A DYNEIN-LIKE PROTEIN ASSOCIATED WITH NEUROTUBULES

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Received 28 January 1974

1. Introduction

While the chemical investigation of microtubules and their subunit proteins – the tubulins – has progressed rapidly over the past decade, little progress has been made in relating these observations to the physiological role of these organelles. One major exception to this is the case of microtubules in cilia and flagella. In flagella, Gibbons has shown that on one tubule of each of the outer doublet tubule pairs there are two 'arms' which are composed of a divalent ion activated, EDTA-inhibited ATPase known as dynein [1, 2]. Recently, it has been shown that the tubules and dynein interact in a 'sliding filament' mechanism to produce flagellar bending [3] which is dependent on ATP as an energy source. Cytoplasmic microtubules exist singly rather than in the higly ordered axonemes of cilia and flagella. However, they too have been reported to have small 'arms' [4, 5] which, in the mitotic apparatus, have been proposed as an element in a sliding filament type of motile structure [6].

The definition of the conditions for the reassembly of tubulin subunits into microtubules by Weisenberg [7] has enabled the development of a method for the purification of microtubules by repeated cycles of assembly and disassembly [8]. Since these conditions are likely to reflect in some manner the conditions for microtubule assembly in the cell, we have undertaken the analysis of such preparations for the presence of a dynein-like ATPase and for the presence of 'arms' on the microtubules.

2. Materials and methods

2.1. *Purification of tubulin*

Tubulin was prepared from brain (guinea pig, calf and pig brains were used with approximately the same results) as previously described [8]. All manipulations were carried out in the reassembly buffer (RB) – 100 mM MES ((2-(*N*-morpholino) ethanesulfonic acid) buffer, pH 6.5 at 4°C; 1 mM EGTA; 1 mM GTP and 0.5 mM $MgCl_2$ – unless otherwise specified. Subunits were stored in RB with 4 M glycerol. The references to 1st, 2nd, 3rd etc. assembly are to the number of cycles of assembly at 37°C and disassembly at 4°C the sample has gone through starting with the original 100 000 *g* supernatant of the brain homogenate. Each disassembly step was followed by centrifugation at 100 000 *g* for one hour to remove aggregates. The assembled tubules were collected by centrifugation at the same speed but with the rotor temperature at 20°C rather than 4°C.

2.2. *Disc gel electrophoresis*

Samples were run in 5% polyacrylamide gels using the discontinuous SDS-Urea system [9, 10]. Gels were fixed, stained in one of the following stains: fast green, amido schwarz or commassie blue, destained by diffusion and scanned in a Gilford spectrophotometer with a linear gel transport. Areas under the curves were determined by tracing the peaks on graph paper, cutting them out and weighing them.

Molecular weight calibrations were done with immunoglobulins and ovalbumin. The molecular weights used were: ovalbumin, 43 000; IgG γ-chain, 55 000; IgM μ-chain, 70 000; IgG, 160 000; IgA, 175 000; IgA dimer, 350 000 (fig. 2).

2.3. *Density gradient centrifugation and column chromatography*

Discontinuous 10 to 30% sucrose gradients were made in 4 M glycerol-RB without GTP and centrifuged for 2 hr at 41 000 rpm in an SW-41 rotor. Centrifugations were carried out at both 4°C and 20°C with no difference in result. Fractions were collected with a Buchler Densi-Flow fractionator and proteins were analysed by determination of A_{280} and by the method of Lowry [11]. Inorganic phosphate was determined by the method of Martin and Doty [12]. The samples were incubated with 1 mM ATP for 20 min at room temperature and the reaction stopped by the addition of silicotungstate. Calcium, Magnesium and EDTA were added to final concentrations of 1 mM as described below. The presence of microtubules was determined by electronmicroscopic examination of negatively stained material.

Gel filtration was carried out on Sepharose 4B columns 2.5 cm in diameter with a bed length of 90 cm. The column was equilibrated with 0.1 M MES buffer at pH 6.5 with 2.5 mM Dithiothreotol.

3. Results

Preparations of tubulin subunits were prepared by three cycles of reassembly and prevented from assembling further by the addition of colchicine (10^{-4} M.) or by maintaining the concentration below the critical concentration for assembly of 0.18 mg/ml [13]. Under either of these conditions a Mg^{2+}-ATPase activity of 1 μmole P_i/mg protein/min was observed. This activity was decreased 50% in the presence of calcium and abolished if the divalent ions were replaced by EDTA. The activity of intact microtubules assembled from 3X purified subunits was 1.7 μmoles P_i/mg prot/min. Since the tubules depolymerised rapidly under the calcium or EDTA conditions, no values were obtained under these conditions.

When preparations of microtubules are examined by electrophoresis on SDS-Urea gels the tubulin double band (fig. 1) was invariably the major component accounting for at least 80% of total protein in all cases. The other constant component was a very tight double band near the origin of the gel. In calibrated gells these two bands had molecular weight of 370 000 and 355 000 respectively and together made up between 4.4% and 12% of the total protein on the gel. A small number of minor components are also variably present.

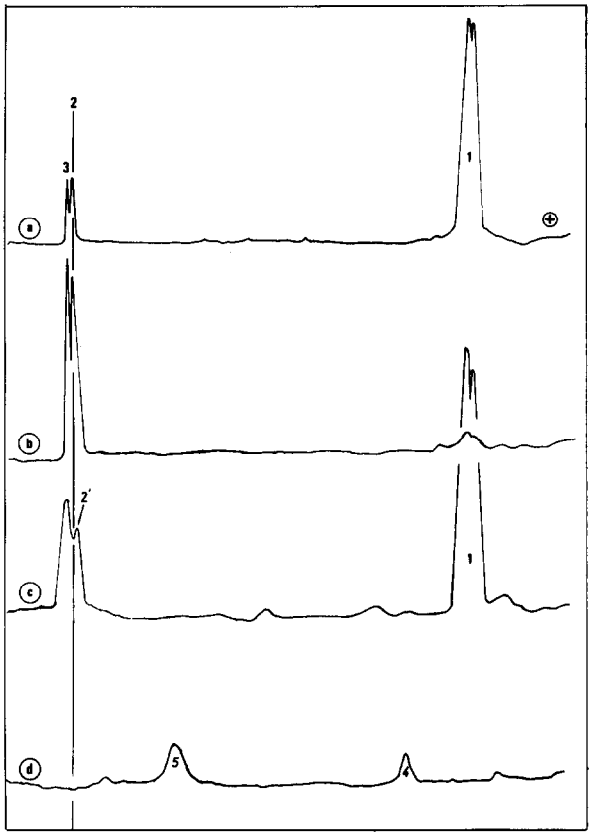

Fig. 1. Polyacrylamide gel patterns of (a) microtubules purified by three cycles of reassembly. (b) Brain dynein peak from Sepharose column – peak 2 in fig. 4. (c) Low ionic strength extract of sea urchin sperm flagella. (d) Concentrated ATPase-rich fractions from sucrose–glycerol gradient. Peak #1 is tubulin; vertical line labeled #2 marks the position of the more rapidly migrating band in the brain dynein; 2' marks the position of the more rapidly migrating band is sperm tail dynein; peaks #3 and #4 are the major components in the ATPase rich fraction. Migration is toward the anode (+) on the right. All scans at 550 nm.

Microtubule preparations were subjected to cen-

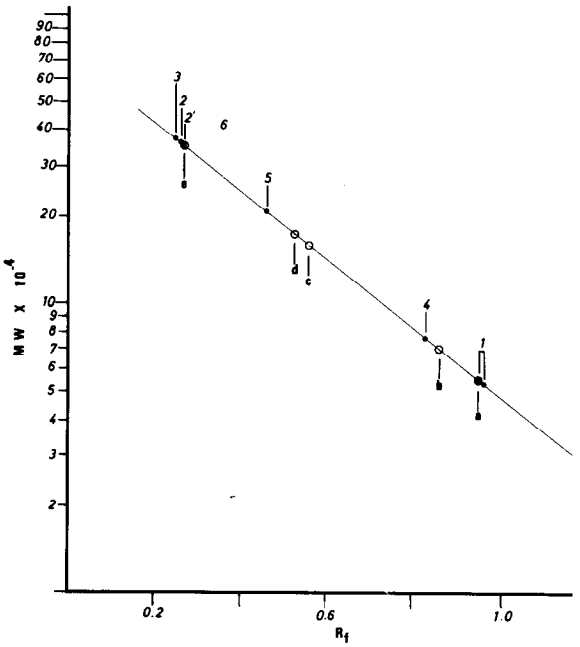

Fig. 2. Molecular weight calibration for the gel in fig. 1. Solid points are the experimental data and are numbered as in fig. 1. The point #3 is the same for brain and sperm dyneins. The open points are the calibration markers as described in the text.

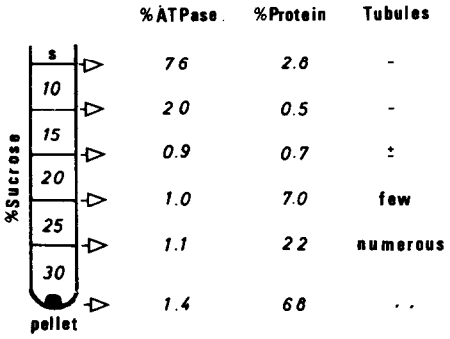

Fig. 3. Discontinuous sucrose gradient in 4 M glycerol. The ATPase and Protein figures represent the percentage of total amount recovered from the gradient. The tubules are estimated from negatively stained samples.

trifugation on discontinuous gradients in 4 M glycerol. Prior to this step the tubules were pelleted in 4 M glycerol and resuspended in the same buffer at room temperature in order to remove the bulk of the unassembled subunits [8]. The ATPase activity of the tubules in glycerol was unchanged from the value cited above. After centrifugation the fractions were

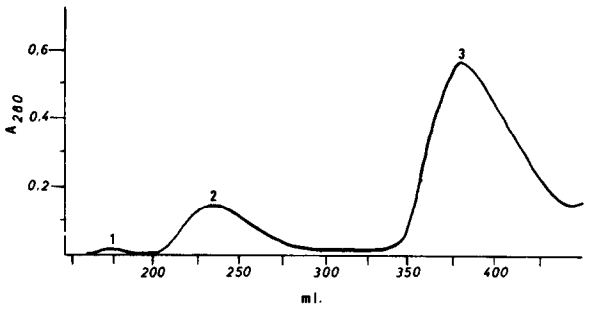

Fig. 4. Elution profile of 2.5 X 90 cm. Sepharose 4B column monitored continuously at 280 nm. Peak #1 is in the void volum peak #2 represents the brain dynein which has been examined electrophoretically in fig. 1b. Peak #3 is tubulin.

collected and assayed for protein, ATPase and microtubules. More than 95% of the ATPase activity is found at the 10:15% interface and above with the highest concentration at the sample: 10% interface. Though these fractions contained the bulk of the Mg-ATPase they contained only three percent of the total protein. Disc electrophoresis of pooled and concentrated ATPase-rich fractions showed major bands at 77 000 and 210 000 mol.wt. (fig. 1d). The first traces of microtubules are seen at the 10:15% sucrose interface where they are very short and number no more than one tubule per ten 400 mesh grids in the electronmicroscope. Microtubules are more numerous, 5 to 10 per grid square, at the 15:20% interface though still short in length. The number of tubules and protein increase in the next two interfaces and reach a maximum value in the pellet where 68% of the protein is recovered (fig. 3). Electrophoretic patterns of the pellet did not differ markedly from the starting preparation. Only 4% of the total ATPase activity was associated with the tubule-rich fraction and the specific activity decreased as the total protein in the fraction increased though the total activity in each of the tubule-rich fractions is about the same. The specific activity of the pooled fractions (15:20% interface and denser including the pellet) in the presence of Mg^{2+} was 10^{-2} micromoles P_i/mg protein/min.

Pelleted tubules from the gradient were dissociated at 4°C in RB without GTP and centrifuged at 100 000 *g* for one hour to remove aggregates prior to filtration on Sepharose 4B. The void volume of the column used was 160 ml and the salt volume 460 ml.

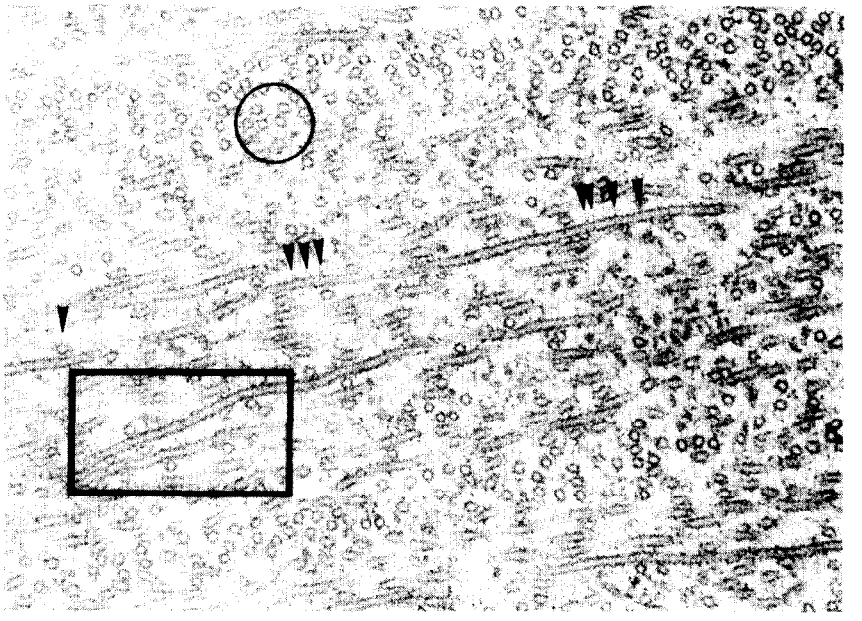

Fig. 5. Thin-section electron micrograph of tubules pelleted through a discontinuous sucrose–glycerol gradient. Arrows indicate 'arms' on tubules in longitudinal and transverse section. In the circled area an 'arm' pattern similar to that of the sperm A-tubule is seen. The tubules within the rectangular block demonstrate 'bridging' between tubules as well as 'arms' (× 54 000, Hitachi HU-12).

A trace of protein was seen in the void volume followed by a peak eluting at 230 ml and containing 13% of the total protein and a major peak at 360 ml (fig. 4). The ATPase activity of the peak at 225 ml was 3×10^{-2} μmoles/mg/min. The major peak showed no detectable activity. The gel electrophoretic pattern of the concentrated 225 ml peak showed marked enrichment in the high molecular weight doublet and some small amount of tubulin (fig. 1b).

Axonemes prepared from sea urchin flagella [14] were treated in 0.5% digitonin to remove membranes and then extracted for 1 hr in 10^{-3} molar Tris, pH 7.8 to extract Dynein [1]. This material had a Mg-ATPase activity of 0.8 μmoles/mg/min and on gel electrophoresis tubulin and two Dynein components with apparent molecular weights of 370 000 and 350 000 were resolved. The more slowly moving of these bands comigrated with the heavier of the brain high molecular weight bands while the more rapidly moving moved consistantly a bit ahead of the lighter brain protein (fig. 1c).

4. Discussion

Preparations of microtubules purified from brain by reassembly [8] show considerable ATPase activity and the presence of considerable amounts – 4 to 12% – of high molecular weight polypeptides which migrate as a tight doublet on disc gels. This figure is roughly in agreement with the value of 0.18% of total brain protein obtained by Burns and Pollard [15] for a microtubule associated peptide with a molecular weight of 380 000. They have called this polypeptide, which gives only a single band on disc gels, brain α-dynein. We shall adopt this terminology and apply the term 'brain dynein' to the doublet polypeptides observed here.

4.1. *Molecular weight*

Brain dynein has two subunits with apparent molecular weights of 370 000 and 355 000 as determined by disc gel electrophoresis. On the same gel system the two components of sperm dynein have molecular weights of 370 000 and 350 000. These values for dynein are considerably lower than have been previously reported [16,17] but have been repeated numerous times in this gel system with the same result. A potential problem might exist in the use of unreduced IgA dimer as a standard.

4.2. *Association with microtubules*

The amount of brain dynein in preparations of microtubules purified by reassembly varies from preparation to preparation from 4 to 12% of the protein in the preparation. However, for a given preparation the ratio of dynein to tubulin is constant through up to ten cycles of assembly and disassembly and remains constant on gradient centrifugation of the intact tubules. High speed centrifugation of disassembled microtubules reveals no pelleted dynein except that present in proportion to pelleted tubulin. Direct proof must rest on the reassociation of dynein with the microtubules.

4.3. *ATPase activity*

While neurotubules prepared by reassembly show an active Mg-ATPase activity, the majority of this activity can be separated from the tubules on a sucrose gradient. This activity is of low density suggesting that it is membrane associated. Gel electrophoresis of these fractions shows no dynein-like peptides. Only 4 to 5% of the total ATPase activity enters the gradient with the microtubules and even this small portion decreases in specific activity as the concentration of microtubules increases (fig. 3). The specific-activity of the tubule-rich fractions (pooled) is 80-fold less than sperm dynein and use of a wide variety of ionic conditions did not increase the activity in the brain samples.

4.4. *Possible location of brain dynein*

The constant ratio of brain dynein to tubulin in our preparations suggests the possible copurification of another structure linked to the microtubule. Careful examination of sections of samples such as the gradient pellet reveals the constant presence of small 'arms' on the microtubules when viewed in either longitudinal or cross section (fig. 5). Often these small projections are seen evenly spaced along a region of a tubule and at other times seem to run between adjacent tubules. Similar projections have been seen on axonal microtubules in vivo [18]. The cross sectional view is similar to what is seen on sperm A-tubules in some cases.

4.5. *Functional considerations*

Should our conclusion that brain dynein is the structural component of the arms on the microtubule be correct, it is tempting to postulate a motile function for them in a sliding tubule mechanism [6]. However, in the case of the flagellum this mechanism depends on arms with ATPase activity, while in our case the activity is extremely low. It is possible that the ATPase activity in the brain protein is very labile or activated by conditions which we have not found. This possibility remains attractive pending further study of the protein. The second possibility is that these structures are purely structural serving to interconnect a microtubular cytoskeleton or to maintain connections between microtubules and other organelles such as synaptic vesicles [19] or mitochondria [20].

No matter what the final results are, it is likely that this protein plays an important part in the biological role of the microtubule.

Acknowledgements

The authors would like to thank Dr. Marshall Nirenberg for his support and encouragement of this work and Mrs. Alice Ling for her aid with electron microscopy. FG is supported by NINDS Special Fellowship NS-55647. MLS is the recipient of Teacher-Investigator Award NS-11011. CRC and MLS thank the Guggenheim Foundation for their generous support during the preparation of this paper.

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