for 30 min to remove insoluble material. The clarified lysate was incubated with 1 mg ml^{-1} ribonuclease A (Sigma, Type II-A) for 30 min at 4 °C and chromatographed on Superose 6 (Pharmacia) in 50 mM imidazole, pH 6.7, 50 mM NaCl, 4 mM $MgCl_2$, 2 mM EGTA. The peak 1–2 ml was pooled and supplemented with 1 mM DTT to yield the purified protein preparation, which was stored at 0 °C. Under these conditions, the microtubule-stimulated ATPase-specific activity of the soluble fraction of the protein was constant for 3 weeks.

Microtubules—Calf brain tubulin was prepared by the method of Weisenberg *et al.* (28) as modified by Na and Timasheff (29) and stored at −70 °C until use. Tubulin at 2–10 mg ml^{-1} in 80 mM Na^+-PIPES, pH 8.0, 30% glycerol, 1 mM EGTA, 10 mM $MgCl_2$, and 1 mM GTP was polymerized by incubation for 30 min at 37 °C. Taxol (gift of N. R. Lomax, National Cancer Institute) was added to 40 μM, and the microtubules were incubated for 20 min at 37 °C, pelleted (21,800 × *g*, 20 min, 25 °C), and resuspended in the appropriate buffer for ATPase or motility assays. Tubulin concentration in the microtubule preparation was determined spectrophotometrically in 6 M guanidine-HCl using $\epsilon^{274}_{0.1\%} = 1.03$ (29).

Sedimentation of Microtubule-K612-BIO Complexes—14 μl of 1.1 mg ml^{-1} K612-BIO (in 50 mM imidazole, pH 6.7, 50 mM NaCl, 4 mM $MgCl_2$, 2 mM EGTA) was mixed with 17.5 μl of 3.4 mg ml^{-1} microtubules (in 100 mM Na^+-PIPES, pH 6.6, 1.0 mM EGTA, 1.0 mM $MgSO_4$, 40 μM taxol, 2 mM DTT) and 2 mM ADP or AMP-PNP. Microtubules and bound K612-BIO were pelleted at 30,200 × *g* for 20 min at 25 °C.

ATPase Assays—K612-BIO steady-state ATPase activity rate was measured spectrophotometrically in a coupled assay system (30). Assay conditions were as follows: 0.13 mg ml^{-1} pyruvate kinase (Sigma, type III), 0.2 mg ml^{-1} lactic dehydrogenase (Sigma, type XI), 0.12 mM NADH, 1 mM phosphoenolpyruvate, 20 μM taxol, 0.1 mg ml^{-1} bovine serum albumin (BSA) in 10 mM imidazole-$CH_3CO_2^-$, pH 6.7, 5 mM KCH_3CO_2, 2 mM EGTA, 4 mM $Mg(CH_3CO_2)_2$ at 25 °C. All assays contained 2 mg ml^{-1} microtubules and 0.1 mM ATP unless otherwise specified. To reduce light scattering from microtubules, a 5-mm path-length cuvette was used. Microtubules and ATP were added immediately prior to the commencement of the assays by the addition of K612-BIO. All reported activities were corrected for the rate of ATP hydrolysis by the microtubules; this correction was always <20%.

Motility Assays—We examined microtubule gliding driven by K612-BIO in two kinds of sample preparations. In the first kind, K612-BIO was specifically bound to a streptavidin-coated surface. Acid-treated (2.4 N HCl for 20 min, rinsed with H_2O) borosilicate glass no. 0 cover slips were rinsed in ethanol and dried. Flow cells of volume < ~10 μl were constructed by using two thin lines of silicone vacuum grease to form a channel between two of the cover slips. The cell was rinsed with 1 mg ml^{-1} biotinamidocaproyl bovine albumin (Sigma), incubated, rinsed four times with H_2O, rinsed twice with 1 mg ml^{-1} streptavidin, incubated, rinsed three times with H_2O, rinsed with 0.8 mg ml^{-1} α-casein in motility buffer (50 mM imidazole-Cl^-, pH 6.7, 50 mM KCl, 2 mM EGTA, 4 mM $MgCl_2$), incubated, rinsed once with 1 mg ml^{-1} BSA in motility buffer, incubated, rinsed once with 5.6 μg ml^{-1} K612-BIO, 0.1% Tween 20, 1 mg ml^{-1} BSA in motility buffer, incubated for 3 min, rinsed three times with 1 mg ml^{-1} BSA, 2 mM ATP in motility buffer, and rinsed with 0.072 or 0.018 mg ml^{-1} microtubules, 2 mM ATP in motility buffer. In control experiments, the H_2O rinses after streptavidin addition were

replaced with biotin-saturated H_2O and the motility buffer was supplemented with 0.01 volumes biotin-saturated H_2O in all rinses except the final one. In the second kind of sample preparation, we examined microtubule gliding driven by K612-BIO bound nonspecifically to an untreated surface. A flow cell (prepared as described above, except that the glass was not acid-treated) was rinsed with 28 μg ml^{-1} K612-BIO in motility buffer, incubated for 3 min, rinsed three times with 1 mg ml^{-1} BSA and 2 mM ATP in motility buffer, and rinsed once with 0.018 mg ml^{-1} microtubules, 1 mg ml^{-1} BSA, 2 mM ATP in motility buffer. In the preparation of both kinds of specimens, all rinses were 10 μl and all incubations were 10 min unless otherwise specified.

The sample preparations were examined by video differential interference contrast microscopy as previously described (31, 32), and images were recorded and processed with the hardware used before (33). Randomly chosen microscope fields were recorded on an sVHS video recorder. Recorded video sequences were transferred without averaging to the image processor for measurement of microtubule gliding velocities using custom written software. To estimate the mean surface density of moving microtubules, we randomly selected time points within video recordings. At each time point, the number of microtubules moving in each field was determined by viewing a continuously repeating loop of 40 frames (1.33 s) of recorded video centered at the selected time point. The mean surface density of moving microtubules was calculated by averaging such counts for several time points in two fields selected at random in the sample and dividing by the area of the video field. Image magnification was calibrated with a diffraction grating standard (Ernest F. Fullam, 10380).

SDS-Polyacrylamide Gel Electrophoresis and Biotin Blots—10.5% SDS-polyacrylamide gels (34) were stained for total protein with Coomassie Brilliant Blue R-250 or were transferred to nitrocellulose membranes for detection of biotinated proteins. The membranes were stained with an avidin/biotinated alkaline phosphatase complex and a chromogenic substrate (Vectastain ABC-Alkaline Phosphatase and alkaline phosphatase substrate kit II, Vector Laboratories).

Densitometry—Reflected light densitometry on gels and blots was performed with a Scanjet II (Hewlett-Packard) and Image 1.10 software (National Institutes of Health).

Non-denaturing Polyacrylamide Gel Electrophoresis—5% polyacrylamide gels were run under non-denaturing conditions (35) and stained with Coomassie Brilliant Blue R-250. All samples were in 10-μl volumes. Samples were incubated 30 min at 0 °C prior to electrophoresis to allow the formation of the K612-BIO-streptavidin complexes.

Protein Concentration Assay—Protein concentrations were measured using the method of Bradford (36). Assays were standardized with BSA. BSA concentrations were determined assuming $\epsilon^{274}_{0.1\%} = 0.67$ (37).

RESULTS

Expression and Purification of a Biotinated Kinesin Derivative—In order to prepare a functional biotinated kinesin derivative, we first tried to express in *E. coli* a recombinant protein, K612-BIO, consisting of the head and a portion of the rod domain of *Drosophila* kinesin fused to the biotin acceptor domain of *E. coli* BCCP. Overexpressed K612-BIO was the most abundant protein in the bacterial lysate, as judged by SDS-polyacrylamide gel electrophoresis. However, most or all of the protein pelleted upon centrifugation of the lysate, suggesting that K612-BIO forms insoluble aggregates or that it binds to insoluble material present in the lysate (38).

In contrast to the results obtained with bacteria, expression of K612-BIO in Sf9 cells using the baculovirus expression vector system produces a high yield of soluble protein. Lysates of Sf9 cells transfected with a recombinant baculovirus strain directing the synthesis of K612-BIO contain as their most prominent protein a species (Fig. 1*a*, *lane LYS(pSK3)*) at approximately the expected M_r = 90,687 of K612-BIO. This protein is not detected in control lysates (Fig. 1*a*, *lane LYS(pVL1392)*) from Sf9 cells transfected with a recombinant baculovirus strain that lacks the K612-BIO cDNA insert. Nearly all of the expressed K612-BIO is soluble (Fig. 1*a*, *lane CLAR*). Because of the large amount of K612-BIO present in the clarified lysate, K612-BIO can be isolated free from most contaminating protein in a single gel filtration step (Table I). The product of this step (Fig. 1*a*, *lane PUR*) is ~75% pure as estimated from densitometry of the stained electrophoresis gel.

Biotination and Binding by Streptavidin—A nitrocellulose blot stained for biotinated proteins shows that K612-BIO is biotinated (Fig. 1*b*). Biotinated K612-BIO is present in the lysate at a much higher concentration than any endogenous biotinated proteins, and no contaminating biotinated proteins are detected in the purified K612-BIO preparation.

The fraction of K612-BIO molecules that bind streptavidin was determined by gel electrophoresis of K612-BIO/streptavidin mixtures under non-denaturing conditions (Fig. 2). K612-BIO migrates predominantly as a single band. The staining intensity in the K612-BIO band is diminished by >90% when the enzyme is mixed with a 10-fold by weight excess of streptavidin, demonstrating that essentially all of the native K612-BIO molecules are biotinated. New bands are observed in the mixture lane, consistent with the formation of at least two distinct K612-BIO-streptavidin complexes. In control experiments, the electrophoretic mobility of K612-BIO was not affected by the addition of streptavidin that had been pretreated with excess biotin (data not shown). The binding of K612-BIO

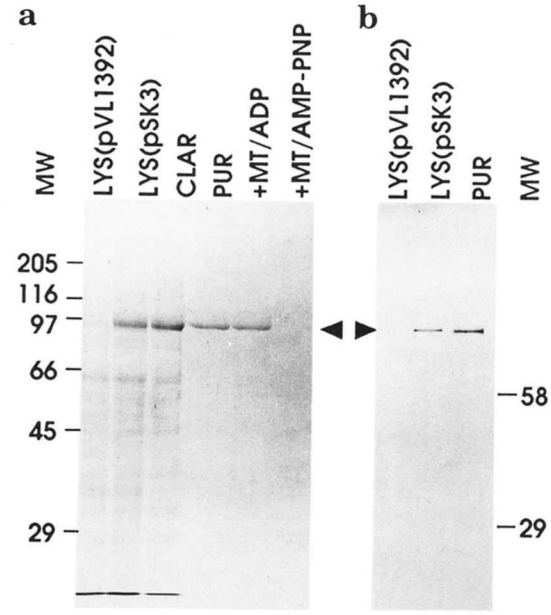

FIG. 1. *a*, K612-BIO visualized by Coomassie Blue staining of an SDS polyacrylamide electrophoresis gel. *MW*, 10^{-3} × standard protein molecular weight. Expression and solubility: *LYS(pVL1392)*, 0.13 μl of lysate from Sf9 cells infected with recombinant baculovirus lacking the K612-BIO cDNA insert; *LYS(pSK3)*, 0.13 μl of lysate from Sf9 cells infected with recombinant baculovirus containing the K612-BIO sequence from pSK3; *CLAR*, 0.13 μl of clarified LYS(pSK3). Supernatants after sedimentation: *PUR*, 0.22 μg of purified K612-BIO plus 2 mM ADP; *+MT/ADP*, 0.22 μg of K612-BIO plus 2 mM ADP and microtubules; *+MT/AMP-PNP*, 0.22 μg of purified K612-BIO plus 2 mM AMP-PNP and microtubules. *b*, K612-BIO visualized on a nitrocellulose blot stained for biotinated proteins. *LYS(pVL1392)*, 0.0025 μl of lysate from Sf9 cells infected with recombinant baculovirus lacking the K612-BIO cDNA insert; *LYS(pSK3)*, 0.0025 μl of lysate from Sf9 cells infected with recombinant baculovirus containing the K612-BIO sequence from pSK3; *PUR*, 0.025 μg of purified K612-BIO. *Arrows*, K612-BIO.

TABLE I
K612-BIO purification

	Clarified lysate	Purified protein
Total protein (mg)	19.8	2.1
Protein concentration (mg ml^{-1})	20.8	1.0
Total ATPase activity[a] (μmol min^{-1})	41.6	21.8
Specific ATPase activity[a] (μmol min^{-1}/mg total protein)	2.1	10.4

[a] 25 °C, 2 mg ml^{-1} microtubules, 0.1 mM ATP.

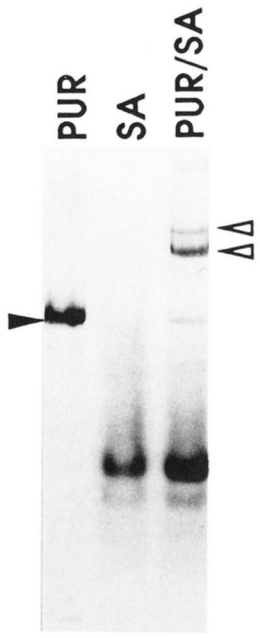

FIG. 2. **Non-denaturing polyacrylamide gel electrophoresis of K612-BIO and K612-BIO-streptavidin complexes.** *PUR*, 6.5 μg of K612-BIO; *SA*, 63 μg of streptavidin; *PUR/SA*, mixture of 63 μg of streptavidin and 6.5 μg of K612-BIO. *Solid arrow*, K612-BIO. *Open arrows*, K612-BIO-streptavidin complexes.

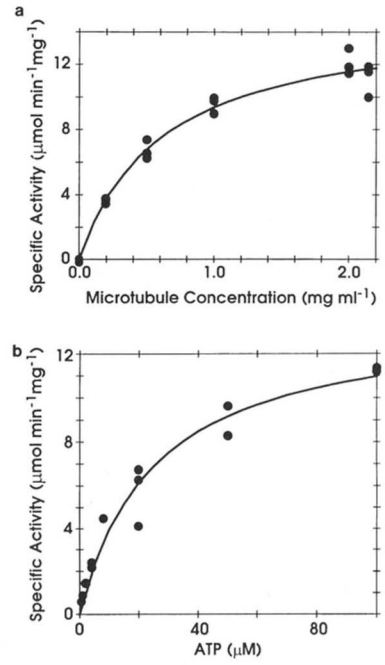

FIG. 3. **K612-BIO ATPase activity at 25 °C.** In *a*, ● indicates specific activity dependence on microtubule concentration at 0.1 mM ATP. *Line* shows computer fit to the Michaelis-Menten equation with V_{max} = 15.0 ± 0.7 (S.E.) μmol min^{-1} mg^{-1}, $K_{0.5,microtubule}$ = 0.61 ± 0.08 mg ml^{-1}. In *b*, ● indicates specific activity dependence on ATP concentration at 2 mg ml^{-1} microtubules. *Line*, shows computer fit to the Michaelis-Menten equation with V_{max} = 13.6 ± 1.1 μmol min^{-1} mg^{-1}, $K_{M,ATP}$ = 24 ± 5 μM.

to streptavidin was also confirmed spectrophotometrically by monitoring the displacement of the reporter dye 2-(4′-hydroxyazobenzene)benzoic acid (39) from the biotin binding site of streptavidin by K612-BIO (data not shown).

Nucleotide-dependent Binding to Microtubules—Kinesin binds tightly to microtubules in the presence of AMP-PNP (1, 40) but dissociates in the presence of ADP (41). This behavior is also shown by K612-BIO, which cosediments with microtubules in the presence of the ATP analog AMP-PNP, but not in the presence of ADP (Fig. 1*a*).

ATPase—Intact kinesin has a low basal ATPase rate; this rate is stimulated 10^3-fold upon the addition of microtubules (30). Like intact kinesin, purified K612-BIO has no detectable ATPase activity in the absence of microtubules under the conditions of our measurements (Fig. 3*a*). The ATPase activity shows a hyperbolic dependence on microtubule concentration (Fig. 3*a*) and on ATP concentration (Fig. 3*b*). Fitting these data to the Michaelis-Menten equation gives $K_{0.5,microtubules}$ = 0.61 ± 0.08 (S.E.) mg ml^{-1} and $K_{M,ATP}$ = 24 ± 5 μM. Determination of V_{max} for several independent enzyme preparations gave values in the range 9.6–15.3 μmol min^{-1}/mg total protein. This range corresponds to 19–30 s^{-1} ATPase turnovers per head based on the estimated purity (75%) of the enzyme preparation. These kinetic parameters are similar to those found for intact kinesin (42). The addition of a 10-fold by weight excess of streptavidin over K612-BIO to the assay does not affect the rate of ATP hydrolysis at 0.1 mM ATP, 2 mg ml^{-1} microtubules (data not shown). This observation suggests that the catalytic properties of K612-BIO will not be altered upon binding to streptavidin-coated surfaces.

Motility—To determine if we could study microtubule motility driven by K612-BIO molecules bound specifically to a surface by their biotin moieties, we performed preliminary experiments in which we nonspecifically adsorbed streptavidin to a glass coverslip and then treated the surface with a solution of K612-BIO. When the free enzyme was rinsed from the cell and replaced with a solution containing microtubules and ATP, we observed microtubule gliding. However, the gliding was also seen in samples in which the streptavidin-coated surface had been pretreated with excess biotin prior to the addition of K612-BIO. This suggests that some or all of the observed motility arises from nonspecifically adsorbed enzyme. To eliminate this nonspecific binding, we adopted a protocol in which we saturated residual nonspecific protein binding sites on the streptavidin-coated surface with a high concentration of carrier protein prior to the incubation with K612-BIO (see "Materials and Methods"). In such samples, we observed a high surface density of moving microtubules (1.6 ± 0.16 (S.E.) × 10^{-2} μm^{-2}; Fig. 4*a*). Pretreatment of such samples with excess biotin prior to the addition of K612-BIO almost completely abolished motility; the mean surface density of moving microtubules was reduced 29-fold. This experiment demonstrates that essentially all of the motility seen in the samples without biotin treatment is due to K612-BIO that is bound specifically to the streptavidin-coated surface. Samples with specifically bound K612-BIO exhibited a distribution of microtubule gliding velocities (Fig. 4*b*) with a mean of 7.5 ± 1.3 (S.D.) × 10^2 nm s^{-1}. A similar value (8.4 ± 0.7 × 10^2 nm s^{-1}) was obtained from K612-BIO that was nonspecifically adsorbed to a glass surface.

DISCUSSION

This work is the first reported use of animal cells to express a cloned kinesin cDNA. This method has two advantages over bacterial expression. First, the baculovirus expression system used here produced milligram quantities of *soluble* K612-BIO. In contrast, bacterial expression produced little soluble K612-BIO although the protein was expressed at high levels. Bacterial expression has failed to produce fully soluble product in previous studies of kinesin constructs that contain a substantial portion of the rod domain (9, 43). Second, kinesin derivatives expressed in eukaryotic cells may have functionally important post-translational modifications that are missing in bacterially expressed enzymes. While such modifications may be present in the purified K612-BIO, our inability to produce

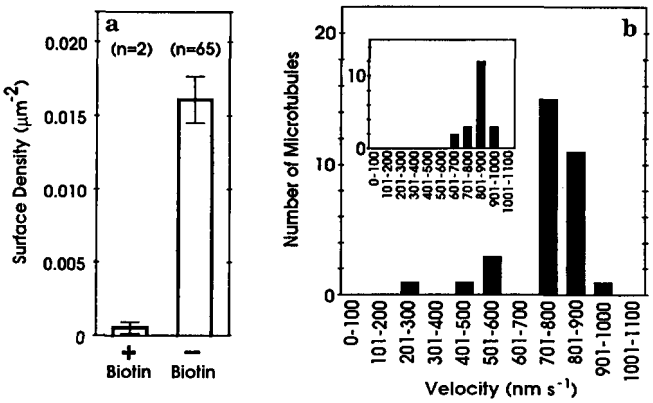

FIG. 4. **Microtubule motility driven by K612-BIO at 2 mM ATP.** *a*, mean ± S.E. surface density of moving microtubules on streptavidin-coated glass with specifically bound K612-BIO (*–BIOTIN*; a total of 65 moving microtubules observed in 10 time points) or in a control sample in which the streptavidin-coated glass was saturated with excess biotin before the addition of K612-BIO (*+BIOTIN*; a total of 2 moving microtubules observed in 9 time points) at 0.072 mg ml^{-1} microtubules, 25 °C. *b*, distribution of gliding velocities for microtubules driven by K612-BIO specifically bound to streptavidin-coated glass at 0.018 mg ml^{-1} microtubules, 26 °C. *Inset*, distribution of gliding velocities for microtubules driven by K612-BIO nonspecifically adsorbed to glass at 0.018 mg ml^{-1} microtubules, 26 °C.

sufficient soluble bacterially expressed enzyme precluded direct comparison of the functional properties of K612-BIO derived from *E. coli* with that from Sf9 cells. To our knowledge, the work presented here is also the first use of an animal cell expression system to prepare biotinated fusion proteins. The DNA sequence that directs synthesis of the biotin acceptor domain of K612-BIO is from a bacterial gene. Nevertheless, K612-BIO is efficiently biotinated by the Sf9 cells. Recognition of the bacterial biotin acceptor domain by eukaryotic cells is undoubtedly a consequence of the high sequence similarity between such domains in enzymes from phylogenetically diverse species (20). Previously, a fusion protein containing a bacterial biotin acceptor sequence was shown to be post-translationally biotinated in yeast cells (13, 44).

Intact kinesin α subunits form a dimer containing an α-helical coiled-coil rod and two globular heads (14, 15, 52). The position of the head/rod junction in the sequence is not precisely known, but is thought to be between 350 and 450 amino acids from the N terminus of the α subunit (8, 9, 16). K612-BIO includes the 612 N-terminal amino acids of the kinesin α subunit. Since it contains >160 amino acids of the rod domain, it is expected to form dimers. Surprisingly, sedimentation equilibrium, sedimentation velocity, and diffusion coefficient measurements indicate that this protein forms a single, higher molecular weight species, probably a tetramer.[2] From the appearance of rotary-shadowed K612-BIO molecules in the electron microscope, we believe that the tetramers result from self-association of dimers at their rod ends. It is unlikely that the biotin acceptor domain alone is responsible for the formation of tetramers because a proteolytic fragment of BCCP similar to the domain used in K612-BIO is itself monomeric (45, 46). Also, there is no evidence that the binding of K612-BIO to streptavidin affects the aggregation state of the protein. The formation of the tetramers could be due to exposure of hydrophobic residues due to misfolding of K612-BIO near the junction of the kinesin and BCCP sequences. Nevertheless, all characterized functional properties of K612-BIO are similar to those of intact kinesin and also to those of previously studied kinesin α chain derivatives.

[2] K. Shearwin, E. Berliner, H. K. Mahtani, and J. Gelles, unpublished data.

Fig. 4*b* reports the velocity of microtubule gliding driven *in vitro* by K612-BIO attached to a surface using the biotin-streptavidin interaction. Stewart *et al.* (8) observed that the addition of an antibody against glutathione *S*-transferase stimulated microtubule gliding by kinesin/glutathione *S*-transferase fusion proteins. However, it is unclear whether the stimulation arose from specific binding of the fusion protein by the antibody. The data of Fig. 4 allow us to directly compare the velocities of microtubule movement driven by a kinesin derivative when it is specifically and nonspecifically bound to a surface. *In vivo*, kinesin is thought to drive the anterograde fast transport of axonal organelles (47, 48) at velocities up to 1.5 μm s^{-1} or more (49, 50). This velocity is higher than the velocities reported for intact kinesin and for kinesin fragments in motility assays based on nonspecific surface adsorption of enzyme (8, 9, 40). Previously, it was hypothesized that the lower velocities in such assays might be explained by partial inhibition of the kinesin as a result of the nonspecific surface adsorption. However, our results show that, at least for K612-BIO, the velocity of kinesin-driven microtubule gliding is similar when it is specifically attached and when it is nonspecifically adsorbed.

Kinesin and its derivatives are inactivated to varying extents by nonspecific adsorption to surfaces. Pretreatment of a surface with carrier proteins largely eliminates inactivation of intact kinesin but not of K447-SP, a derivative that unlike K612-BIO lacks most of the rod domain (2, 3, 51). Specific attachment of such a derivative using the biotin/streptavidin methods described here results in greatly reduced inactivation.[3] Eliminating or limiting enzyme inactivation is essential to establish that microtubule movements observed at high enzyme dilution are driven by single enzyme molecules (51).

We now know of many cellular enzymes with a head domain structurally similar to that in one of the three canonical cytoskeletal motor ATPases myosin, dynein, and kinesin. However, relatively few of the motor homologs have been shown to drive movement *in vitro*. In some cases, failure to observe movement may be caused by enzyme inactivation due to surface adsorption or by adsorption of enzyme molecules in orientations that cannot interact with F-actin or microtubules. Enzymes that show no activity in motility assays based on nonspecific adsorption may produce movement in an assay like that described here in which the tail of the enzyme is linked to a protein-coated surface by a specific, stable chemical interaction.

Acknowledgment—We thank Dr. Susan Lowey for comments on the manuscript.

REFERENCES

1. Vale, R. D., Reese, T. S., and Sheetz, M. P. (1985) *Cell* **42,** 39–50
2. Howard, J., Hudspeth, A. J., and Vale, R. D. (1989) *Nature* **342,** 154–158
3. Block, S. M., Goldstein, L. S. B., and Schnapp, B. J. (1990) *Nature* **348,** 348–352
4. Kuo, S. C., and Sheetz, M. P. (1993) *Science* **260,** 232–234
5. Hall, K., Cole, D. G., Yeh, Y., Scholey, J. M., and Baskin, R. J. (1993) *Nature* **364,** 457–459
6. Hunt, A., and Howard, J. (1993) *Biophys. J.* **64,** a263 (abstr.)
7. Kachar, B., Albanesi, J. P., Fujisaki, H., and Korn, E. D. (1987) *J. Biol. Chem.* **262,** 16180–16185
8. Stewart, R. J., Thaler, J. P., and Goldstein, L. S. B. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5209–5213
9. Yang, J. T., Saxton, W. M., Stewart, R. J., Raff, E. C., and Goldstein, L. S. B. (1990) *Science* **249,** 42–47
10. Svoboda, K., Schmidt, C. F., Schnapp, B. J., and Block, S. M. (1993) *Nature* **365,** 721–727
11. Green, N. M. (1975) *Adv. Protein Chem.* **29,** 85–133
12. Wilchek, M., and Bayer, E. A. (1988) *Anal. Biochem.* **171,** 1–32
13. Cronan, J. E. (1990) *J. Biol. Chem.* **265,** 10327–10333
14. Yang, J. T., Laymon, R. A., and Golstein, L. S. B. (1989) *Cell* **56,** 879–889
15. Hirokawa, N., Pfister, K. K., Yorifuji, H., Wagner, M. C., Brady, S. T., and Bloom, G. S. (1989) *Cell* **56,** 867–878

[3] E. Berliner and J. Gelles, unpublished observations.

16. Scholey, J. M., Heuser, J., Yang, J. T., and Goldstein, L. S. B. (1989) *Nature* **338,** 355–357
17. Gilbert, S. P., and Johnson, K. A. (1993) *Biochemistry* **32,** 4677–4684
18. de Cuevas, M., Tao, T., and Goldstein, L. S. B. (1992) *J. Cell Biol.* **116,** 957–965
19. Yang, J. T., Saxton, W. M., and Goldstein, L. S. B. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1864–1868
20. Li, S., and Cronan, J. E., Jr. (1992) *J. Biol. Chem.* **267,** 855–863
21. Tabor, S., and Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 1074–1078
22. Studier, F. W. (1991) *J. Mol. Biol.* **219,** 37–44
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Summers, M. D., and Smith, G. E. (1987) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experiment Station, College Station, TX
25. Luckow, V. A., and Summers, M. D. (1988) *Biotechnology* **6,** 47–55
26. Hink, W. F. (1970) *Nature* **226,** 466–467
27. Sarvari, M., Csikos, G., Sass, M., Gal, P., Schumaker, V. N., and Zavodszky, P. (1990) *Biochem. Biophys. Res. Commun.* **167,** 1154–1161
28. Weisenberg, R. C., Borisy, G. G., and Taylor, E. W. (1968) *Biochemistry* **7,** 4466–4479
29. Na, G. C., and Timasheff, S. N. (1980) *Biochemistry* **19,** 1347–1354
30. Hackney, D. D. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 6314–6318
31. Schnapp, B. J. (1986) *Methods Enzymol.* **134,** 561–573
32. Gelles, J., Schnapp, B. J., and Sheetz, M. P. (1988) *Nature* **331,** 450–453
33. Schafer, D. A., Gelles, J., Sheetz, M. P., and Landick, R. (1991) *Nature* **352,** 444–448
34. Laemmli, U. K. (1970) *Nature* **227,** 680–685
35. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121,** 404–427
36. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
37. Fasman, G. D. (1976) *Handbook of Biochemistry and Molecular Biology*, CRC Press, Cleveland, OH
38. Frankel, S., Sohn, R., and Leinwand, L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 1192–1196
39. Green, N. M. (1965) *Biochem. J.* **94,** 23c-24c
40. Saxton, W. M., Porter, M. E., Cohn, S. A., Scholey, J. M., Raff, E. C., and McIntosh, J. R. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1109–1113
41. Romberg, L., and Vale, R. D. (1993) *Nature* **361,** 168–170
42. Wagner, M. C., Pfister, K. K., Bloom, G. S., and Brady, S. T. (1989) *Cell Motil. Cytoskel.* **12,** 195–215
43. Gilbert, S. P., de Cuevas, M., Goldstein, L. S. B., and Johnson, K. A. (1990) *J. Cell Biol.* **111,** 416a (abstr.)
44. Ackerman, S. H., Martin, J., and Tzagoloff, A. (1992) *J. Biol. Chem.* **267,** 7386–7394
45. Nervi, A. M., Alberts, A. W., and Vagelos, P. R. (1971) *Arch. Biochem. Biophys.* **143,** 401–411
46. Fall, R. R., and Vagelos, P. R. (1973) *J. Biol. Chem.* **248,** 2078–2088
47. Hirokawa, N., Sato-Yoshitake, R., Kobayashi, N., Pfister, K. K., Bloom, G. S., and Brady, S. T. (1991) *J. Cell Biol.* **114,** 295–302
48. Amaratunga, A., Morin, P. J., Kosik, K. S., and Fine, R. E. (1993) *J. Biol. Chem.* **268,** 17427–17430
49. Allen, R. D., Metuzals, J., Tasaki, I., Brady, S. T., and Gilbert, S. P. (1982) *Science* **218,** 1127–1129
50. Vale, R. D., Schnapp, B. J., Reese, T. S., and Sheetz, M. P. (1985) *Cell* **40,** 559–569
51. Schnapp, B. J., Block, S. M., Goldstein, L. S. B., Stewart, R. J., and Godek, C. P. (1991) *Biophys. J.* **59,** 567a (abstr.)
52. Hackney, D. D., Levitt, J. D., and Wagner, D. D. (1991) *Biochem. Biophys. Res. Commun.* **174,** 810–815