increased about 5000-fold above wild-type cells by cloning on plasmid pBR322, by the deletion of some *E. coli* DNA from in front of the *araC* gene, and by the insertion of the *lac* promoter in front of the gene.

Acknowledgments

We thank Tom Roberts for performing the exonuclease III and S1 digestions and Ed Cannon and William Lane for performing the N-terminal sequencing of *araC* protein.

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Potent Microtubule Inhibitor Protein from *Dictyostelium discoideum*†

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ABSTRACT: A novel potent protein factor capable of inhibiting the in vitro polymerization of mammalian brain microtubule protein and of breaking down preformed microtubules has been partially purified from cell extracts of *Dictyostelium discoideum*. The factor has an apparent M_r of around 13 000 and is trypsin resistant but heat and pepsin sensitive. When soluble microtubule protein was fractionated into tubulin and microtubule-associated proteins and each fraction was assayed independently for its susceptibility toward inhibition, it was clearly demonstrated that the tubulin but not the associated protein fraction was rendered nonpolymerizable. Soluble tubulin was inactivated at ratios of 1 mol of inhibitor to 100 mol of tubulin, estimated conservatively. Quantitative separation of tubulin and inhibitor after inactivation did not result in reactivation of tubulin's polymerizing capacity, suggesting a catalytic modification. The biochemical properties tested of the inactive tubulin argue against a mechanism involving simple proteolysis, N-site GTP hydrolysis or release, or general denaturation.

Cytoplasmic microtubules have been shown to play a dynamic role in such diverse motile processes as mitosis, axonal transport, protein secretion, and changes in cell shape (Dustin, 1978). The elucidation of the mechanisms of these events and of the means by which they are regulated depends therefore upon detailed investigations of microtubule components at the molecular level. Since Weisenberg's initial demonstration of the in vitro polymerization of microtubules from mammalian brain (Weisenberg, 1972), much progress has been made in identifying the components and defining their biochemical roles. The two components that have been studied most extensively, guanosine triphosphate and microtubule-associated proteins, are both thought to have positive control functions in the assembly of microtubules, i.e., they facilitate the formation of tubules from soluble tubulin dimers (Kirschner, 1978). Although these factors have been shown to be integral parts of microtubules, evidence suggesting their roles in the dynamic control of microtubule assembly and disassembly in vivo has not been forthcoming.

Regulation by factors acting as negative controls should also exist; they could trigger the disassembly of microtubules and/or maintain large pools on nonpolymerized tubulin in a

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0006-2960/82/0421-0782$01.25/0

state readily convertible for subsequent polymer formation. The rapid disassembly of cytoplasmic microtubules during the cell cycle progression from G2 to mitosis and the presence of large amounts of apparently unpolymerized tubulin in sea urchin oocytes are just two examples of systems suggestive of regulation by such mechanisms (Weber et al., 1975; Raff et al., 1971). Potential negative control factors have, however, been identified in only a few instances. Plant alkaloids have long been known to be inhibitors of microtubules (Dustin, 1978), and a number of mammalian brain proteins apparently share the same tubulin binding site with these alkaloids (Lockwood, 1979; Sherline et al., 1979). None of these protein inhibitors, however, have comparable potency in inhibiting microtubule polymerization and/or inducing their depolymerization. Similarly, calmodulin (Means & Dedman, 1980) and a recently reported M_r 94000 polypeptide (Nishida & Sakai, 1980) from mammalian brain both show inhibitory effects of microtubules, but again with relatively low efficiencies. Polyanions like RNA and DNA (Bryan et al., 1975; Corces et al., 1980) have also been found to inhibit polymerization in vitro, but as in the former cases, their importance in vivo remains unclear. Finally, free calcium ions per se seem unlikely candidates for active negative in vivo control of microtubules, with their cellular concentration being below that reported for effective in vitro inhibition (Solomon, 1976). The general lack of success in isolating potent inhibitory factors is probably attributable in part to the nature of the commonly used purification scheme for microtubule protein, which selects for tubulin polymerizability and therefore eliminates such factors and any tubulin species which is specifically deficient in the ability to polymerize.

Recently, Unger et al. (1979) found that vegetatively growing amoeboid cells of *Dictyostelium discoideum* apparently contain considerable amounts of nonpolymerized tubulin, as judged by fluorescent antibody staining techniques. This system therefore seemed a potential source for potent microtubule inhibitory activities. We report here the partial purification and characterization of a novel, potent low molecular weight inhibitory protein from *Dictyostelium discoideum* that specifically inactivates the polymerizing ability of brain tubulin dimers in an apparent catalytic fashion.

Materials and Methods

Enzymes and Chemicals. Trypsin and trypsin soybean inhibitor were purchased from Sigma, Munich; phospholipase C, RNase A, and DNase I were from Boehringer Mannheim; pepsin and phenylmethanesulfonyl fluoride were from Merck, Darmstadt. Radiochemicals, [^{32}P]phosphate, [^{3}H]$NaBH_4$, and [^{3}H]colchicine were from the Radiochemical Center, Amersham. [γ-^{32}P]GTP was prepared according to the method of Glynn & Chappell (1964).

Preparation of Microtubule Inhibitory Activity from Dictyostelium discoideum. Dictyostelium discoideum AX2 was grown axenically on HL5 medium containing 86 mM glucose (Cappuccinelli et al., 1978). Amoebas were grown to a density of (3–5) × 10^6 cells/mL, harvested by centrifugation, and washed twice with 25 mM sodium 2-(*N*-morpholino)ethanesulfonate (Mes),[1] pH 6.4, 0.5 mM EGTA, and 1.0 mM $MgCl_2$ (buffer A) supplemented with 1 mM phenylmethanesulfonyl fluoride and 1% dimethyl sulfoxide. The washing and all following procedures were carried out in the cold. Two milliliters of buffer A plus 0.25 M sucrose was added per gram of washed cells, and the cells were broken by 30 passes in a tightly fitting glass Teflon homogenizer using a high-speed rotor. The homogenate was then centrifuged at 100000*g* for 1 h, the supernatant decanted off and saved, and the pellet (cell debris) again extracted by repeating the homogenization and centrifugation procedure. Supernatants were pooled and clarified by centrifugation at 100000*g* for 2 h. Milky liquid floating at the top of the tubes was removed carefully, and the supernatants were drawn off, strained through glass wool or a glass-filter membrane, and either used immediately or stored at −70 °C at a protein concentration of 10–12 mg/mL. Aliquots (60 mL) of the extracts were chromatographed on phosphocellulose (PC)[1] columns (3 × 10 cm) equilibrated with buffer A, at a flow rate of 20 mL/h, and 6-mL fractions were collected. After passage of the flow through, the column was washed with 60 mL of buffer A and then developed with a linear gradient of 0–0.8 M NaCl, and 3.5-mL fractions were collected. Fractions containing inhibitory activity were pooled, and the protein was precipitated by the addition of $(NH_4)_2SO_4$ to saturation. Precipitated protein was resuspended in 100 mM Mes, pH 6.4, 0.5 mM EGTA, and 1 mM $MgCl_2$ (buffer B) plus 10% sucrose at an approximate concentration of 6 mg/mL and chromatographed on Sephadex G-50 columns (2 × 140 cm) which were calibrated with bovine serum albumin (68000 daltons) and cytochrome *c* (12000 daltons). The fractions containing peak levels of inhibitory activity were used for experiments directly or were first concentrated by ultrafiltration using Amicon U2 filters. In some cases, the latter step resulted in concentration of activity but loss in specific activity of up to 5-fold (see next section).

Microtubule Protein. Microtubule protein was purified from hog or bovine brain according to the methods of Shelanski et al. (1973) or Berkowitz et al. (1977), respectively. Hog brain microtubule protein was stored (for up to 4 weeks) in 8 M glycerol at −20 °C and bovine brain microtubule protein in 4 M glycerol at −80 °C. Stored microtubule protein was subjected to a cycle of polymerization and depolymerization before use in polymerization experiments. Phosphocellulose-purified tubulin (PC-tubulin) and microtubule-associated proteins (PC-MAPs) from hog brain were prepared essentially according to Weingarten et al. (1975). PC fractions were frozen in liquid nitrogen and stored at −70 °C either immediately (tubulin) or after concentration by ultrafiltration methods and desalting on Bio-Gel P-6 columns (MAPs). Aliquots of fractions were thawed and centrifuged for 1 h at 200000*g* to remove aggregated protein before use. The source of microtubule proteins (hog or bovine brain) was irrelevant for the results reported below.

Turbidity Assay of Microtubule Polymerization and Inhibition. Turbidity measurements were made routinely by using a Gilford 240 spectrophotometer equipped with a thermostat and an automatic chart recorder. Preincubations and assays were carried out in 0.25-mL cuvettes as follows: Protein samples (microtubule protein plus inhibitor fraction) were diluted into buffer B to a final volume of 0.19 mL and final concentrations as indicated in the text. The temperature of the mixtures was allowed to equilibrate to 30 °C unless otherwise stated, the turbidity was set at zero, and polymerization was initiated by adding 10 μL of a 20 mM GTP stock in buffer B. The solution was rapidly mixed, and the turbidity change was followed at 350 nm. The extent of turbidity increase or decrease was stable after 15 min in all cases. Preincubations were carried out routinely for 5 min before

[1] Abbreviations: Mes, sodium 2-(*N*-morpholino)ethanesulfonate; PC, phosphocellulose; MAPs, microtubule-associated proteins; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate.

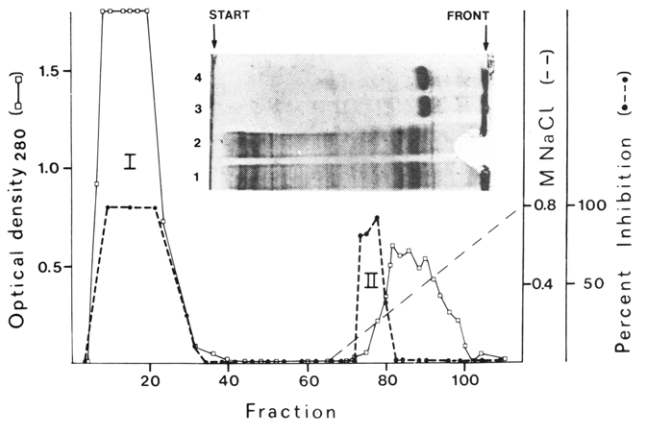

FIGURE 1: Phosphocellulose chromatography of crude cytoplasmic extracts. Soluble extracts of *Dictyostelium discoideum* were prepared and chromatographed as described under Materials and Methods. Fractions were assayed for protein by measuring the optical density at 280 nm (□), and aliquots (50 μL) were assayed for inhibition of microtubule polymerization (●). Fractions eluted by salt (>0.2 M) were dialyzed before assaying. (Inset) $NaDodSO_4$–polyacrylamide (7.5%) gel electrophoresis of PC-tubulin (slots 3 and 4) and PC-MAPs (slots 1 and 2) fractions. Samples of slots 1 and 3 were incubated with peak II material (see legend to Figure 7) prior to electrophoresis; those of slot 2 and 4 were untreated.

initiation of polymerization. Percent inhibition was calculated from the extent of polymerization vs. a control of microtubule polymerization without inhibitor. The specific activity was estimated from the amount of protein needed to achieve the observed level (percent) of inhibition. In these assays there was a linear relationship between amount of inhibitor and inhibition for a given concentration of microtubule protein (see below).

Miscellaneous Procedures. In vitro labeling of proteins by reductive methylation and tritiation was carried out essentially as described by Kumarasamy & Symons (1979) and colchicine binding as described by Wiche & Furtner (1980). For the in vivo labeling of N-site GTP, the procedure of Spiegelman et al. (1977) was followed, with a few minor modifications, including the use of rat glial C_6 cells instead of CHO cells. $NaDodSO_4$–polyacrylamide gel electrophoresis in one dimension was carried out by using the discontinuous buffer system of Laemmli (1970). For two-dimensional gel electrophoresis, the method of O'Farrell (1975) was followed. Gels were stained with Coomassie Brilliant Blue and destained according to Fairbanks et al. (1971). The protein was measured according to Lowry et al. (1951) or by the Bio-Rad method (Bradford, 1976).

Results

Partial Purification of a Low Molecular Weight Inhibitory Protein. In initial experiments, we observed a potent inhibition of hog brain microtubule assembly by crude cytoplasmic extracts of *Dictyostelium discoideum*. As tested by gel electrophoresis of assembly components before and after incubation with extracts, at least part of this inhibition was due to breakdown of microtubule-associated proteins (MAPs)[1] by proteolytic activities present in the crude extracts. In an attempt to isolate any nonproteolytic inhibitory activities, crude extracts were chromatographed on phosphocellulose (PC)[1] columns (Figure 1). Two distinct peaks of inhibitory activities were resolved, one of which, peak II, was free of any MAP or tubulin degradation activity, as judged by electrophoresis of inhibitor-treated tubulin and MAP preparations (inset). Rechromatography of both peaks on freshly prepared PC columns demonstrated that there was no cross-contamination

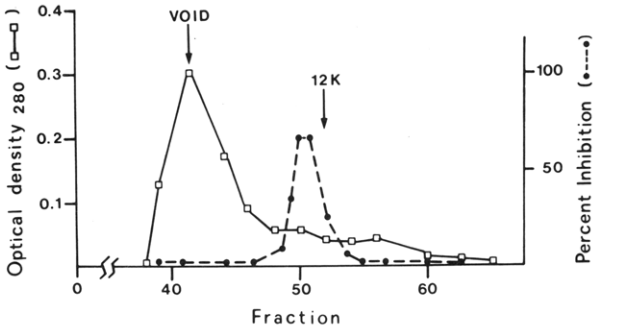

FIGURE 2: Sephadex G-50 chromatography. Pooled fractions of peak II activity (Figure 1) were concentrated and chromatographed on Sephadex G-50 columns as described under Materials and Methods, collecting 2-mL fractions. Fractions were assayed for protein (□) and aliquots (30 μL) tested for inhibition of microtubule polymerization (●).

Table I: Chemical Properties of Inhibitory Activity[a]

treatment	inhibition
none	+
trypsin (1 mg/mL)[b]	+
pepsin (10 μg/mL)[c]	−
heat (80 °C, 5 min)	−
RNase (20 μg/mL)	+
DNase (20 μg/mL)	+
phospholipase C (20 μg/mL)	+
glycine hydrochloride, pH 2.5	+

[a] Aliquots (20 μL) of an inhibitory fraction from a Sephadex G-50 column (see Figure 2) were treated in the ways shown in the table and then assayed for retention of inhibitory activity turbidimetrically. Enzymatic digests were for 30 min at 37 °C. [b] After treatment, a 3-fold excess of trypsin soybean inhibitor was added to inhibit the reaction. [c] Prior to pepsin treatment, the inhibitor was desalted into 50 mM glycine hydrochloride, pH 2.5, on a Bio-Gel P-6 column. After treatment, NaOH was added to pH 6.0; no residual proteolysis remained under these conditions. The pH change did not affect the inhibitory activity.

of one activity in the other (data not shown).

The presumably nonproteolytic inhibitory activity of peak II was further purified by column chromatography on Sephadex G-50 (Figure 2). The inhibitor eluted as a single symmetrical peak with a M_r slightly greater than that of cytochrome *c*. The specific activity increased about 7-fold over that of the material eluted from the phosphocellulose column. Peak I activity of the PC column eluted in the void volume on Sephadex G-50, indicating a M_r greater than 30 000 (data not shown). The behavior of peak I and peak II activities in both chromatographic steps strongly suggested that they were separate and distinct molecular entities. Because of its apparent nonproteolytic nature, the peak II inhibitory activity, further purified by the Sephadex G-50 step, was used in this study.

Because of the proteolytic inhibitory activity in the crude extracts, resolved as peak I on phosphocellulose, it was impossible to accurately estimate the initial activity attributable to the peak II material. Assuming its complete recovery from the crude supernatants in the peak II fraction, we calculated a purification of about 500-fold after the two chromatographic steps.

The inhibitor was shown to be sensitive to both heat and pepsin treatment (Table I). It was insensitive to trypsin, DNase, RNase, and phospholipase C. The inhibitor therefore is a trypsin-resistant polypeptide.

An attempt was made to identify the inhibitor among the other contaminating proteins in the Sephadex G-50 fraction with the aim of estimating the stoichiometry of microtubule

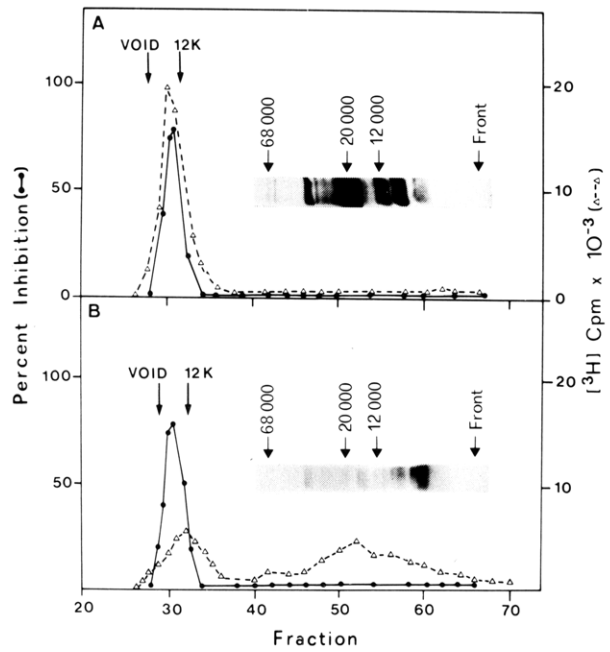

FIGURE 3: Bio-Gel P-10 chromatography of trypsin-treated and untreated samples. A 100-μL aliquot of a partially purified inhibitor preparation at 100 μg/mL was desalted into 0.2 M sodium borate, pH 9.0, and labeled with [^{3}H]$NaBH_4$ essentially as described by Kumarasamy & Symons (1979). Tritiated (5×10^4 cpm/μL) inhibitor fractions (0.8 mL) were incubated in buffer A with and without trypsin for 45 min at 37 °C and then chromatographed separately in the same buffer on Bio-Gel P-10 columns (1.8 × 40 cm) at a flow rate of 5 mL/h. Fractions of 1 mL were collected, and aliquots were either counted for radioactivity (Δ) or assayed for inhibitory activity (●). Samples with trypsin were treated with trypsin soybean inhibitor before assaying. (A) Inhibitor without trypsin. (B) Trypsin-treated inhibitor. 12K designates the peak fraction of eluted cytochrome *c*. (Insets) Aliquots (10^6 cpm) of samples (A or B) analyzed by electrophoresis on 15% polyacrylamide slab gels and subsequent fluorography (Chamberlain, 1979); standards were cytochrome *c* (M_r 12 000), trypsin soybean inhibitor (M_r 20 000), and bovine serum albumin (M_r 68 000).

protein inhibition (see below). An inhibitor preparation was therefore radioactively labeled by reductive methylation and divided into two parts, one of which was exposed to trypsin to select for trypsin-resistant factors. Both the trypsin-treated and the untreated samples were then analyzed separately by chromatography on Bio-Gel P-10 (Figure 3). In both cases the elution profiles and the recovery of the inhibitory activities were identical. However, in the trypsin-treated sample, the protein content of the peak fraction (no. 30) was about 10-fold reduced. This led us to conclude that no more than 10% of the protein in the Sephadex G-50 fraction is attributable to the inhibitor. $NaDodSO_4$–polyacrylamide gel electrophoresis of the untreated sample (inset, Figure 3A) revealed a number of protein bands, predominantly in the M_r range between 10 000 and 20 000. Since most of these bands were still detectable in the trypsin-treated sample, although some at drastically reduced amounts, it was not possible to identify any particular band as the inhibitor.

Characteristics of Microtubule Protein Inhibition. The effects of the inhibitor on the polymerizing ability of microtubule proteins were observed in several ways. First, preincubations of microtubule proteins with sufficient amounts of inhibitor before initiation of assembly resulted in the complete inhibition of polymerization (Figure 4, curve IV). Second, addition of inhibitor to preformed microtubules caused a rapid breakdown of polymers, as shown by the sudden drop in turbidity (curve II). Third, when inhibitor and the assembly initiator, GTP, were added simultaneously to the soluble

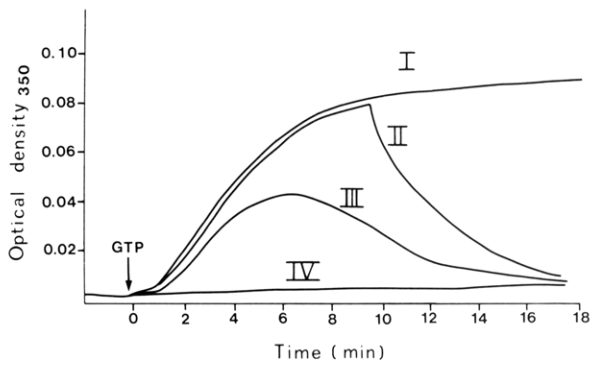

FIGURE 4: Inhibition of microtubule polymerization. Hog brain microtubule protein (0.6 mg/mL) in buffer B was preincubated in the absence of GTP at 30 °C for 5 min with inhibitor fractions from the Sephadex G-50 column. Polymerization was initiated by addition of GTP as described under Materials and Methods. The inhibitor, at a final concentration of 10 μg/mL, was either absent during the assay (control, curve I), added 10 min after polymerization had been initiated (curve II), added simultaneously with GTP (curve III), or preincubated with microtubule protein before addition of GTP (curve IV).

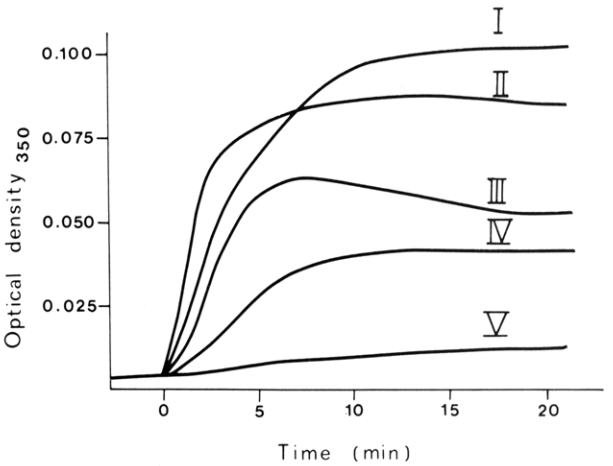

FIGURE 5: Relationship between extent of polymerization and inhibitor concentration. Hog brain microtubule protein (0.7 mg/mL) was preincubated for 5 min at 30 °C with inhibitor fractions from Sephadex G-50 which had been concentrated as described under Materials and Methods. Polymerization was initiated by addition of GTP. No inhibitor (curve I); 5 μg/mL (curve II); 10 μg/mL (curve III); 15 μg/mL (curve IV); 50 μg/mL (curve V).

microtubule protein (curve III), a burst of polymerization occurred before subsequent breakdown of microtubules. In this case initially the rate of polymerization was evidently faster than the rate of inhibition, indicating that the inhibitor required some time to bring about inhibition of microtubule components.[2] Lower ratios of inhibitory activity to microtubule protein than those used in the experiments of Figure 2 led to a corresponding decrease in the extent of inhibition of polymerization (Figure 5). The observation that in these cases the optical densities did not decrease to base levels but remained more or less constant after maximum values were reached would have been consistent with a stoichiometric relationship between the amount of inhibitor and the extent of polymerization.[3]

[2] It should be noted that in additional experiments where the period of preincubation of microtubule protein with inhibitor was varied (1, 2, or 3 min), the initial rates of polymer formation upon addition of GTP were inversely proportional to the length of preincubation.

[3] The significance of the "overshoot" kinetics (Figure 5, curve II) observed repeatedly at low inhibitor to microtubule protein ratios remains unclear. Similar unclarified observations have been reported by others (Vallee & Borisy, 1977).

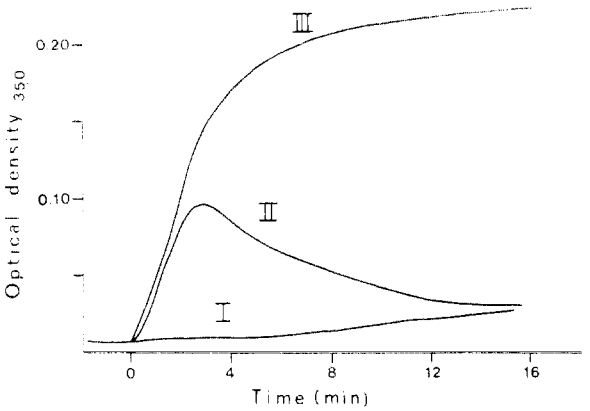

FIGURE 6: Effects of inhibitor on microtubule protein components. PC-tubulin and PC-MAPs prepared as described under Materials and Methods were individually incubated (37 °C, 5 min) with inhibitor (10 μg/mL) of unconcentrated Sephadex G-50 fractions, at final concentrations of 1.2 mg/mL PC-tubulin, 0.4 mg/mL PC-MAPs, and 1 mM GTP. (Curve III) Preincubation of PC-tubulin without inhibitor; initiation of polymerization by addition of MAPs (control). (Curve II) Preincubation of PC-MAPs with inhibitor; initiation of polymerization by PC-tubulin. (Curve I) Preincubation of PC-tubulin with inhibitor; initiation by PC-MAPs. GTP was present throughout the incubations.

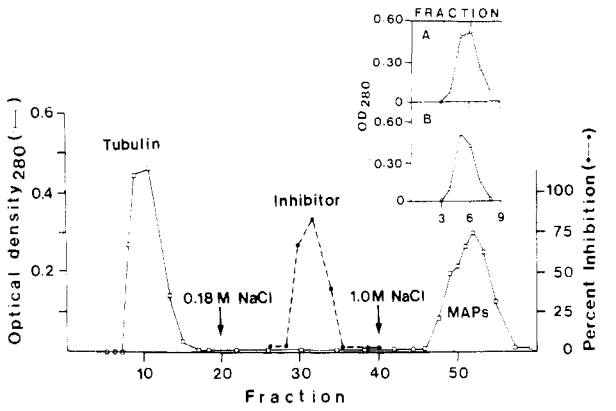

FIGURE 7: Chromatographic separation of inhibitor, tubulin, and MAPs after incubation. Hog brain microtubule protein (6 mg/mL) in buffer B with 0.1 mM GTP and 2 mM 2-mercaptoethanol was preincubated with inhibitor (80 μg/mL) for 60 min at 4 °C. The mixture (1.7 mL) was chromatographed on phosphocellulose columns (5 mL) equilibrated with the same buffer, at a flow rate of 2 mL/h, and 0.4-mL fractions were collected. After elution of tubulin, the column was first developed with 8 mL of 0.18 M NaCl in buffer B and subsequently with 1 M NaCl. Aliquots of fractions were assayed for protein (□) and inhibition of microtubule polymerization (●). (Inset) Microtubule protein (0.6 mL, 6 mg/mL) with (A) or without (B) inhibitor (50 μg/mL) was incubated as above and chromatographed under identical conditions on 2-mL columns at a flow rate of 1.5 mL/h, collecting 0.25-mL fractions. Each column required about 1 h for loading and elution of the tubulin.

Site of Inhibition and Evidence for a Catalytic Mechanism. For identification of which of the two major components of in vitro polymerized microtubules, the tubulin, or the microtubule-associated proteins (MAPs)[1] was being inactivated by the inhibitory factor, a series of mixing experiments with phosphocellulose purified tubulin and MAPs was carried out. One component was preincubated with the inhibitor and GTP, and the second was added to initiate polymerization. Conditions were chosen such that a burst of polymerization, like that in Figure 4 (curve III), would occur only if the preincubated components were unaffected by the inhibitor. (Preliminary experiments showed that free GTP was not cleaved by the inhibitor and therefore appeared not to be directly involved in the mechanism of inhibition.) In fact, when PC-MAPs, GTP, and inhibitor were preincubated for 5 min at 37 °C and PC-tubulin was added to initiate polymerization, an initial burst of polymerization occurred (Figure 6, curve II) which was identical in slope with that seen in the control sample (curve III). However, when PC-tubulin, GTP, and inhibitor were preincubated and PC-MAPs added later, no polymerization ensued (curve I). These results suggested very strongly that tubulin was the specific site of inhibitory action and that MAPs were unaffected.

Assuming a M_r of around 13000 for the inhibitor and complete inactivation of the tubulin in the experiments shown in Figure 6 (a conclusion supported in experiments shown below) and considering that the inhibitor constitutes at most only 10% of the protein in the partially purified fractions, we estimated from the data shown in Figure 6 a minimum stoichiometry of 100 mol of tubulin being inactivated by 1 mol of inhibitor. This ratio suggested that the inhibitor either acts effectively through some substoichiometric poisoning mechanism, whereby only a few tubulin–inhibitor complexes are needed to block and depolymerize microtubules, or acts by inactivating tubulin in a catalytic-like manner.

To distinguish between these two possibilities, we designed an experiment to examine whether inhibitor-treated tubulin would regain its polymerizability after removal of the inhibitor. Microtubule protein was rendered nonpolymerizable by addition of inhibitor, and this mixture was subsequently chromatographed on phosphocellulose columns under conditions where the inhibitor and MAPs, but not the tubulin, should bind to the column. As shown in Figure 7, the inhibitory activity adhered to the column and eluted in low salt, as was the case in its purification. Its recovery from the starting material was 92%, which is essentially complete recovery within the experimental uncertainty inherent in the turbidity measurements. The tubulin recovery from the PC columns was unaffected by the preincubation with the inhibitor, eluting in the same yield as an untreated tubulin sample (insets in Figure 7). As expected, the MAPs eluted from the column retained their ability to stimulate polymer formation of control tubulin, as demonstrated both by turbidity and electron microscopic methods (data not shown).

The polymerizability of the recovered tubulin (inhibitor-treated tubulin) was then tested by addition of PC-MAPs and GTP. In contrast to control tubulin, isolated under identical conditions (Figure 8, curve I), the inhibitor-treated tubulin was incompetent for polymerization (curve II). Upon addition of fresh, non-inhibitor-treated PC-tubulin at a final concentration approximating that of the control incubation (curve I) to a mixture of inhibitor-treated tubulin, PC-MAPs, and GTP, polymerization ensued immediately (curve III). The kinetics and extent of polymerization, being identical with those seen in the control, demonstrated that no detectable amounts of inhibitor were present in the inhibitor-treated PC-tubulin. This, together with the complete recovery of the inhibitor from the phosphocellulose column (Figure 7), showed that the inhibitor-treated PC-tubulin is lacking its ability not only to initiate polymerization (curve II) but also to elongate onto existing microtubules (curve III). If the inhibitor-treated PC-tubulin could participate in elongation, the extent of turbidity seen in curve III would have been much higher than that of the control (curve I). These experiments suggested that the activity rendering tubulin nonpolymerizable was of an enzymatic nature.

Further Characterization of Polymerization-Inhibited Tubulin. In a preliminary effort to unravel the molecular basis of the inhibitory reaction rendering tubulin nonpolymerizable, a few characteristics of the tubulin species were studied.

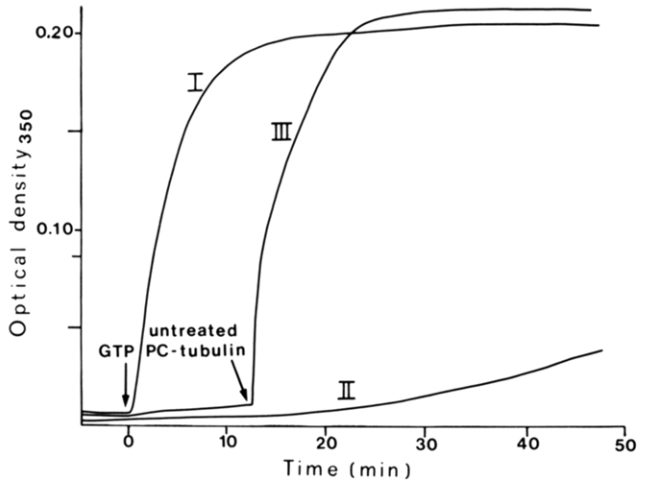

FIGURE 8: Polymerizability of inhibitor-treated and control PC-tubulin. For polymerization, inhibitor-treated PC-tubulin and control PC-tubulin prepared by phosphocellulose chromatography as described in Figure 7 were incubated with PC-MAPs and GTP, at final concentration of 1 mg/mL PC-tubulin, 0.4 mg/mL PC-MAPs, and 1 mM GTP. (Curve I) Control PC-tubulin plus PC-MAPs; (curve II) inhibitor-treated PC-tubulin plus PC-MAPs; (curve III) as in curve II, but with the later addition of a concentrated solution of untreated PC-tubulin to a final concentration of 1 mg/mL.

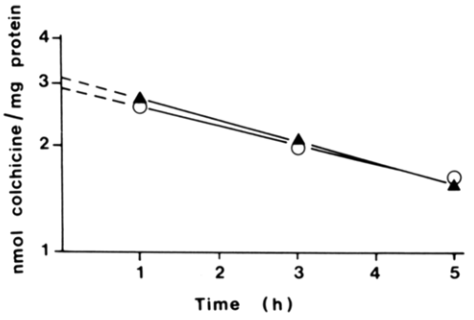

FIGURE 9: Colchicine binding of inhibitor-treated and untreated tubulin. Control PC-tubulin and inhibitor-treated PC-tubulin were prepared as described in the legend to Figure 7. Aliquots were assayed for colchicine binding essentially as described by Wiche & Furtner (1980). All assays were run in triplicate at a final protein concentration of 0.25 mg/mL. Decay curves were constructed by linear least-squares fit to averaged data points. (○) Untreated tubulin, half-life of decay 5.6 h; (▲) inhibitor-treated tubulin, half-life 5.0 h.

Colchicine binding activity is considered a measure of tubulin's native state and conformational integrity. Both the initial colchicine binding capacity and the rate of decay of binding of the polymerization-incompetent tubulin were found to be very similar to those of control PC-tubulin (Figure 9) and agreed with published data (Wiche & Furtner, 1980).

The polypeptide length and gross charge of the protein subunits also remained unaltered after treatment with inhibitor, as judged by two-dimensional gel electrophoresis (Figure 10). Thus proteolytic activity does not seem likely to be involved, although it cannot be rigorously ruled out.

The conclusion drawn from the experiments shown in Figures 7 and 8 was that tubulin is irreversibly altered by the inhibitor. This rendered simple cleavage of GTP bound to the exchangeable (E) site of tubulin a highly unlikely mechanism of inactivation. In addition, the following experiment clearly established that the inhibitor fractions were lacking GTPase activity in any sufficient amount to account for tubulin's incapability to polymerize. An inhibitor fraction (2 μg of protein) and, as a control, a MAPs fraction (20 μg of protein) were incubated with 0.3 mM [γ-^{32}P]GTP in conditions similar to those used for turbidity measurements of microtubule polymerization and inhibition (see Materials and Methods). After adsorption of nucleotides with charcoal and centrifugation, released $^{32}P_i$ was measured in the supernatants. It was determined that in both cases less than 1% of the total GTP present in the incubation mixtures was being cleaved during 5 min, the preincubation period applied in most of the inhibition experiments (e.g., Figure 5). This negligible GTPase activity of the inhibitor fraction was independent of the presence of tubulin in the incubation mixtures.

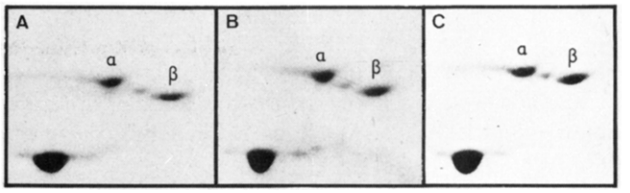

FIGURE 10: Two-dimensional gel electrophoresis of untreated and inhibitor-treated tubulin. Bovine microtubule protein (2.4 mg/mL) was preincubated in the presence or absence of inhibitor (75 μg/mL) for 15 min on ice. The samples were then placed in cuvettes, the temperature was raised to 32 °C, and polymerization was allowed to proceed to completion. (The plateau levels of optical density at 350 nm were 0.255 for control microtubule protein and <0.01 for inhibitor-containing samples.) Aliquots were then taken and prepared for two-dimensional gel electrophoresis according to the procedure of O'Farrell (1975). Electrophoresis was performed with a pH 5–7 gradient in the first dimension and a 10% polyacrylamide gel (Laemmli, 1970) in the second dimension. Actin (8 μg) was added to all samples before isoelectric focusing to aid in comparison of mobilities. (A) Untreated (8 μg), (B) inhibitor-treated (8 μg), and (C) mixture of untreated (4 μg) and inhibitor-treated (4 μg) microtubule protein. Only the tubulin region of the gels is shown. The large spot in the lower left corner of each picture is actin. Spots of α- and β-tubulin are indicated. Note the resemblance in size and relative mobilities of tubulin spots in all three pictures.

To determine whether nonexchangeable (N-site) GTP was altered upon inactivation of tubulin, we radioactively labeled N-site GTP by in vivo methods (Spiegelman et al., 1977) and showed that there was no detectable release of $^{32}P_i$ from a tubulin sample upon incubation with the inhibitor. Moreover, it was shown by extensive dialysis of either mixtures of inhibitor and radioactively labeled tubulin or tubulin alone, followed by analysis of bound nucleotide by thin-layer chromatography, that the amounts of protein-bound GTP and GDP were the same in the control and inhibitor-treated sample (data not shown). Thus, N-site GTP was neither cleaved nor released from tubulin upon inactivation by inhibitor.

Discussion

In the present study we used purified porcine brain microtubule protein as an in vitro assay system to search for inhibitors of microtubule polymerization in extracts of *Dictyostelium discoideum*. By fractionating crude cell extracts of *Dictyostelium discoideum* on phosphocellulose and Sephadex G-50 columns, we partially purified a novel inhibitory protein of M_r around 13 000. The inhibitor bears no resemblance to previously reported inhibitory proteins, including those from mammalian brain (Lockwood, 1979; Sherline et al., 1979) and the ubiquitously found calmodulin (Means & Dedman, 1980), as it differs from these factors either in size, or heat resistance and Ca^{2+} requirement. It is the most potent inhibitor reported to date, inhibiting microtubule assembly conservatively estimated at a stoichiometry of 1 inhibitor to 100 tubulin molecules.

Two lines of evidence clearly established that among the two major components of the in vitro assay system used, the tubulin and the MAPs, only the former was affected by the inhibitor. First, when the two components were chromato-

graphically separated and then incubated independently with the inhibitor, the polymerizability of the tubulin but not that of the MAPs was abolished. Second, after incubation of microtubule protein containing both components with the inhibitor, only the MAPs were recovered in active (polymerizable) form.

Tubulin was susceptible to inactivation by the inhibitor both in its soluble free dimer and in its polymerized form. Brief incubation with the inhibitor at the proper proportions was sufficient to render soluble tubulin completely nonpolymerizable, and a rapid breakdown of microtubules was induced upon addition of inhibitor to preformed polymers. However, complete suppression of microtubule polymerization was only observed above a certain ratio of inhibitor to tubulin. At lower rates, the extent of inhibition was reversely proportional to inhibitor concentration (Figure 5). Thus, in general, the characteristics of the inhibiting activity would have been compatible with a mechanism similar to that of microtubule poisons, such as colchicine or podophyllotoxin, which have been suggested to inhibit microtubule polymerization via a substoichiometric end-blocking mechanism (Margolis & Wilson, 1977; but Sternlicht & Ringel, 1979). The finding that the inhibitor could be chromatographically removed from the tubulin and the isolated tubulin remained nonpolymerizable, however, argues very strongly against such a mechanism. Therefore it appears most likely that the inhibitor catalytically alters the tubulin molecule by some as yet undefined enzymatic mechanism. Given the enzymatic nature of the inactivation, the apparent "stoichiometric" relation between inhibitor and unfractionated microtubule protein seen in Figure 5 remains to be explained. Conceivable, though still untested, explanations would be either some reactivation or protecting activities present in the unfractionated microtubule preparations.

Among the various possible enzymatic mechanisms that could render tubulin nonpolymerizable, a few were tested experimentally. We considered the enzymatic modification of tubulin-bound guanine nucleotides as attractive candidates, since both the exchangeable and nonexchangeable GTPs have been suggested to be possible control sites for regulating the polymerizability of tubulin (Penningroth et al., 1976; Weisenberg & Deery, 1976). Two different experiments rule out the direct involvement, i.e., cleavage, of the E-site GTP in this inhibition mechanism. First, tubulin remained inactive even after complete removal of the inhibitor, and second, inhibitor preparations showed only negligible GTPase activity when tested under polymerization assay conditions either in the absence or in the presence of tubulin. An effect of the inhibitory activity on the exchangeable GTP binding site per se cannot be excluded. It could be that, due to a primary modification of the tubulin molecule by the inhibitor, GTP no longer bound to the tubulin with the same affinity as to the control tubulin. Further GTP binding experiments are necessary to clarify this point. N-site guanosine nucleotide in polymerizable tubulin is apparently always in the triphosphate form (Spiegelman et al., 1977), and though selective cleavage has not been reported, alterations of this tightly bound GTP initially could not be ruled out. However, by labeling N-site GTP in vivo with ^{32}P and comparing the protein-bound nucleotides of inhibitor-treated and untreated tubulin, any possible effects of inhibitor on the N-site GTP could be eliminated.

One- and two-dimensional gel electrophoreses also failed to demonstrate differences in polypeptide length or charge between the treated and untreated tubulin polypeptides. The possibility of an unusual specific limited exopeptidase activity rendering tubulin nonpolymerizable could not be ruled out by these experiments, for that terminal amino acid analysis will be necessary. Similarly, a potentially novel mechanism involving sulfhydryl group rearrangement cannot be excluded, although the inhibitory activity is not affected by the presence of reducing agents (unpublished data), nor is the inhibitor-treated tubulin reactivated in their presence. Finally, an attractive inhibitory mechanism would be the covalent modification of tubulin, for instance, by phosphorylation. This could render tubulin molecules nonpolymerizable either by directly impeding their interaction with each other and/or associated proteins or by causing irreversible conformational changes.

General denaturation of the tubulin molecule was ruled out by the colchicine binding assays which revealed full retention of initial binding capacity and decay rates of the inhibitor-treated tubulin. The observation of unaffected colchicine binding but total lack of polymerizability is, as far as we know, the first direct demonstration that these two parameters specific for tubulin are not necessarily related. Thus, estimating tubulins conformational integrity by the criteria of colchicine binding apparently does not reflect its intrinsic ability to polymerize.

In summary, the potent microtubule inhibitory protein partially purified from extracts of *Dictyostelium discoideum* appears to render microtubule protein nonpolymerizable in a fashion reminiscent of an enzymatic process. Although the precise mechanism of action has yet to be resolved, the tubulin molecule was clearly identified as the inhibitor's target. Finding the exact nature of the inhibitory mechanism could lead to new formulations and approaches to the problem of in vivo control of microtubule assembly and disassembly.

Acknowledgments

We wish to thank Dr. E. Unger for advice and help in the initial phase of this work and the members of the Viennese laboratory for help, stimulating discussions, and critical comments on the manuscript. Also we greatfully acknowledge the assistance of E. Goni in typing the manuscript and a kind gift of actin from Ed Bonner.

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Multiplicity, Strain Differences, and Topology of Phenobarbital-Induced Cytochromes P-450 in Rat Liver Microsomes†

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ABSTRACT: The multiplicity of phenobarbital-induced cytochromes P-450 in liver microsomes from male rats was investigated by using two-dimensional gel electrophoresis, peptide fingerprinting, and immunoaffinity chromatography. Two colonies each of Holtzman and Long-Evans rats were studied. Four molecular forms of phenobarbital-induced cytochromes P-450 were distinguished as polypeptides (designated PB3, variant PB3, PB4, and PB5) which showed apparent immunochemical identity and ≥95% fingerprint homology. Two of these polypeptides corresponded to cytochrome P-450b [Ryan, D., Thomas, P. E., Korzeniowski, D., & Levin, W. (1979) *J. Biol. Chem. 254*, 1365–1374] and cytochrome P-450e [Ryan, D., & Levin, W. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1640] which had been purified from Long-Evans rats (variant PB3 and PB5, respectively). Each rat colony was characterized by unique combinations of two or three of these immunochemically related forms of cytochrome P-450. Cytochrome P-450e was present in rats from all four colonies, but cytochrome P-450b was only found in Long-Evans rats. Polypeptide PB3 was only found in the two colonies of Holtzman rats, whereas polypeptide PB4 was present in one colony each of Holtzman and Long-Evans rats. In addition to these forms of cytochrome P-450, rats from each colony also evidenced three other major phenobarbital-induced polypeptides which gave unique fingerprints, and one of these was identified as representing epoxide hydrolase. Proteolytic digestion studies of intact microsomes demonstrated that the four immunochemically identical forms of cytochrome P-450 were partially exposed on the outer (cytoplasmic) surface of microsomes. However, polypeptide PB3 was characterized by the greatest rate of proteolytic degradation. These results clearly demonstrate that phenobarbital-induced cytochromes P-450 include microheterogeneous proteins which show remarkable variations related to rat strain and/or colony.

It is generally accepted that the ability of mammalian liver monooxygenase systems to metabolize a remarkably wide range of xenobiotic and endogenous substrates results from the participation of multiple forms of cytochrome P-450, each of which may exhibit a broad but selective substrate specificity (Lu & Levin, 1974). The number of unique cytochromes P-450 that potentially constitute the liver monooxygenase system in a single mammalian species is currently unknown. Nevertheless, at least six distinct forms of this enzyme have already been purified from rat liver (Guengerich, 1978; Ryan et al., 1979; Elshourbagy & Guzelian, 1980; Ryan et al., 1980; Ryan & Levin, 1981), and four of these have been shown to represent different gene products (Ryan et al., 1980; Botelho et al., 1979). However, in addition to their primary structures, it is also possible that cytochrome P-450 polypeptides differ as a result of posttranslational modifications [e.g., by glycosylation (Haugen & Coon, 1976; Hiwatashi & Ichikawa, 1980) and/or phosphorylation (Sharma et al., 1978)] which

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0006-2960/82/0421-0789$01.25/0