The Motile β/IC1 Subunit of Sea Urchin Sperm Outer Arm Dynein Does Not Form a Rigor Bond

Anthony G. Moss, Jean-Luc Gatti, and George B. Witman

Cell Biology Group and Male Fertility Program, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

Abstract. We used in vitro translocation and cosedimentation assays to study the microtubule binding properties of sea urchin sperm outer arm dynein and its β/IC1 subunit. Microtubules glided on glass-absorbed sea urchin dynein for a period of time directly proportional to the initial $MgATP^{2-}$ concentration and then detached when 70–95% of the $MgATP^{2-}$ was hydrolyzed. Detachment resulted from $MgATP^{2-}$ depletion, because (a) perfusion with fresh buffer containing $MgATP^{2-}$ reconstituted binding and gliding, (b) microtubules glided many minutes with an ATP-regenerating system at ATP concentrations which alone supported gliding for only 1–2 min, and (c) microtubules detached upon total hydrolysis of ATP by an ATP-removal system. The products of ATP hydrolysis antagonized binding and gliding; as little as a threefold excess of ADP/P_i over ATP resulted in complete loss of microtubule binding and translocation by the β/IC1 subunit. In contrast to the situation with sea urchin dynein, microtubules ceased gliding but remained bound to glass-absorbed *Tetrahymena* outer arm dynein when $MgATP^{2-}$ was exhausted. Cosedimentation assays showed that *Tetrahymena* outer arm dynein sedimented with microtubules in an ATP-sensitive manner, as previously reported (Porter, M.E., and K. A. Johnson. *J. Biol. Chem.* 258: 6575–6581). However, the β/IC1 subunit of sea urchin dynein did not cosediment with microtubules in the absence of ATP. Thus, this subunit, while capable of generating motility, lacks both structural and rigor-type microtubule binding.

D YNEINS are large, multicomponent ATPases responsible for minus-end–directed microtubule-based cell motility, such as ciliary and flagellar beating, certain types of intracellular transport, and organelle sorting (65). In cilia and flagella, they form the inner and outer rows of arms which connect the outer doublet microtubules. Several lines of evidence indicate that the arms are permanently bound to the A-tubule of each doublet, and that, concomitant with a cycle of ATP binding and hydrolysis, they undergo a cycle of attachment to and detachment from the B-tubule of the adjacent doublet, giving rise to interdoublet sliding (42, 43, 52). When this sliding is resisted by structures within the axoneme, axonemal bending results (49). Because the arms appear to push on an adjacent doublet only in a base-to-tip direction (6, 41), it has been proposed that at any one time the arms on only one side of an axonemal bend are actively generating sliding, while arms on the opposite side of the axoneme must be disengaged to permit passive interdoublet sliding in the opposite direction (44). When the axoneme bends back the other way, the previously active arms must be switched off and the previously disengaged arms switched on. Bend formation, coordinated wave propagation, and cyclic beating must be achieved by controlling the timing and positioning of dynein arm activity and resistance to sliding (3, 4, 51, 68).

The best understood of all dyneins is the outer arm dynein, which is composed of three ATPase-containing subunits in *Chlamydomonas* and *Tetrahymena*, but only two such subunits in sea urchins and vertebrates (7, 67). In all cases, each subunit is organized around a single copy of a high molecular weight polypeptide (α, β, and in *Chlamydomonas* and *Tetrahymena*, γ heavy chains); each subunit also contains one or more intermediate and/or light chains (for reviews see references 67, 69, 70). In cases where the individual subunits have been isolated and characterized, they have been found to differ substantially in their enzymatic and structural properties (1, 20, 21, 22, 35, 36, 54, 55). However, little is known about the specific roles of the different subunits in flagellar movement.

A complete understanding of how ciliary and flagellar movement is generated and controlled will require detailed knowledge of how the inner and outer arms and their individual subunits interact with outer doublet microtubules to produce force and regulate both active and passive sliding. The microtubule-binding properties of axonemal dynein have been investigated in the intact axoneme (8, 9, 10, 29, 30, 33, 50, 62), but the presence of both inner and outer

arms, and of multiple ATPase-containing subunits within each arm, has made the results of these studies difficult to interpret in terms of the function of individual dyneins and dynein subunits. Therefore, to better understand dynein-microtubule interactions at the molecular level, these interactions have been investigated in progressively simpler systems, including rebinding of isolated dynein to extracted axonemes or outer doublet microtubules (Fay and Witman, 1977. *J. Cell Biol.* 75:286(abstract); and 25, 26, 53, 63, 64), and ultimately to microtubules assembled in vitro from purified tubulin (for reviews see references 5, 14). These studies, all of which were carried out using dynein isolated either from *Chlamydomonas* or *Tetrahymena*, indicated that dynein binds to microtubules in a tight "rigor" complex in the absence of ATP, and this has been accepted as a fundamental property of dynein.

We demonstrated previously that the force-transducing activity of purified dynein could be examined in vitro using a gliding microtubule assay (32). This assay revealed for the first time that outer arm dynein by itself was capable of force production, and the assay subsequently has been used for investigating the force-producing properties of one of the subunits of the outer arm (40, 58, 59). We now show that the same assay can be used to investigate directly the microtubule-binding properties of motility-competent dynein and dynein subunits. A major advantage of this assay is that binding and force production can be monitored simultaneously in precisely the same population of dynein molecules. Moreover, because the dynein is immobilized on a glass surface and bound microtubules are held in close proximity to that surface, the physical conditions of the assay resemble those that exist in the axoneme. Finally, this solid-phase assay requires lesser amounts of both dynein and microtubules than binding assays based on cosedimentation or turbidometric methods, and thus should be very useful for studying the dyneins of those organisms from which only limited amounts of material are available.

The results presented here show that microtubules bind to and are translocated by isolated sea urchin outer arm dynein and its purified β/IC1 subunit absorbed to a glass coverslip as long as sufficient ATP is present, but are released upon depletion of MgATP^{2-}. These observations are contrary to the generally accepted notion that dynein forms "rigor cross-bridges" to microtubules in the absence of ATP. In the accompanying paper (27), we show that intact sea urchin outer arm dynein does form rigor cross-bridges in solution; presumably this property is not evident in the solid-state binding assay because the α subunit, which appears to be responsible for rigor binding, is not available to interact with the microtubules. Thus, the β/IC1 subunit of sea urchin outer arm dynein has microtubule-binding properties significantly different from those of the intact outer arm dynein of either sea urchin or *Tetrahymena* (37). It is possible that one or more force-generating subunits of all dyneins have a low affinity for microtubules in the absence of ATP, but that in solution assays this has been masked by the properties of the other subunits in the intact dynein.

Materials and Methods

Isolation of Axonemal Dynein

Sea urchins (*Strongylocentrotus purpuratus*) were spawned by intracoe-lomic injection of 0.5 M KCl, and their sperm collected either dry onto a plastic Petri dish on ice, or into ice-cold unbuffered 0.5 M NaCl. Intact 21S outer arm dynein was extracted as described previously (32) by a one-step homogenization in 1% Triton-X100 or by the sucrose homogenization/NP-40 method of Sale and Fox (40), followed by sedimentation in a 5–20% sucrose gradient prepared in 10 mM Tris/HCl, pH 7.3, 200 mM NaCl, 4 mM MgSO$_4$, 0.2 mM EDTA, 0.2 mM PMSF. The β/IC1 subunit was isolated by the method of Sale and Fox (40). Motile *Tetrahymena* 22S outer arm dynein was generously provided by Drs. S. Marchese-Ragona and K. Johnson and Mr. K. Facemyer (Pennsylvania State University, State College, PA). Motile *Tetrahymena* 14S dynein was prepared by the method of Johnson (19) except that cultures were grown in 4-liter diptheria toxin flasks aerated at 32°C, and the crude high salt extract was layered directly on a 5–25% sucrose gradient for ultracentrifugation. The sucrose gradient-purified dyneins were used fresh, or frozen in 100–200-μl aliquots by immersion in liquid nitrogen and stored in liquid nitrogen until use. The ATPase and motile properties of the frozen dynein were unchanged from fresh material.

Taxol-stabilized Microtubules

Taxol-stabilized bovine brain microtubules were routinely prepared as described by Vallee (60). Briefly, microtubule protein was carried through three cycles of assembly/disassembly and DEAE-Sephadex column purification, and frozen as purified tubulin in 50-μl aliquots by immersion in liquid nitrogen for subsequent storage at -80°C. Aliquots were thawed, and microtubules polymerized at 37°C for 20 min and then stabilized by the addition of 250–300 μl of motility buffer (see below) containing 20 μM taxol. Microtubules used for the motility assay were typically 2–10 μm in length. Microtubules used for sedimentation assays were washed once by centrifugation at 39,000 g for 30 min at 22°C in taxol-containing motility buffer, and resuspended to $\sim$6 mg/ml stock concentration, as determined by protein dye binding (2).

Solid Phase In Vitro Motility Assay

Sucrose gradient-purified dynein was routinely diluted to $\sim$200 μg/ml in cold TEMK (10 mM Tris/HCl, pH 7.8, 0.5 mM EDTA, 4 mM MgSO$_4$, 25 mM KCl) or TEMA (10 mM Tris/HCl, pH 7.8–8.0, 0.5 mM EDTA, 4 mM MgSO$_4$, 100 mM CH$_3$COOK) motility buffer before application to the motility chamber. The dynein was then perfused through a $\sim$20 μl motility chamber consisting of a clean cover glass and slide sealed on two sides with vacuum grease (Dow Corning, Corning, NY) (In early trials, we found that Vaseline petroleum jelly inhibited motility). After 3–5 min, the chamber was flushed with 200 μl of TEMK containing 20 μM taxol. Subsequently, 200 μl of the test solution in TEMA with or without 200–300 μg/ml taxol-stabilized bovine brain microtubules (previously shown to have no contaminating ATPase activity) were similarly perfused through the chamber. All perfusions and observations were carried out at 28°C. For experiments in which ADP was added to the motility buffer, the concentrations of MgATP^{2-} and MgADP$^-$ were computed as described previously (16). Both slides and cover slips were detergent washed, using a 1:100 dilution of Contrad 70 (Curtin Matheson Scientific, Wilmington, MA) with sonication for 15 min, followed by extensive rinsing with 18 MOhm deionized water.

Video Microscopy

Microtubules were imaged with an inverted microscope (IM-35; Carl Zeiss, Oberkochen, Germany) equipped with a 100× planachromat oil immersion objective (1.25 NA), differential interference contrast optics, and a 16× projection ocular. The final magnification to the video monitor (12-inch screen measured diagonally) was 7,500×. Illumination was provided by a high pressure mercury arc lamp model 910426; Carl Zeiss. Images were digitized, irregularities in illumination subtracted, and contrast enhanced using computer-driven video processing software and hardware (Image-1; Universal Imaging, Media, PA). Sequences were recorded on a high resolution 3/4 inch U-Matic (model 5800H; Sony, Montvale, NJ) video recorder. Successive fields were permanently numbered using a field/frame counter (model VFF6030; QSI Systems, Newton, MA).

Analysis of Translocating Microtubules

The number of gliding and "nongliding-bound" microtubules was determined by counting the number of each class of microtubules per field over a 30-s or 1-min period. The field of view was changed every 30 s or 1 min by the operator. "Nongliding-bound" microtubules were not moving and adhered closely along their length to the cover slip. A few microtubules were

attached by their tips; these could not be reliably quantified because of their variable orientation and weak image. Gliding rates were determined without regard to microtubule length, since it was previously shown that there is no relationship between length and gliding rate (32).

Measurement of MgATP^{2-} Hydrolysis by Glass-adsorbed Dynein

ATP hydrolysis was measured in motility chambers as follows: chambers were coated with dynein as described above and washed with 100 μl of TEMA. [γ-^{32}P]ATP in TEMA with microtubules was perfused into the chamber as usual, and allowed to incubate until the microtubules released from the cover slip. 2 μl of the solution was then removed from the motility chamber, immediately spotted on a 0.1-mm thick polyethyleneamine-impregnated cellulose-coated plate and chromatographed using a 0.5 M LiCl, 1.0 M formic acid solvent system. The locations of the ATP and phosphate on the plate were determined by autoradiography, the spots cut out, and the radioactivity in each determined by liquid scintillation counting. Percent hydrolysis was expressed as the cpm in the phosphate spot divided by the total cpm in the ATP and phosphate spots. This system separates phosphate (R_f=0.604 $\pm$ 0.006, n=9) very effectively from ADP (R_f=0.478 $\pm$ 0.009, n=7) and ATP (R_f=0.069 $\pm$ 0.004, n=17), and provides accurate quantification of the relative hydrolysis of ATP.

Cosedimentation Assays

Cosedimentation assays were performed as follows: 10–20 μl of dynein-containing solution (1 mg/ml stock, diluted as necessary) and 10–20 μl of microtubule-containing solution ($\sim$5 mg/ml) in TEMA plus 20 μM taxol were mixed in 1.5-ml microcentrifuge tubes with additional TEMA to bring the total volume to 10 times the dynein volume. The tubes then were capped and allowed to incubate at room temperature for 30 min. In some samples, MgATP^{2-} was added to a final concentration of 1 mM (pH 7.8–8.0) 5 min before the end of the incubation period. Samples were then centrifuged at 39,000 g (18,000 rpm) (SS-34 rotor; Sorvall Instruments, Newton, CT) for 30 min at 20–22°C. The supernatant was carefully pipeted away from the pellet, and samples brought to identical final volumes in gel electrophoresis sample buffer. Samples were boiled before gel electrophoresis.

Gel Electrophoresis

Polypeptide composition was assayed on discontinuous SDS–polyacrylamide gels (23). 8-cm long, 8% polyacrylamide slab gels were used to assay tubulin and dynein content of pellets and supernatants from cosedimentation experiments. 15-cm gels consisting of 3–6% linear gradients of acrylamide (12) were used to resolve the dynein heavy chains.

Reagents

Hepes, SDS, apyrase, and hexokinase were from Sigma Chemical Co. (St. Louis, MO), as were all nucleotides and analogs, except adenosine 5'-triphosphate (ATP) and adenosine 5'-o-thiotriphosphate (ATP-γS), which were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Tris and ultra-pure sucrose were from Schwartz/Mann (Spring Valley, NY). Phosphocreatine and phosphocreatine kinase were from Boehringer Mannheim Biochemicals. Potassium acetate was from Fisher Scientific Co. (Pittsburg, PA). All remaining salts were from Mallinkrodt Inc. (St. Louis, MO). To eliminate contaminating ATP (34), AMP-PNP was treated with apyrase (10 U/ml) for 1 h before use. Taxol was kindly provided by Dr. M. Suffness of the National Cancer Institute (NIH, Bethesda, MD).

For experiments investigating the effects of ADP on ATP-dependent binding and gliding, ADP was further purified by anion exchange chromatography using a Mono-Q column (FPLC system; Pharmacia, Uppsala, Sweden). The ADP sample was applied at $\sim$1 mM in 500-μl increments to a 4-cm-long by 0.5-cm-diam MonoQ column equilibrated with 10 mM ammonium bicarbonate (pH 8.0). The column was then flushed with a 80–250 mM ammonium bicarbonate gradient (pH 8.0). The ADP eluted at $\sim$160 mM ammonium bicarbonate. After determining purity by thin-layer chromatography (see above), samples were vacuum evaporated in a centrifugal rotary evaporator (model RH40-12, Speed Vac concentrator; Savant, Farmingdale, NY). ADP solutions were reconstituted from the dried sample and concentration determined by direct measurement at A_{260} using a molar extinction coefficient of 15.4 $\times$ 10^3 M^{-1}cm (66). ATP concentrations were similarly determined for the competition experiments.

Results

### Microtubule Gliding Rate Is Dependent upon MgATP^{2-} Concentration

Perfusion of the chamber with microtubule- and MgATP^{2-}-containing buffer resulted in binding of microtubules to the sea urchin dynein–coated cover slip. Immediately upon binding, microtubules began gliding across the cover slip. The number of bound microtubules increased rapidly during the first few minutes (Fig. 1, A and B); after this time gliding microtubules were probably in equilibrium with free microtubules, as detachment of bound microtubules and attachment of free microtubules was commonly observed. Gliding microtubules underwent net unidirectional displacement by a series of starts and stops and transient backward steps (Moss, A. G., J.-L. Gatti, and G. B. Witman. 1988. *J. Cell Biol.* 107:245a) (59), and exhibited considerable lateral mobility (32).

Gliding rate was dependent upon the concentration of MgATP^{2-} in buffer containing an ATP-regenerating system (10–25 mM phosphocreatine, 0.1–0.2 mg/ml phosphocreatine kinase) (Fig. 2, A and B). Both the intact outer arm dynein and its β/IC1 subunit had an apparent K_m for gliding of $\sim$17 μM MgATP^{2-}. The intact outer arm had a V_{max} of $\sim$6.9 μm/s, whereas V_{max} was $\sim$11.5 μm/s for the β/IC1 subunit, confirming the observation of Sale and Fox (40) that the isolated β/IC1 subunit translocates microtubules at a rate faster than that of the intact dynein.

Both the intact outer arm dynein and its β/IC1 subunit supported motility even at 5 μM MgATP^{2-} in the presence of an ATP-regenerating system, but fewer microtubules were observed to glide at such low MgATP^{2-} concentrations, and those that did moved in a more saltatory manner. Microtubules did not bind to the cover slip at MgATP^{2-} concentrations <5 μM. The translocation rate for both the intact dynein and its β/IC1 subunit increased up to 250 μM free MgATP^{2-} and then leveled off (Fig. 2 A). Microtubules translocated by the β/IC1 subunit had relatively more lateral mobility, and progressed in a more discontinuous manner than those being translocated by the intact dynein (data not shown). A consistent feature of the microtubule–sea urchin dynein interaction was that there were very few nonmotile, bound microtubules (Fig. 3 A). Those that did bind without gliding were usually attached at only one point, or by one end, not along their full lengths, as were gliding microtubules.

Binding of Microtubules by Sea Urchin Dynein Requires MgATP^{2-}

In the absence of an ATP-regenerating system, the microtubules eventually detached from the cover slip. The length of the motile period before detachment was directly proportional to the amount of added MgATP^{2-} (Figs. 1 A and B). Motility became less steady over time, and shorter microtubules were released first, so that the observed population was composed progressively of longer and longer microtubules. These results strongly suggest that the number of dynein-microtubule cross-bridges per unit length of microtubule decreased over time; longer microtubules could interact with more dynein arms, and so remain attached to the glass for a longer period of time.
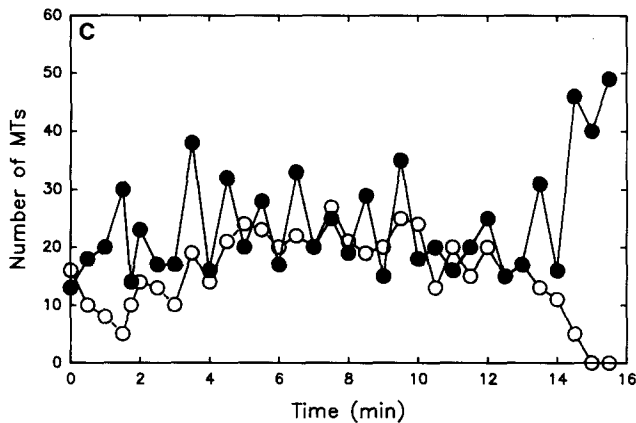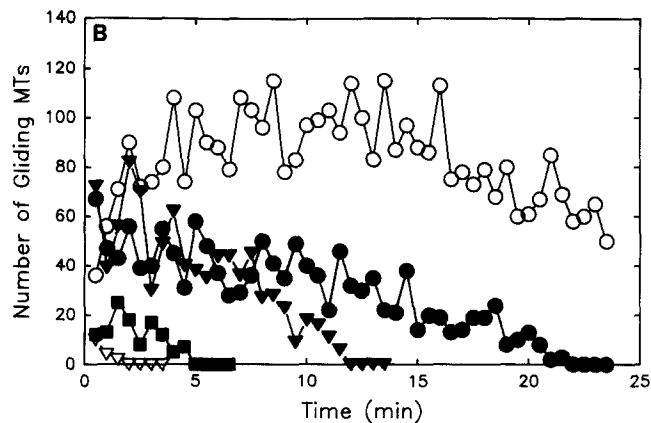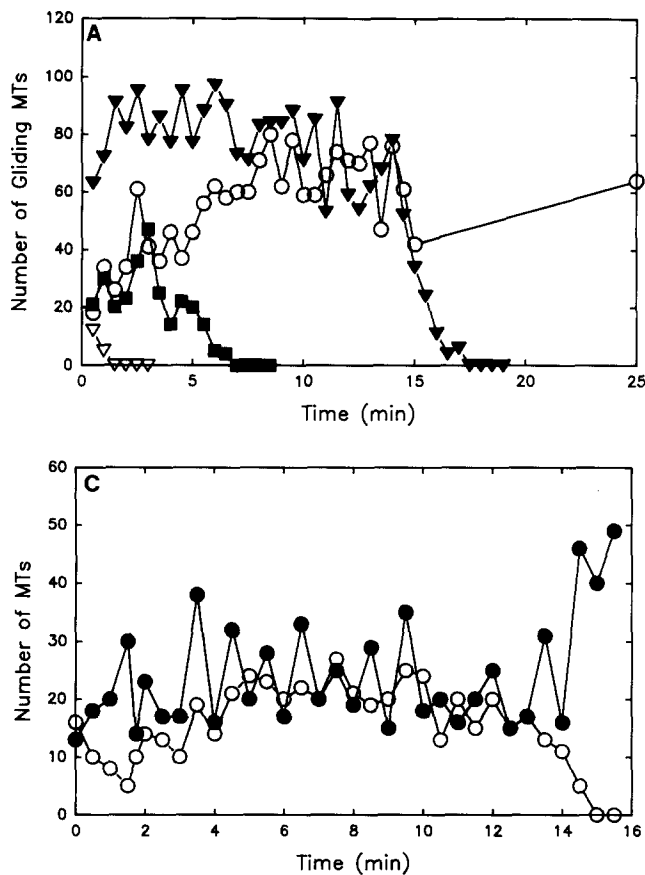
Figure 1. Number of microtubules bound to glass-absorbed dynein as a function of time for different initial concentrations of MgATP^{2-}. (A) Intact sea urchin 21S outer arm dynein. Gliding continued for 33 min in 2 mM initial MgATP^{2-} (not shown). (B) Sea urchin outer arm β/IC1 subunit. MgATP^{2-} concentrations, in millimolar: (-•-), 2; (-▼-), 1; (-■-), 0.5; (-▽-), 0.1; (-○-), 0.1 plus phosphocreatine (20 mM) and phosphocreatine kinase (0.2 mg/ml). (C) Tetrahymena outer arm dynein at 2 mM MgATP^{2-}. (-•-) Non-gliding, bound microtubules (present in significant numbers only in Tetrahymena dynein preparations); (-○-) gliding microtubules. Ordinate: Number of microtubules observed per 30-s interval.

The time course of decrease in the number of gliding microtubules differed between the intact dynein and its β/IC1 subunit. With millimolar initial ATP the number of microtubules gliding upon intact dynein was stable over a long period and then dropped quickly (Fig. 1 A). Nearly all of the microtubules completely detached from the glass, with a few remaining attached by one end. In contrast, the number of microtubules gliding upon the β/IC1 subunit decreased linearly over time until again only a few microtubules remained attached by one end (Fig. 1 B).

Inclusion of an ATP-regenerating system greatly prolonged movement (Fig. 1, A and B). For example, in the presence of 100 μM MgATP^{2-}, 10 mM phosphocreatine, and 0.2 mg/ml creatine kinase, microtubule gliding continued unabated for at least 25 min, whereas without the regenerating system, 100 μM MgATP^{2-} supported motility for only 2-3 min. When no regeneration system was used, and microtubules detached from the coverslip, subsequent perfusion of the chamber with motility buffer containing MgATP^{2-} and microtubules reconstituted motility, confirming that the dynein was still functional. Perfusion of the chamber many times with motility buffer with or without microtubules caused no diminution in the capacity of the dynein subsequently to translocate microtubules. Chambers could be stored for several days at 4°C at high humidity with little or no loss of motility-generating activity.

Addition of a droplet of buffer containing 1 mM MgATP^{2-} without microtubules to the edge of the coverslip reconstituted motility in the vicinity of the droplet, with mo-

tility spreading away from the site of the applied droplet. Therefore, detachment was not due to an irreversible change in the microtubules.

When the sea urchin dynein-coated chamber was perfused with microtubule-containing TEMA without ATP (Table I), or TEMA containing an ATP-depleting system (10 U/ml apyrase) (Fig. 3 A), neither gliding nor lengthwise binding of microtubules was observed. Binding and gliding were highly specific for ATP; millimolar P_i, AMP, ADP, ADP/P_i, GTP, CTP, UTP, ITP, ATP-γS, and AMP-PNP supported neither binding nor translocation (Table I). However, binding with little or no net translocation was observed if micromolar concentrations of vanadate accompanied a concentration of ATP that by itself supported net translocation. Microtubules under these conditions "shuttled", or moved axially backwards and forwards (59), in an ATP-dependent manner (Moss, A. G., J.-L. Gatti, and G. B. Witman. 1988. J. Cell Biol. 107:245a).

Direct Measurement of MgATP^{2-} Hydrolysis in Motility Chambers

The above observations strongly suggested that cessation of gliding and detachment of microtubules was a direct consequence of the reduction of MgATP^{2-} concentration as a result of dynein hydrolytic activity. To determine the extent of MgATP^{2-} hydrolysis under the conditions of our experiments, ATP and P_i were measured in aliquots removed from the motility chamber at the time of microtubule detach-

A
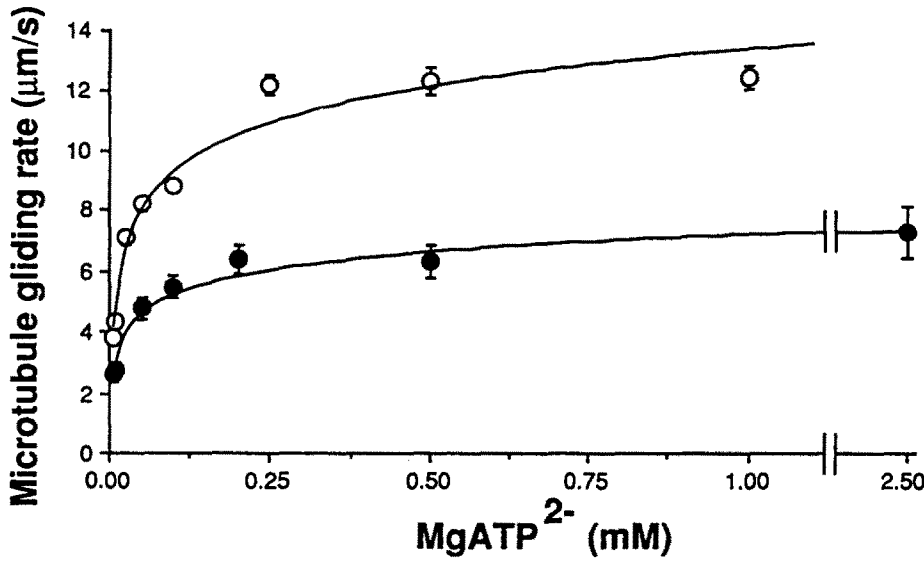
B
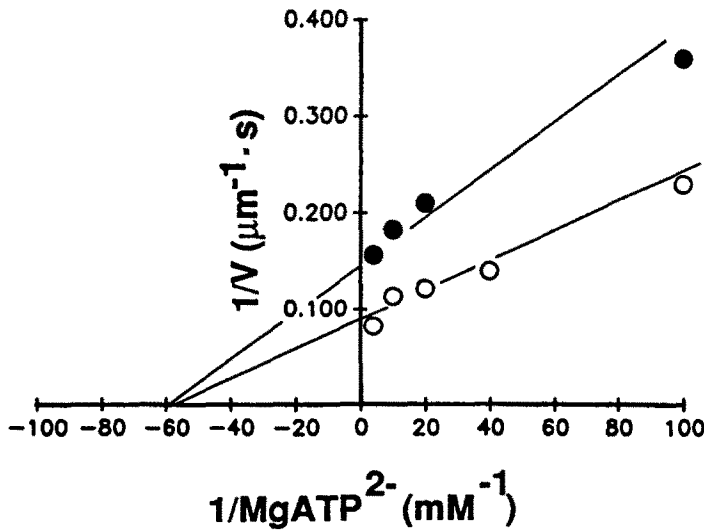
Figure 2. (*A*) Rate of microtubule gliding as a function of MgATP^{2-} concentration for the intact sea urchin outer arm dynein (-●-) and its β/IC1 subunit (-O-). All cases included 25 mM phosphocreatine and 0.2 mg/ml phosphocreatine kinase to maintain the MgATP^{2-} concentration at a constant level throughout the period of rate measurements. Microtubules do not bind at concentrations of MgATP^{2-} below 5 μM. (*B*) Double reciprocal plot of MgATP^{2-} concentration versus velocity of microtubule gliding. Same key as in *A*. K_m was 17 μM for both the intact dynein and the β/IC1 subunit. Data are from two representative experiments in TEMK buffer; each point is the average of 14 measurements for the intact outer arm, and 50 measurements for the β/IC1 subunit. Error bars depict standard error of the mean (71). Points fitted by eye.

ment (see Materials and Methods). Beginning with an initial MgATP^{2-} concentration of 2.0 mM, the intact dynein had hydrolyzed nearly 95% of the MgATP^{2-} by the time the microtubules were released (Table II). Thus the concentration of MgATP^{2-} at the time of microtubule detachment was ~100 μM. In the case of the β/IC1 subunit, when the initial concentrations of MgATP^{2-} ranged from 0.5 to 2.0 mM, only 64-78% of the nucleotide was hydrolysed at microtubule release (Table II).

Effects of Products of Hydrolysis on Microtubule Binding and Translocation

For intact dynein, and certainly for its β/IC1 subunit, the residual MgATP^{2-} concentration at the time of microtubule release should have been sufficient, by itself, to support

microtubule binding and translocation (Fig. 1). This finding suggested that other components in the solution, most likely the products of ATP hydrolysis, were facilitating microtubule release. We therefore examined the effects of ADP and inorganic phosphate (P$_i$) on ATP-supported microtubule binding and gliding (Table III). In these experiments, total ATP was kept constant at 1.0 mM while various amounts of ADP and/or P$_i$ were added. The concentration of total Mg^{2+} was maintained at two millimolar greater than the concentration of total nucleotide to ensure that nearly all of the nucleotide was complexed with Mg^{2+}. With the intact dynein, binding and gliding were completely eliminated by addition of 10 mM ADP; under these conditions, the concentrations of MgATP^{2-} and MgADP$^-$ were 0.96 and 8.0 mM, respectively. In the case of the β/IC1 subunit, reduced motil-
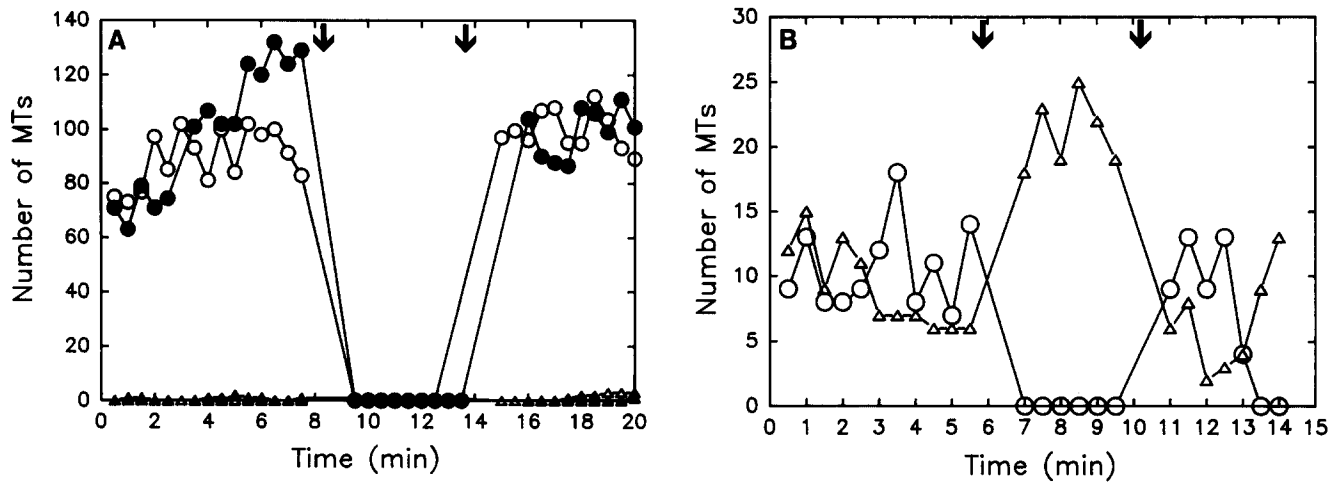
Figure 3. Effects of depletion of free MgATP^{2-} by perfusion with 10 U/ml apyrase-containing motility buffer (*first arrow*) on binding and gliding of microtubules on sea urchin (*A*) and *Tetrahymena* (*B*) dyneins. Perfusion was incomplete to prevent removal of all microtubules from the chamber. Subsequently, the chamber was washed with motility buffer and the ATP-depleted solution replaced with motility buffer containing microtubules and an ATP-regenerating system (*second arrow*). (-O-) Microtubules gliding on intact sea urchin or *Tetrahymena* outer arm dynein; (-●-) microtubules gliding on the β/IC1 subunit of sea urchin dynein; (-△-) non-gliding microtubules bound to intact sea urchin or *Tetrahymena* outer arm dynein; (-▲-) non-gliding microtubules bound to the β/IC1 subunit of sea urchin dynein. (*A*) Depletion of ATP by apyrase caused gliding microtubules to detach from the surface of sea urchin dynein-coated cover slips. Perfusion of the ATP/regeneration system buffer resulted in microtubules becoming reattached to the surface and gliding as before treatment. (*B*) Depletion of MgATP^{2-} resulted in the recruitment of many nonmoving microtubules from the motility buffer to the cover slip when the latter was coated with *Tetrahymena* outer arm dynein. During the period of depleted ATP, microtubules became closely attached to the dynein-coated surface of the coverslip, and displayed no lateral or longitudinal movement. Microtubules so bound could withstand repeated perfusion of the chamber with hundreds of microliters of ATP-free buffer without being washed away, thus demonstrating that they were tightly bound. Upon perfusion with 2 mM MgATP and an ATP-generating system, many microtubules began gliding. The apyrase was not completely washed out in this case, and microtubules were rapidly recruited again to the cover slip surface near the end of the record.

Table 1. Effect of Various Nucleotides on the Binding and Motility Properties of Axonemal Dyneins from Sea Urchin and Tetrahymena

| Condition | Concentration | Sea Urchin | | Tetrahymena |
		21S	β/IC1	22S
Buffer alone	N/A	–	–	b
MgATP^{2-}	5 μM–5 mM	*G*	*G*	*G*
MgATP^{2-} run-down	variable	–	–	b
AMP	1 mM	–	–	b
AMP-PNP	1 mM	–	–	N.T.
Pi (phosphate)	1 mM	–	N.T.	b
ADP	1 mM	–	–	b
MgADP/P$_i$	1 mM each	–	–	b
MgATP-γS^{2-}	1 mM	–	–	*G*
GTP	1 mM	–	–	N.T.
CTP	1 mM	–	–	N.T.
ITP	1 mM	–	–	N.T.
UTP	1 mM	–	–	N.T.
Vanadate/MgATP^{2-}	5–100 μM/1–5 mM	s	s	s

G, Gliding; *b*, binding (no gliding); -, no binding; *s*, shuttle; *N.T.*, not tested. For explanation of "shuttle" see Results.

Table II. Hydrolysis of MgATP^{2-} at Time of Microtubule Release

Dynein	Initial MgATP^{2-} concentration	Percent MgATP^{2-} hydrolysis
Intact 21S dynein	2.0 mM MgATP^{2-}	95
β/IC1 subunit	2.0 mM MgATP^{2-}	77.5
	1.0 mM MgATP^{2-}	66.6
	0.5 mM MgATP^{2-}	63.6

Table III. *Effect of ADP and P_i on the Motility and Binding Properties of Sea Urchin Dynein*

Concentration of added nucleotide	Free cation-nucleotide complex (mM)		MgADP	Enzyme	
	MgATP^{2-}	MgADP$^-$	MgADP + MgATP	Intact Dynein	β/IC1
1 mM ATP	>0.95	0	0*	+++	+++
Inorganic (sodium) phosphate (P$_i$) alone	1	not applic.	0*	−	N.T.
1 mM ADP alone	0	>0.95	1	−	−
1 mM ADP/P$_i$	0	>0.95	1	−	−
For each of the following, 1 mM ATP added, and [P$_i$] = [ADP]:					
1 mM ADP	0.92	0.7	0.432	+	+
2 mM ADP	0.95	1.5	0.612	+/−	+/−
3 mM ADP	0.94	2.2	0.701	N.T.	−/+
4 mM ADP	0.94	3.0	0.761	−/+	−
8 mM ADP	0.95	6.3	0.869	−/+	N.T.
10 mM ADP	0.96	8.0	0.893	−	N.T.

+/−, indicates more robust binding and motility than −/+. +, binding; −, no binding; N.T. = not tested; [Mg^{2+}]$_{total}$ maintained at 2 mM > [ATP]$_{added}$ + [ADP]$_{added}$.
* ADP content of the commercial ATP was not determined; it is typically very low (Moss, A.G., unpublished results).

ity and diminished binding were observed when as little as 2.0 mM ADP/P$_i$ were added, corresponding to 0.95 mM MgATP^{2-}, 1.5 mM MgADP$^-$, 2 mM P$_i$. In both cases, the concentration of MgATP^{2-} was well above the minimum required to support motility. These ratios of ATP/product are very similar to those that would have been in the chamber at the time of microtubule release (see Table III). Therefore, ADP and possibly P$_i$ antagonize microtubule attachment and translocation, probably by competing with ATP for a nucleotide binding site. This effect is likely to be enhanced in the boundary layer at the glass surface, where dynein-microtubule interactions are occurring and the products of hydrolysis are most concentrated.

The release of protons during hydrolysis of ATP was not responsible for detachment. Direct measurement of the pH of motility buffer following complete hydrolysis of 1 mM initial ATP by apyrase revealed that the pH dropped only 0.4 U, from pH 8.0 to 7.6. The latter pH is still permissive for motility (40). Moreover, if a decrease in pH was responsible for detachment of the microtubules, then the microtubules should have detached at least as rapidly in the presence of high as in low concentrations of ATP. In fact, the converse was true.

Microtubules Bind to Glass-adsorbed Tetrahymena Dynein without ATP

Tetrahymena 22S outer arm dynein has been shown to bind to brain microtubules with very high affinity in a "rigor" state upon depletion of free MgATP^{2-} (37). To determine if the contrasting and unexpected result obtained with sea urchin dynein was due to a species or subunit difference or to our assay method, we examined *Tetrahymena* outer arm dynein in the same solid-phase assay. As previously reported (57), *Tetrahymena* dynein adsorbed to a glass cover slip binds microtubules and translocates them over the surface of the cover slip in the presence of millimolar MgATP^{2-}. In contrast to the results with sea urchin dynein, microtubules remained bound to *Tetrahymena* dynein upon hydrolysis of ATP by the glass-bound dynein (cf. Fig. 1, *A* and *B* vs. *C*) or by an exogenously applied ATP-depletion system (cf. Fig. 3, *A* vs. *B*); indeed, the number of bound nonmoving micro-

tubules substantially increased upon removal of ATP. If the chamber was subsequently perfused with fresh buffer containing MgATP^{2-} and microtubules, gliding resumed. *Tetrahymena* dynein also differed from sea urchin dynein in that it bound microtubules to the glass coverslip in the presence of added AMP, ADP, P$_i$, or ADP and P$_i$ together (Table I). Although microtubules did not bind to sea urchin dynein–coated coverslips in the presence of MgATP-γS^{2-}, MgATP-γS^{2-} supported microtubule gliding with *Tetrahymena* 22S outer arm dynein. This is consistent with studies of *Tetrahymena* outer arm substrate specificity (46, 47).

Effects of Ionic Strength on Microtubule Binding and Translocating Activity of Axonemal Dyneins

We wished to determine whether the β/IC1 subunit might exhibit rigor binding under different ionic strength conditions than those used in our standard assay, and also to compare the ionic strength dependency of the β/IC1 subunit's microtubule-translocating activity with that of other axonemal dyneins. Therefore, we examined the binding and motility of that subunit, of the intact sea urchin outer arm dynein, and of *Tetrahymena* 22S and 14S dyneins over a wide range of potassium acetate concentrations. Potassium acetate was chosen as the variable salt in these experiments because acetate is the preferred anion for supporting reactivation of demembranated sea urchin sperm (11), and also because it is the anion in greatest abundance in our standard in vitro motility assay.

For the β/IC1 subunit, the rate of microtubule translocation increased from 10 to 120 mM potassium acetate (Fig. 4 *A*); above and below these concentrations no binding was observed. Microtubules were released from the β/IC1 subunit upon depletion of ATP at all salt concentrations that supported binding and motility, and no binding was observed in the absence of ATP at any of the salt concentrations. Therefore, the lack of rigor bond formation by this subunit in our standard assay was not simply because the ionic strength was not optimal for such binding.

The binding and force-generating properties of the intact sea urchin outer arm dynein showed a similar dependence
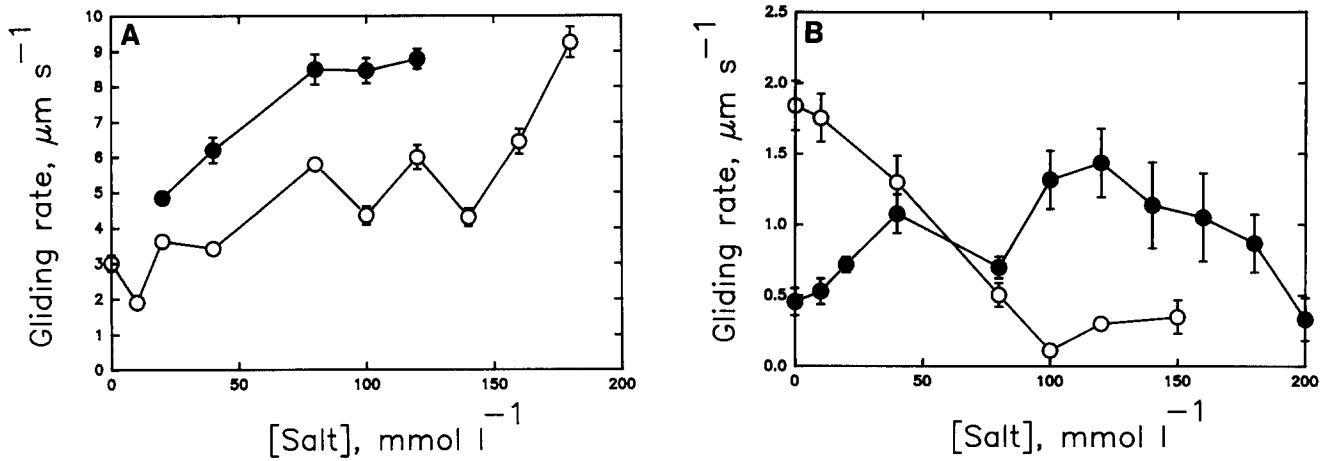
Figure 4. Effect of salt concentration on the gliding rate of microtubules upon glass-adsorbed axonemal dyneins. Abscissa indicates the concentration of potassium acetate. (*A*) Sea urchin dynein: (-o-) intact 21S outer arm; (-●-) β/IC1 subunit. (*B*) *Tetrahymena* 14S (-o-) and 22S dynein (-●-). Error bars, standard error of the mean. Data represent microtubules that were detectably moving; all nonmoving microtubules were ignored. The background of nonmoving microtubules was inversely related to the ionic strength in the intact dynein samples, with the most pronounced effect occurring with *Tetrahymena* 14S dynein.

on ionic strength from 10 to 120 mM potassium acetate (Fig. 4 *A*). However, some microtubules remained bound to the intact dynein and translocated slowly even in the absence of potassium acetate. Above 120 mM potassium acetate, the number of bound microtubules dropped off dramatically but the rate of gliding of the remaining microtubules continued to increase up to 180 mM potassium acetate, at which concentration the gliding rate matched the maximum rates observed for microtubules being translocated by the purified β/IC1 subunit. Above 180 mM potassium acetate, no binding or gliding was observed. These results suggest that as ionic strength increases, sea urchin dynein proceeds through its mechanochemical cycle more rapidly, and its affinity for microtubules diminishes. Like the β/IC1 subunit, the intact arm released microtubules upon depletion of ATP and did not bind microtubules in the absence of ATP at all ionic strengths tested.

Tetrahymena 22S outer arm dynein translocated microtubules at a maximal rate of 1.5 μm/s at 120 mM potassium acetate (Fig. 4 *B*); the rate of gliding decreased above and below this concentration, although many microtubules remained bound but did not glide at both the higher and lower ionic strengths. In contrast, *Tetrahymena* 14S dynein bound and translocated microtubules maximally at low ionic strength, and exhibited only marginal motility at potassium acetate concentrations of 100 mM or higher (Fig. 4 *B*). These results suggest that these two dyneins interact with microtubules via different types of bonds. In contrast to the situation with the sea urchin dyneins, microtubules remained bound to the glass-adsorbed *Tetrahymena* 22S and 14S dyneins in the absence of ATP or upon depletion of ATP at all salt concentrations that supported motility.

All glass adsorbed dyneins recovered translocating activity after high or low salt treatment; therefore, loss of activity at these concentrations did not result in irreversible denaturation of the enzyme.

Cosedimentation Assays

To determine if soluble dynein had the same microtubule-

binding properties as glass-adsorbed dynein, we investigated the microtubule-binding characteristics of sucrose gradient-purified *Tetrahymena* and *S. purpuratus* dyneins using conventional cosedimentation assays.

As previously reported (37), *Tetrahymena* 22S dynein bound to microtubules in the absence of MgATP^{2-}; almost all of the 22S dynein sedimented with microtubules up to a molar ratio of ∼1 dynein/29 tubulin monomers, at which saturation of binding was clearly evident (Fig. 5). Addition of 2 mM MgATP^{2-} released most of the *Tetrahymena* dynein from the microtubules (data not shown; see reference 27), indicating that most of the *Tetrahymena* dynein was bound by ATP-sensitive sites, again as previously reported (37).

Most of the intact sea urchin dynein partitioned into the pellet at low molar ratios of dynein/tubulin, but increasing amounts of dynein remained in the supernatant as the molar ratio increased. Saturation of binding became evident at a molar ratio of ∼1 dynein molecule/39 tubulin monomers (Fig. 5). A portion of this bound dynein was released from the microtubules by MgATP^{2-} (data not shown; see reference 27), indicating that some of the intact arms were bound by rigor bonds whereas the remainder were bound by ATP-insensitive bonds. In the accompanying paper (27), we investigate the origin of ATP-sensitive and -insensitive binding in intact sea urchin dynein, and conclude that both are properties of the α subunit.

In contrast to the situation with the intact sea urchin and *Tetrahymena* dyneins, the sea urchin β/IC1 subunit never sedimented with microtubules, even at a high molar ratio of 1 dynein molecule/8.8 tubulin monomers (Fig. 5). Addition of 2 mM MgATP^{2-} to the mixture had no effect on the sedimentation behavior of the β/IC1 subunit (data not shown; see reference 27). Therefore, the cosedimentation assay, like the solid-phase assay, provides no evidence for rigor bond formation by this subunit. Moreover, these results indicate that the isolated β/IC1 subunit also is incapable of ATP-insensitive, structural binding to microtubules.

Electrophoretic analysis of supernatant and pellet samples
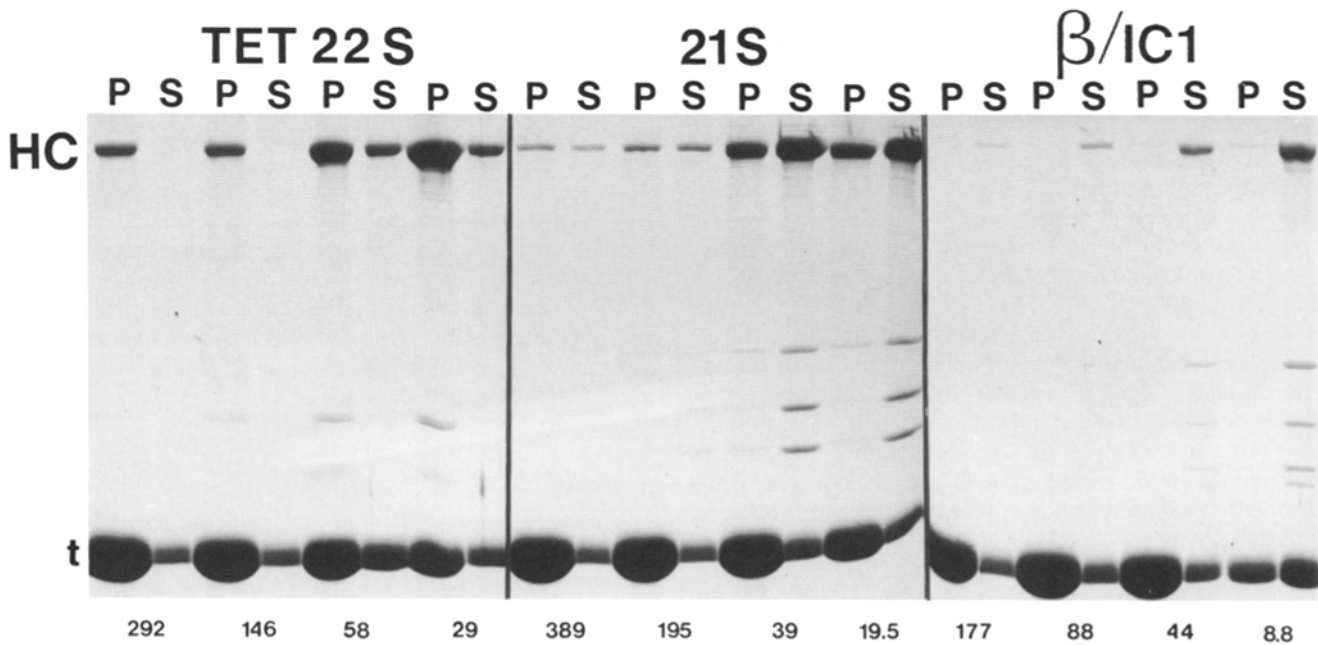
Figure 5. Comparison of the microtubule-binding properties of axonemal dyneins by cosedimentation assay. To determine whether different dynein:tubulin ratios affected the ability of the dynein to bind, outer arm dyneins were added in different final concentrations to the taxol-stabilized microtubules. Tubulin concentration was 1.1 mg/ml for all trials except those represented by the right most pair of lanes of the respective groups, in which the tubulin concentration was 0.55 mg/ml. *P*, microtubule pellet; *S*, supernatant. (*TET22S*) *Tetrahymena* 22S outer arm dynein. Composition of samples is as follows: 143, 290, 710, 710 μg/ml dynein; molar ratio of tubulin to dynein; 292, 146, 58, 29. (*21S*) *S. purpuratus* intact 21S outer arm dynein. Composition of samples: 70, 143, 290, 710 μg/ml dynein; molar ratio of tubulin:dynein 389, 195, 39, 19.5. (*β/IC*) *S. purpuratus* β/IC1 subunit of outer arm dynein. Composition of samples: 70, 143, 290, 710 μg/ml dynein; molar ratio of tubulin to dynein: 177, 88, 44, 8.8.

showed that there was no selective partitioning of either the sea urchin α or β or the *Tetrahymena* α, β, or γ heavy chains into either the pellet or supernatant (not shown). These results indicate that the outer arm dyneins remained structurally intact under our conditions.

Discussion

Dynein–Microtubule Interactions

The outer dynein arm in situ has at least two microtubule-binding sites: an " A-end" or ATP-insensitive structural binding site that permanently binds the dynein to the A-tubule of an outer doublet, and a "B-end" or ATP-sensitive site believed to interact transiently with the B-tubule of the adjacent outer doublet during force generation. In the solid phase in vitro motility assay, sea urchin dynein translocates microtubules in the presence of ATP, and does not bind microtubules in an ATP-insensitive manner. The observed dynein-microtubule interactions are thus those of the B-end of the dynein arm.

Characteristics of B-end Binding

The intact sea urchin sperm outer arm dynein and its β/IC1 subunit bound and translocated microtubules in an ATP-dependent manner, indicating that the glass-bound dynein was functional. Nevertheless, neither the intact dynein nor the β/IC1 subunit formed tight bonds with the microtubules upon depletion of ATP, or in the absence of added nucleotide. That rigor binding was not observed with the intact dynein

in the solid-phase assay does not mean that sea urchin dynein is unable to form such bonds; indeed, the intact dynein can bundle microtubules in an ATP-sensitive manner (Moss, A. G., W. S. Sale, L. A. Fox, and G. B. Witman. 1990. *J. Cell Biol.* 111:26a) (27), but apparently the part of the arm that is responsible for rigor binding (the α subunit) simply is not available to the microtubule in the solid-phase assay. However, we have not observed rigor formation with the isolated β/IC1 subunit in either the solid-phase or cosedimentation assays. Therefore, although this subunit is capable of interacting with and translocating microtubules in the presence of ATP, it appears to lack the ability to form a rigor complex, which has been generally accepted to be a property of all force-producing proteins.

Although the α subunit of the intact arm does not appear to interact directly with the microtubules in the solid phase assay, the rate of translocation, the kinetics of microtubule release, and the affinity of the glass-bound dynein for microtubules as a function of ionic strength differed between the purified β/IC1 subunit and the intact arm. These differences may reflect modulation of the β/IC1 subunit's activity as a result of allosteric interaction with the α subunit and/or the intermediate chain/light chain complex in the intact arm (70). Pfister and Witman (35) showed that the ATPase activity of the isolated β subunit of *Chlamydomonas* outer arm dynein is reduced upon reassembly with the α subunit.

The absence of rigor formation by the functional sea urchin β/IC1 subunit is surprising inasmuch as intact *Tetrahymena* dynein has been shown to form such a bond in this and many previous studies (17, 31, 37, 38; see references 18,

39 for reviews). A possible explanation for the different behaviors is that the β/IC1 subunit of sea urchin dynein and the force-generating subunit(s) of *Tetrahymena* dynein differ in their relative affinities for microtubules during one or more stages of the mechanochemical cycle. Kinetic analysis of the isolated *Tetrahymena* outer arm dynein has clearly established that microtubules bind to the *Tetrahymena* dynein/ADP/P$_i$ complex and remain tightly bound during product release and in the absence of bound products; dissociation of the microtubule/dynein complex occurs rapidly upon addition of ATP (15, 17, 31, 38). Our finding that the β/IC1 subunit of sea urchin dynein releases microtubules when ATP is depleted and does not bind microtubules in the absence of added MgATP^{2-} suggests that this subunit, in contrast to *Tetrahymena* dynein, has a lower affinity for microtubules when its hydrolytic site is empty than when the site is occupied (isolated sea urchin outer arm dynein is unlikely to contain bound nucleotide at its hydrolytic sites, because vanadate-dependent photolysis of the heavy chains is not detected without added ADP or ATP [24]).

Addition of excess ADP and P$_i$ enhanced release of microtubules from sea urchin dynein in the presence of ATP, suggesting that the β/IC1/ADP and β/IC1/ADP/P$_i$ complexes also have relatively low affinities for microtubules. However, it is possible that the complexes formed by the addition of exogenous product differ in their affinities for microtubules from those formed by the hydrolysis of bound ATP (Shimizu, T., S. Ohashi, and T. Katsura. 1990. *J. Cell Biol.* 111:118a).

Schemes for the mechanochemical cycle of the β/IC1 subunit of sea urchin dynein also can be constructed that are in agreement with our data and that differ more profoundly from that of *Tetrahymena* dynein. It will be necessary to determine the rate constants for specific steps in the cycle to know if such schemes are realistic. Nevertheless, it appears that the ability to form a rigor bond is not a prerequisite for microtubule binding and force generation.

An alternative explanation for the observed differences between the β/IC1 subunit of sea urchin dynein and intact *Tetrahymena* dynein is that the activity of the former may be regulated in an ATP-dependent manner not seen in the latter. The β heavy chain contains multiple ATP-binding consensus sequences (13, 28), suggesting that it binds ATP at more than one site. It is possible that loss or hydrolysis of ATP at one site affects the activity at a second site coupled to microtubule binding and force generation. Current models for the mechanism of axonemal bending dictate that the arms must have a low affinity for microtubules during at least one-half of each beat cycle to permit passive sliding of the outer doublet microtubules facing them (51, 61). The existence of a biochemical regulatory mechanism that turned the arms on and off at the appropriate time in the beat cycle would provide more precise control over interdoublet sliding than could be achieved if the "off" time were simply determined by how fast the arm independently proceeded through its mechanochemical cycle. Such active control of dynein/microtubule interactions probably is necessary for the regulation of flagellar beat frequency and curvature.

The above discussion notwithstanding, the differences in the rigor-forming capabilities of intact *Tetrahymena* dynein and the β/IC1 subunit of sea urchin dynein do not necessarily mean that the force-generating moieties of these dyneins are fundamentally different. The intact *Tetrahymena* dynein contains three subunits, and although the head of each subunit appears to interact directly with microtubules (45), increasing biochemical and structural data indicate that there are differences between the subunits (55, 56, 58). If one of the heads binds tightly to microtubules in the absence of ATP, that interaction could prevent detection of weak ATP-dependent binding by another head in the intact particle. Further analysis of isolated subunits of protistan dyneins will be necessary to determine if a low affinity for microtubules in the absence of ATP is a property that is common to at least one subunit (possibly the motility-generating subunit) of all outer arms but which has gone unnoticed because of the rigor-forming properties of one or more other subunits in the intact particle.

A-end Binding

A substantial proportion of the intact outer arm dynein of sea urchin sperm flagella bound to microtubules in the cosedimentation assay. In the accompanying paper (27), we show that some of this bound dynein is not released by ATP, suggesting that these molecules are interacting with the microtubules via their structural A-end binding site. Such binding was not observed with the isolated β/IC1 subunit, indicating that structural A-end binding requires a dynein component either not present or not functional in the β/IC1 preparation. This component appears to be the α subunit (27).

The Utility of Various Assays for Determining Dynein/Microtubule Binding Properties

In general, comparable qualitative results were obtained with the solid-phase and solution microtubule-binding assays. In the absence of ATP, the β/IC1 subunit of sea urchin dynein neither bound microtubules to the glass coverslip nor cosedimented with microtubules. Similarly, intact *Tetrahymena* dynein bound microtubules tightly to the coverslip in the absence of ATP and cosedimented with microtubules in an ATP-sensitive manner. However, some dynein/microtubule interactions were apparent in one assay but not the other. For example, ATP-insensitive A-end binding of the intact sea urchin dynein was apparent in the solution assay but not the solid phase assay (27). Conversely, because the sea urchin β/IC1 subunit does not form a rigor bond, its transient B-end interactions with microtubules could be detected only in the solid-phase assay. Therefore, both assays are of value for studying dynein–microtubule interactions. The solid-phase assay is quicker and can be carried out with much less dynein and tubulin, and allows real-time observation of molecular interactions, whereas the solution assay is at present more readily quantified.

Implications for the Isolation of Novel Microtubule-associated Motors

An increasingly popular method for isolating microtubule-associated motors takes advantage of the rigor-forming properties of the motor: the motor is bound to microtubules in the absence of nucleotide, sedimented with the microtubules, and specifically released into the supernatant in the presence of nucleotide. Our findings suggest that not all microtubule motors may be identified and isolated on the basis of rigor formation. Some mechanochemical transducers

may remain in the original homogenate under ATP-depleted conditions. This should be kept in mind when attempting to isolate uncharacterized force-generating molecules from cytosolic extracts.

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