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COLCHICINE BINDING TO AN OLIGOMER OF TUBULIN

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SUMMARY: An oligomeric form of tubulin present in microtubule protein prepared from mammalian brain, the 36S double ring containing tau protein, is reported to bind colchicine. Colchicine binds to each individual 6S tubulin subunit in the 36S ring without apparent effect on quarternary structure. The colchicine-oligomer complex forms by colchicine binding directly to the tubulin ring; alternatively, complexes formed by colchicine with 6S tubulin subunits associate in the presence of tau protein to form the colchicine-oligomer complex.

INTRODUCTION

One approach to elucidating the mechanism of action of the antimitotic drug colchicine is to analyze the inhibitory effect of colchicine on the assembly in vitro of microtubules purified from mammalian brain (1-4). Initially, colchicine was shown to bind to tubulin, the 6S dimeric subunit protein of microtubules (5), and most investigations of the mechanism of colchicine action have focussed on the colchicine-dimer complex (1-7). In other contexts, a number of investigators have reported that purified microtubule protein from mammalian brain (8,9) and from tissue culture cells (10) contains, in addition to 6S tubulin, one or more species of tubulin oligomers comprising up to half of the total tubulin in the repolymerization mixture (8,9). The tubulin oligomers present in depolymerized microtubule protein are ring-shaped,

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but their exact structure as well as their component of MAP's[2] appear to vary depending on specific buffer systems used to prepare microtubule protein (11-13). There is considerable evidence that different species of microtubule protein oligomers either facilitate (12,14,15) or are essential for (16-18) microtubule assembly in vitro. In view of the postulated role of MAP's in the microtubule assembly reaction, we asked whether the mechanism of colchicine action could involve a complex of colchicine with a MAP-induced tubulin oligomer. Such a colchicine-oligomer complex might inhibit the polymerization of microtubules more effectively than a colchicine-dimer complex (9,16,18). As a first step toward elucidating the possible role of a colchicine-oligomer complex in the mechanism of colchicine action, colchicine binding to the 36S tubulin double ring structure containing the MAP referred to as tau protein (9,16,17,19) has been investigated.

METHODS

Preparation of microtubule protein: Microtubule protein was purified from porcine brain by alternate cycles of assembly/disassembly in the presence of glycerol (9,20). Experiments were conducted in a simple buffer system (0.1 M MES (2N-morpholino ethane sulfonic acid), 0.5 mM $MgCl_2$, pH 6.4 adjusted with NaOH at 21°C) unless otherwise indicated.

Analytical ultracentrifugation: All runs were at 47,660 rpm at 18°C in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner. An AND rotor and 12 mm double sector cells were used. Schlieren and absorbance data were recorded within 1.5 minutes of each other. Scanner traces included an internal calibration for optical density. Schlieren images were photographed at a bar angle setting of 75° on metallographic plates (Kodak). Peak areas, calibrated for protein concentration by the synthetic boundary method using bovine serum albumin as a standard, were measured with a Nikon microcomparator.

Other methods: Large pore gel filtration was performed on a 1.5 cm x 25 cm column using agarose 15 m, 100-200 mesh (Biogel) at 4°C. Colchicine was purchased from Sigma. [C ring methoxy-^3H] colchicine was obtained from New England Nuclear, and its purity was established by paper chromatography (21). Protein concentration was determined by the method of Lowry et al (22) using bovine serum albumin as a standard, which has been shown to give accurate results for tubulin (19).

RESULTS

Previous work has shown that depolymerized microtubule protein is fractionated into 6S tubulin and 36S tubulin rings by large pore gel filtration

[2]Microtubule Associated Proteins

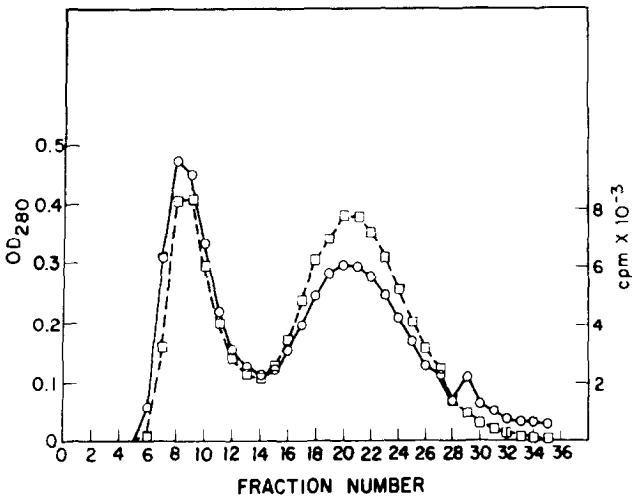

Figure 1. Formation of a ^{3}H-colchicine-36S ring complex from ^{3}H-colchicine-6S dimer complexes. Microtubule protein suspended in 1.0 ml buffer plus 1 mM $CaCl_2$ was mixed with 0.75 M NaCl to reversibly dissociate 36S rings into 6S tubulin subunits. The suspension was centrifuged at 180,000 x g for 30 minutes at 25°C to remove large, non-salt-dissociable aggregates shown to be incapable of binding colchicine (24). A small pellet was discarded, and the supernatant, containing salt-dissociated 6S tubulin, was incubated with 1 mM ^{3}H-colchicine for 50 minutes at 37°C. Following quenching of the colchicine-binding reaction at 4°C, the reaction mixture was rapid-dialyzed at 4°C against 400 volumes of buffer in order to remove NaCl and thus reform 36S tubulin rings as well as remove unbound ^{3}H-colchicine. The dialyzate (7.4 mg/ml) was applied to an agarose column, and 1.0 ml fractions were collected and analyzed for OD_{280} (o——o) and bound ^{3}H-colchicine (□---□).

(9,23). Figure 1 presents the finding that following incubation with ^{3}H-colchicine, microtubule protein was fractionated into two peaks of ^{3}H-colchicine binding activity on an agarose column. The results in Figure 1 indicate that ^{3}H-colchicine binds to 36S tubulin rings as well as to 6S tubulin.

In the experiment presented in Figure 1, ^{3}H-colchicine was bound initially to microtubule protein, the 36S ring component of which had been dissociated to 6S tubulin subunits by treatment with 0.75 M NaCl (8,9). This concentration of NaCl does not block colchicine-binding to 6S tubulin (24). The tubulin rings isolated by column chromatography in Figure 1 were reformed after the colchicine-binding reaction to 6S tubulin had been quenched by lowering the temperature to 0°C. Therefore, under the conditions of this experiment, the colchicine-36S ring complex could only have arisen by the association of colchicine-6S dimer complexes which had formed previously at 37°C in the presence

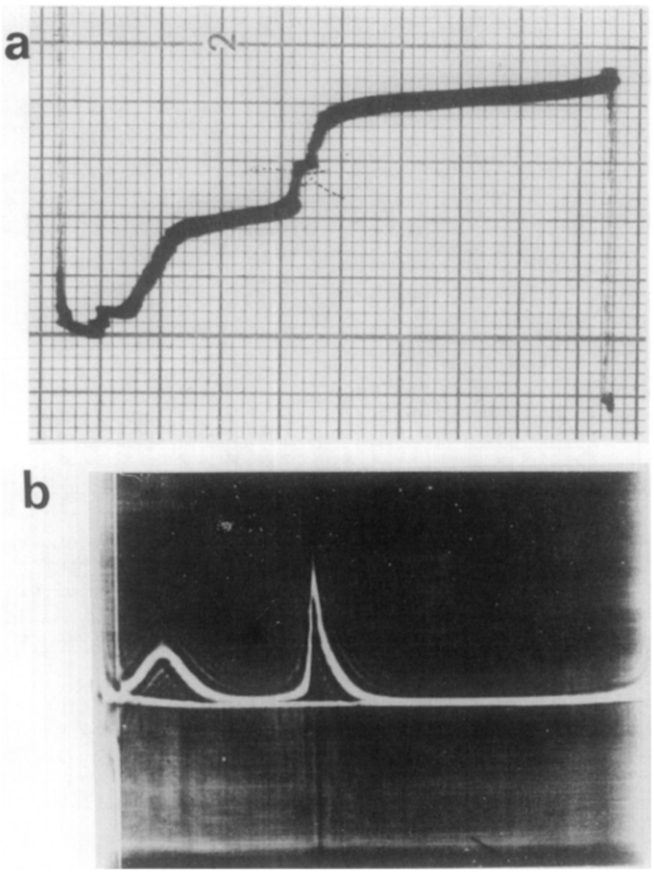

Figure 2. Colchicine binding to 36S rings and 6S tubulin dimers in the analytical ultracentrifuge. Microtubule protein was incubated with 0.5 mM colchicine for 45 minutes at 37°C, and free colchicine was removed by adding 50 microliters of a 100 g/ltr suspension of charcoal per ml of microtubule protein (28). Charcoal was removed by centrifugation at 12,000 x g for five minutes, and the charcoal treatment was repeated one time. The colchicine-microtubule protein complex was fractionated in the analytical ultracentrifuge. Data were recorded 35 minutes after reaching operating speed. Direction of centrifugation is from left to right.
2a. Photoelectric scanner trace of the absorbance at a wavelength of 351 nanometers. Each small division represents an absorbance of 0.051. Approximately 27% of the height of the leading peak is due to light scattering from the 36S tubulin rings.
2b. Schlieren peaks migrating at 36S and 6S which correspond to the absorbance peaks in Figure 2a.

of 0.75 M NaCl. Tau protein is, of course, a requirement for ring formation (16,17) as are HMW[3] proteins in other in vitro microtubule assembly systems (11,15).

Efforts to use agarose column chromatography to quantitate the stoichiometry of colchicine-binding to 36S tubulin rings proved unsatisfactory,

[3]High Molecular Weight

probably due to the elution of varying amounts of denatured tubulin aggregates (24-26) with native 36S oligomers from the column. Inactive aggregates eluting with the active 36S rings would have the effect of lowering the apparent colchicine-binding stoichiometry of the rings, and, indeed, this now appears to be the explanation for an earlier report from this laboratory that 36S rings bound less colchicine than 6S tubulin dimers (27).

In order to differentiate between active, 36S oligomers and inactive microtubule protein aggregates, colchicine-binding stoichiometry was measured in the analytical ultracentrifuge using a combination of absorbance optics to determine colchicine concentration and schlieren optics to determine protein concentration (25,26). Early in each ultracentrifuge run, a broad peak was observed the sedimentation coefficient of which was estimated at $\geq$ 400S (data not shown). This heterogeneous material was tentatively identified as the denatured aggregates which eluted at or near the void volume of the agarose column (24). Typical traces from one ultracentrifuge experiment are presented in Figures 2a and b. Figure 2a shows two peaks of bound colchicine which corresponded to two schlieren peaks sedimenting at 6S and 36S (9) in Figure 2b. Table 1 presents the results of six stoichiometry determinations (two at different time points in each of three ultracentrifuge runs). The apparent colchicine concentration in the 36S ring fraction had to be corrected for the intrinsic light-scattering properties of the ring (9) (Table 1, column 4); the exact correction factor depended on protein concentration and was determined in a separate set of measurements in the ultracentrifuge omitting colchicine (data not shown). The results in Table 1 indicate that, within experimental error, 36S tubulin rings and 6S tubulin dimers bound equivalent amounts of colchicine on a mass basis. This is consistent with the observation that tau protein present in the rings competitively inhibits, but does not block, colchicine-binding to tubulin (29). The colchicine-binding stoichiometry of 0.7-0.8 moles colchicine per mole tubulin dimer, though less than unity (30), compares favorably with

TABLE I

Stoichiometry of Colchicine Binding to 36S Tubulin Rings and 6S Tubulin[a]

Experiment	Stoichiometry[b] 36S Rings	6S Tubulin	Protein Concentration[c] (mg/ml)	Correction for Light Scattering[d]
1	0.88	0.63	1.95	20%
2	0.67	0.81	5.73	27%
3	0.67	0.76	5.42	27%
Average Values[e]	0.75 ± 0.14	0.73 ± 0.09		

[a]Determined by analytical ultracentrifugation as described in the text and legend to Figure 2.

[b]Average of two determinations at different times in each experiment. Units are moles of colchicine bound per 1.1×10^5 grams of protein

[c]Sum of 36S and 6S schlieren peaks.

[d]Fraction of 36S absorbance peak due to light scattering from rings (cf. Figure 2a).

[e]Means of six stoichiometry determinations with standard deviation.

other values reported in the literature which range from 0.2 to 0.9 moles/mole (1,10,26,27,31) in the absence of corrections for first-order decay of colchicine-binding activity (32).

DISCUSSION

Although a preliminary report of this work has appeared elsewhere (33), this paper documents for the first time that colchicine binds to tubulin rings. That colchicine bound to Mg^{++}-induced tubulin oligomers was shown previously (25), but neither the structure of these oligomers nor their possible role in microtubule assembly was clear. The significance of colchicine binding to 36S rings lies in the postulated role of rings in the microtubule assembly reaction (9-12,14-18,34). Our observation of a stable colchicine-ring complex in the analytical ultracentrifuge (Figure 2a) has been corroborated by Weisenberg (R. Weisenberg, personal communication).

Of the many ways in which a colchicine-oligomer complex could conceivably inhibit microtubule assembly, only one will be elaborated here. Assuming uncoiled rings add as protofilament segments directly to the nascent microtubule (19), a substoichiometric colchicine-oligomer complex could account for the observed substoichiometric "poisoning" effect of colchicine on microtubule assembly (1,35) if the presence of one or a few colchicine-dimer complexes within the oligomer acted to destabilize lateral, interprotofilament bonding such that the substoichiometric colchicine-oligomer complex could not be incorporated into the microtubule lattice. This view seems to be supported by electron micrographs demonstrating abortive polymerization products in the presence of colchicine (36); it also appears consistent with the cooperative oligomer addition model of microtubule assembly outlined recently (37). This view differs, however, from hypotheses based solely on the colchicine-6S tubulin dimer complex, including the end-poisoning (1,35) and the copolymerization (2,3) models.

In summary, several features of a binding reaction between the antimitotic drug colchicine and 36S tubulin rings have been characterized: (1) The 36S rings are stable to colchicine. (2) A colchicine-tubulin ring complex can form in either of two ways: (a) Colchicine can bind directly to the ring (Figure 2); (b) Alternatively, colchicine can bind first to the 6S tubulin dimer, and the resulting colchicine-dimer complex can then associate, in the presence of tau protein and low salt concentration, to form the colchicine-ring complex (Figure 1). (3) Colchicine binds to each individual tubulin subunit in the 36S ring (Table 1).

REFERENCES

1. Margolis, R.L. and Wilson, L. (1977) Proc. Nat. Acad. Sci. USA 74, 3466-3470.
2. Sternlicht, H. and Ringel, I. (1978) J. Cell Biol. 79, MT 1875.
3. Sternlicht, H. and Ringel, I. (1979) J. Biol. Chem., in press.
4. Deery, W.J., Zackroff, R.V. and Weisenberg, R.C. (1978) J. Cell Biol. 79, MT 1877.
5. Borisy, G.G. and Taylor, E.W. (1967) J. Cell Biol. 34, 535-548.
6. Wilson, L. and Meza, I. (1973) J. Cell Biol. 58, 709-719.
7. Wilson, L. (1975) Life Sci. 17, 303-310.

8. Marcum, J.M. and Borisy, G.G. (1978) J. Biol. Chem. 253, 2825-2833.
9. Weingarten, M.D., Suter, M.S., Littman, D.R. and Kirschner, M.W. (1974) Biochemistry 13, 5529-5537.
10. Wiche, G., Honig, L.S. and Cole, R.D. (1979) J. Cell Biol. 80, 553-563.
11. Vallee, R.B. and Borisy, G.G. (1978) J. Biol. Chem. 253, 2834-2845.
12. Murphy, D.B., Vallee, R.B. and Borisy, G.G. (1977) Biochemistry 16, 2598-2605.
13. Cleveland, D.W., Hwo, S.-Y., and Kirschner, M.W. (1977) J. Mol. Biol. 116, 207-225.
14. Murphy, D.B. and Borisy, G.G. (1975) Proc. Nat. Acad. Sci. USA 72, 2696-2700.
15. Kim, H., Binder, L. and Rosenbaum, J.L. (1979) J. Cell Biol. 80, 266-276.
16. Weingarten, M.D., Lockwood, A.H., Hwo, S.-Y., and Kirschner, M.W. (1975) Proc. Nat. Acad. Sci, USA 72
17. Cleveland, D.W., Hwo, S.-Y., and Kirschner, M.W. (1977) J. Mol. Biol. 116 227-247.
18. Witman, G.B., Cleveland, D.W., Weingarten, M.D. and Kirschner, M.W. (1976) Proc. Nat. Acad. Sci. USA 73, 4070-4074.
19. Kirschner, M.W., Honig, L.S. and Williams, R.C. (1975) J. Mol. Biol. 99, 263-276.
20. Shelanski, M.L., Gaskin, F. and Cantor, C.R. (1973) Proc. Nat. Acad. Sci. USA 70, 765-768.
21. Haber, J.E., Peloquin, J.G., Halvorson, H.W. and Borisy, G.G. (1972) J. Cell Biol. 55, 355-367.
22. Lowry, O.H., Rosebrough, N.J., Farr, A.C. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
23. Kuriyama, R. (1975) J. Biochem. (Japan) 77, 23-31.
24. Penningroth, S.M. (1977) Doctoral dissertation, Princeton University, Princeton, New Jersey.
25. Weisenberg, R.C. and Timasheff, S.N. (1970) Biochemistry 9, 4110-4116.
26. Shelanski, M.L. and Taylor, E.W. (1968) J. Cell Biol. 38, 304-315.
27. Kirschner, M.W., Williams, R.C., Weingarten, M.D. and Gerhart, J.C. (1974) Proc. Nat. Acad. Sci. 71, 1159-1163.
28. Sherline, P., Bodwin, C.K. and Kipnis, D.M. (1974) Anal. Biochem. 62, 400-407.
29. Nunez, J., Fellons, A., Francon, J. and Lennon, A.M. (1979) Proc. Nat. Acad. Sci. USA 76, 86-90.
30. Weisenberg, R.C., Borisy, G.G. and Taylor, E.W. (1968) Biochemistry 7, 4466-4479.
31. Friedman, P.A. and Platzer, E.G. (1978) Biochim. et Biophys. Acta 544, 605-614.
32. Wilson, L. (1970) Biochemistry 9, 4999-5007.
33. Penningroth, S.M. and Kirschner, M.W. (1977) J. Cell Biol. 75, MT 480.
34. Borisy, G.G., Johnson, K.A. and Marcum, J.M. (1976) In Cell Motility (Goldman, R., Pollard, T. and Rosenbaum, J., eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., Vol. 3, pp. 1093-1108.
35. Olmsted, J.B. and Borisy, G.G. (1973) Biochemistry 12, 4282-4289.
36. Kirschner, M.W. and Williams, R.C. (1974) J. Supramol. Struc. 2, 412-428.
37. Weisenberg, R.C. (1979) In The Cytoskeleton: Membranes and Movement, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p. 110 (Abst.).