Decoration and Stabilization of Intact, Smooth-Walled Microtubules with Microtubule-Associated Proteins†

Roger D. Sloboda* and Joel L. Rosenbaum

ABSTRACT: Brain microtubules assembled in vitro are composed of four major proteins: high molecular weight microtubule-associated proteins (MAPs) 1 and 2 and α- and β-tubulin. MAPs 1 and 2 have been shown to be associated with tubulin as filaments projecting from the wall of the microtubule and were required for the assembly of low concentrations of purified tubulin dimers [Sloboda, R. D., Dentler, W. L., & Rosenbaum, J. L. (1976) *Biochemistry 15*, 4497–4505]. Since it was determined that high concentrations of tubulin dimers in the absence of MAPs could assemble into microtubules which lacked filamentous projections, it was possible to study the association of MAPs with preformed microtubules. The data reported here show that: (a) when resuspended in the presence of MAPs the smooth-walled microtubules became decorated with a filamentous coating analogous to that seen on microtubules assembled in the presence of the MAPs as determined by thin-section electron microscopy; (b) resuspension of the smooth-walled microtubules in increasing concentrations of the MAPs resulted in a linear increase in the amount of MAPs sedimenting with the decorated microtubules, as judged by quantitative SDS–polyacrylamide gel electrophoresis, until a MAP/tubulin ratio characteristic of microtubules assembled in the presence of the MAPs was reestablished; (c) the smooth-walled microtubules, when resuspended in MAPs, did not first depolymerize and then reassemble with the MAPs forming new microtubules with a filamentous coating since the decoration was done in the presence of colchicine (10^{-4} M) sufficient to block the MAP-stimulated assembly of tubulin dimers; (d) the cold (4 °C) stability of microtubules was directly affected by the presence of the MAPs such that the rate of depolymerization in response to cold treatment decreased as the ratio of MAP/tubulin increased. The data are consistent with the observation that the MAPs function in the in vitro microtubule assembly reaction by associating stoichiometrically with the tubulin subunit lattice after tubulin polymerization has occurred; this shifts the equilibrium toward the formation of polymer by stabilizing the assembled tubulin.

Microtubules assembled in vitro from homogenates of chick, calf, or porcine brain contain two high molecular weight microtubule-associated proteins (MAPs[1] 1 and 2; see Sloboda et al., 1975) in addition to the α and β subunits of tubulin. The MAPs are integral components of microtubules assembled in vitro since they have been shown to copurify with the tubulin at a constant stoichiometric ratio through up to five cycles of assembly and disassembly in vitro (Borisy et al., 1975; Sloboda et al., 1975; Berkowitz et al., 1977). Furthermore, the MAPs can be observed morphologically as distinct, periodic side-arm projections when in vitro assembled microtubules are viewed by thin-section (Dentler et al., 1975; Murphy & Borisy, 1975; Kim et al., 1979) or negative-stain (Amos, 1977) electron microscopy; analogous morphological projections have been described on microtubules in situ (Burton & Fernandez, 1973; Ross et al., 1975; Yamada et al., 1971). In vitro assembled microtubules have also been reported to contain another protein, Tau (Weingarten et al., 1975), which has been suggested to be required for both the initiation and elongation phases of microtubule assembly (Witman et al., 1976). Thus Tau may also be a microtubule-associated protein, although Tau has not yet been shown to copurify with or to exist in a reproducible, constant ratio to tubulin during the in vitro assembly process as do MAP 1 and MAP 2 (Berkowitz et al., 1977).

The MAPs can be separated from the tubulin by ether molecular-sieve chromatography or by ion-exchange chromatography using either phosphocellulose or DEAE–Sephadex. The tubulin alone does not readily assemble at low concentration; however, tubulin does assemble to a limited extent at high protein concentrations (Dentler et al., 1975; Murphy & Borisy, 1975; Bloodgood & Rosenbaum, 1976). Microtubules assembled in this manner in the absence of the MAPs have comparatively smooth walls lacking the surface decorations seen on microtubules assembled in the presence of the MAPs (Dentler et al., 1975; Murphy & Borisy, 1975). The purified tubulin can be induced to assemble to a greater extent by recombining it with appropriate aliquots of the MAP fraction obtained by ion-exchange chromatography (Murphy & Borisy, 1975; Sloboda et al., 1976a,b; Weingarten et al., 1975). When this is done, the tubulin assembles at concentrations less than 1 mg/mL, and it has been shown that the MAPs increase both the rate of assembly and the total mass of microtubules produced during the in vitro assembly reaction (Sloboda et al., 1976a,b; Murphy et al., 1977a); therefore, these proteins affect both the initiation and elongation functions of microtubule assembly in vitro. When the microtubules assembled in these reconstitution experiments are viewed in the electron microscope, they are seen to have a filamentous coating similar to that observed on microtubules assembled from unfractionated preparations (Dentler et al., 1975; Murphy & Borisy, 1975; Sloboda et al., 1976a,b).

However, recent evidence has shown that although the MAPs affect initiation and elongation they are not an absolute requirement for microtubule assembly in vitro but rather function merely by lowering the rate of dissociation of the formed microtubule (Murphy et al., 1977a). To study this MAP–tubulin interaction further, the interaction of the MAPs with intact, smooth-walled microtubules in vitro was inves-

† From the Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755 (R.D.S.), and the Department of Biology, Yale University, New Haven, Connecticut 06520 (J.L.R.). *Received February 28, 1978. Revised Manuscript Received September 11, 1978.* This work was supported by National Institutes of Health Grants GM 14642 and AM 19742 (J.L.R.) and National Science Foundation Grant PCM 77-24329 (R.D.S.). R.D.S. was the recipient of National Research Service Award GM 02890 during a portion of this work.

[1] Abbreviations used: MAP, microtubule-associated protein; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; GTP, guanosine triphosphate; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid).

0006-2960/79/0418-0048$01.00/0

tigated to determine if the MAPs had to be present during assembly to attach to the microtubules or if the MAPs could be attached in a stoichiometric manner to the microtubule wall after tubulin assembly occurred ("decoration"). The results presented here show that the MAPs attach stoichiometrically to preassembled, smooth-walled microtubules in vitro, decorating them with a filamentous coating, thus forming microtubules having increased stability. A preliminary report of this work has appeared (Sloboda & Rosenbaum, 1977).

Materials and Methods

Purification of Microtubules and Microtubule-Associated Proteins. Using modifications of the in vitro assembly procedure originally described by Weisenberg (1972), microtubule proteins were isolated from either calf brain homogenates, in the presence of glycerol (Sloboda et al., 1976b), or from chick brain homogenates, in the absence of glycerol (Dentler et al., 1974). Proteins from both sources were purified by two cycles of the assembly–disassembly procedure (2X-microtubule protein) and stored as frozen pellets at −80 °C overlaid with the appropriate buffer. For use in an experiment, the pellets were thawed and carried through one complete cycle of assembly–disassembly to obtain 3X-microtubule protein; this step assured that only protein competent to assemble after a period of storage would be used in an experiment. Protein concentrations were quantitated by the Schacterle & Pollack (1973) modification of the method of Lowry et al. (1951).

Purified tubulin was obtained by chromatography of 3X-microtubule protein on a Bio-Gel A-1.5m column (2.5 × 60 cm) as previously described (Sloboda et al., 1976b) or by chromatography of 3X-microtubule protein on phosphocellulose (Weingarten et al., 1975). It was found that when phosphocellulose-purified tubulin was used in assembly experiments it was necessary to adjust the fractions containing tubulin to 1 mM GTP and 1 mM $MgSO_4$ *immediately* after their elution from the column; otherwise, the protein rapidly lost its ability to assemble in the absence of MAPs. Both procedures yielded tubulin fractions that were greater than 96% α- and β-tubulins, with the remaining 3–4% distributed between 2–3 bands of molecular weight lower than tubulin, as determined by SDS–gel electrophoresis.

The MAPS were obtained by chromatography of 3X-microtubule protein on phosphocellulose as reported previously (Sloboda et al., 1976b); the phosphocellulose column was prepared, equilibrated, and loaded with 3X-microtubule protein, and the column was then washed with buffer to elute the tubulin which does not bind under these conditions. The bound protein was then eluted with two successive steps of 0.18 and 0.80 M KCl in buffer. The 0.18 M KCl fraction contained proteins whose molecular mass corresponded to those previously reported for Tau (i.e., 58 000–75 000 daltons; see Penningroth et al., 1976; Cleveland et al., 1977). The high molecular weight MAPs (MAP 1 and MAP 2) eluted with the 0.80 M KCl step; this fraction, which contained all of the tubulin assembly stimulating activity (see Figure 3), was referred to as the MAP fraction and was used in all the experiments reported here.

Microtubule Decoration Procedures. For studies in which MAPs were added to assembled, purified tubulin, the tubulin from the Bio-Gel or phosphocellulose column was concentrated to 8 mg/mL or greater by pressure dialysis under nitrogen using an Amicon ultrafiltration apparatus with a PM30 filter. This step was necessary so that the tubulin dimers would readily assemble within a reasonable amount of time in the absence of any added associated proteins (see Results). Aliquots (1 mL) of this purified tubulin were incubated at 37 °C for 60 min, and the assembled protein was then collected by centrifugation at 27 000 × *g* for 10 min at 25 °C in conical centrifuge tubes. The microtubules were resuspended at 37 °C in 0.5 mL of buffer alone or buffer containing increasing concentrations of MAPs. The microtubules were then resedimented immediately and analyzed by SDS–polyacrylamide gel electrophoresis and thin-section electron microscopy. In some experiments the decorated microtubules were washed one time by resuspension in fresh 37 °C buffer alone and collected again by centrifugation. The buffer for all the in vitro decoration experiments reported here was 50 mM Pipes (pH 6.9), 0.5 mM GTP, 1 mM EGTA, 0.5 mM $MgSO_4$, and 0.1 mM colchicine (see Results, Figure 1, for explanation).

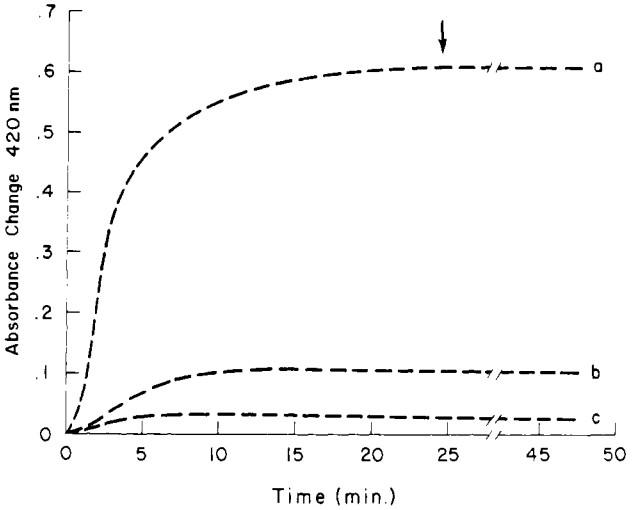

FIGURE 1: Effect of colchicine on the MAP-stimulated assembly of tubulin dimers: curve a, control; curve b, assembly in the presence of 10^{-6} M colchicine; curve c, assembly in the presence of 10^{-4} M colchicine. At the point in the control (curve a) indicated by the arrow the contents of the cuvette were adjusted to 10^{-4} M colchicine. Absorbance in this figure was measured at 420 nm rather than the usual 350 nm due to the absorbance peak of colchicine.

Quantitative Procedures. Where necessary, the assembly reaction was monitored and quantitated turbidimetrically (Gaskin et al., 1974) and verified by negative-stain electron microscopy. The assembly reaction was initiated by raising the temperature of the cuvette from 4 to 37 °C, a process which took 60–70 s. The kinetics of microtubule assembly and disassembly were monitored in a thermal cuvette (Gilford Instruments) providing precise measurements of the cuvette temperature. All turbidity assays were performed in this cell in a Gilford 250 recording spectrophotometer. Gel electrophoresis was performed after the method of Laemmli (1970) except where modified as noted (see legend, Figure 2); the gels were stained with either Coomassie Blue or, where quantitation was necessary, with Fast Green (Gorovsky et al., 1970) using procedures previously described (Sloboda et al., 1975). Quantitatively stained gels were scanned at 650 nm and the areas under the protein peaks determined by planimetry. For thin-section electron microscopy, assembled microtubules were sedimented at 36 000 × *g* for 30 min at 25 °C, fixed in glutaraldehyde, postfixed in OsO_4, embedded, sectioned, and viewed with a Philips EM 201 (Dentler et al., 1975).

Results

The experiments reported here were designed to determine if intact microtubules assembled in the absence of the MAPs and, therefore, having smooth walls (Dentler et al., 1975; Murphy & Borisy, 1975) could be decorated with MAPs after the tubulin assembly reaction had reached an apparent

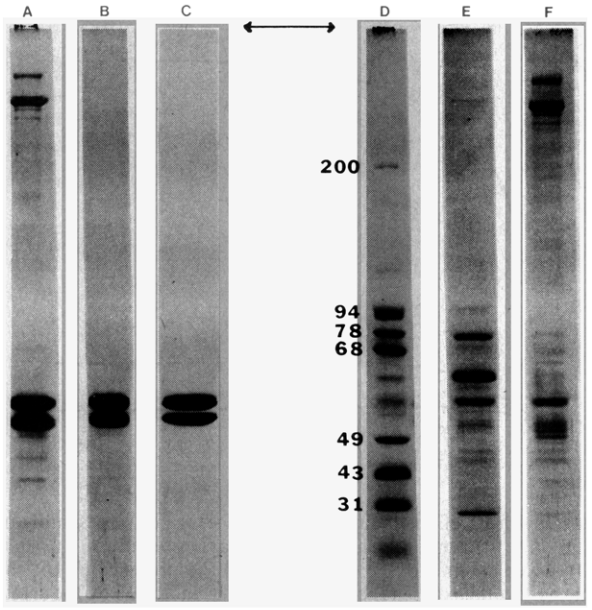

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the protein fractions used in these experiments. The gels shown are channels from a single slab gel cast after the method of Laemmli (1970) but containing a 6–16% acrylamide and 2–8 M urea gradient: (A) 3X-chick brain microtubule proteins; (B) tubulin purified by molecular-sieve chromatography on Bio-Gel A-1.5m; (C) tubulin purified by phosphocellulose chromatography; (D) series of molecular weight standards, in order of increasing molecular weights ($\times 10^{-3}$): DNase I (31), ovalbumin (43), fumarase (49), bovine serum albumin (68), transferrin (78), phosphorylase *a* (94), myosin (200); (E) proteins eluted from the phosphocellulose column with 0.18 M KCl; and (F) proteins (MAP fraction) eluted from phosphocellulose with 0.80 M KCl. The major difference between gels E and F is the presence of the high molecular weight microtubule-associated proteins (MAPs 1 and 2) in gel F. This fraction (gel F) contained all of the tubulin assembly stimulating activity (Figure 3) and was used in all of the experiments reported here. The arrow indicates the interface between the stacking and separating gels.

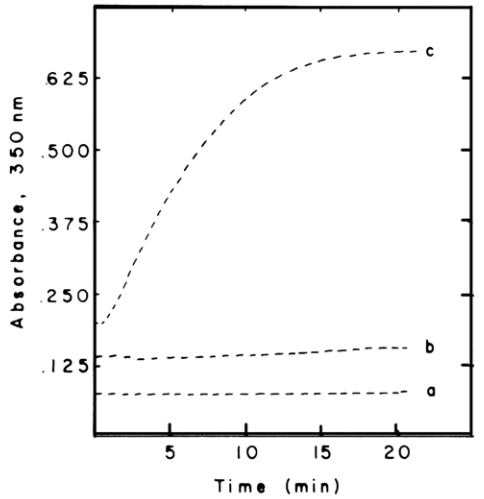

FIGURE 3: Relative tubulin assembly stimulating activity of two fractions eluted from the phosphocellulose column (Figure 2E,F). The tubulin, purified by Bio-Gel chromatography, was constant at 3.35 mg/mL in a–c: (a) tubulin dimers alone; (b) tubulin plus 0.18 M KCl phosphocellulose fraction at 1.35 mg/mL; and (c) tubulin plus 0.80 M KCl phosphocellulose fraction (i.e., the MAP fraction) also at 1.35 mg/mL. For clarity, the starting A_{350} of each sample has been offset along the ordinate using the controls on the spectrophotometer.

equilibrium. That is, were the MAPs capable of associating with the tubulin subunit lattice independent of microtubule polymerization? The basic experimental approach used to answer this question was to assemble tubulin dimers at high concentration in the absence of the MAPs, collect the microtubules by centrifugation, resuspend them in the presence of the MAPs, resediment the microtubules, and use gel electrophoresis and thin-section electron microscopy to determine if the microtubules had MAPs associated with them. The results of such an experiment alone would be inconclusive, however, since it was possible that the smooth-walled microtubules, when resuspended in MAPs, first depolymerized into subunits and then reassembled with the MAPs forming new microtubules with a filamentous coating. To control for this possibility, a concentration of colchicine was determined that completely blocked the MAP-stimulated assembly of tubulin dimers and yet did not depolymerize the assembled microtubules within the period of time in which the experiment was carried out. The results of this experiment are shown in Figure 1. The assembly reaction was almost completely inhibited by 10^{-4} M colchicine (curve c); however, when intact microtubules at the apparent equilibrium point of the reaction were adjusted to 10^{-4} M colchicine (arrow, curve a), no loss in turbidity, and therefore no tubulin disassembly, occurred for at least 25 min. Therefore, all of the microtubule decoration experiments reported in the following section were performed in the presence of 10^{-4} M colchicine in order to prevent the assembly of any tubulin subunits present during the decoration experiment.

Tubulin and MAPs were separated from each other by combinations of molecular-sieve and ion-exchange chromatography as described under Materials and Methods. The resulting protein fractions obtained are shown in Figure 2. Two peaks were eluted from the phosphocellulose column by steps of 0.18 and 0.80 M KCl. Proteins with molecular weights similar to those reported for Tau eluted with the 0.18 M KCl step (Figure 2E; Penningroth et al., 1976; Cleveland et al., 1977) while all of the high molecular weight MAPs (MAP 1 and MAP 2), which form the filamentous coating on in vitro assembled microtubules, were present in the 0.80 M KCl fraction (Figure 2F). All of the tubulin assembly stimulating activity was associated with the 0.80 M KCl fraction as determined turbidimetrically (Figure 3); therefore, this protein sample (Figure 2F), termed the MAP fraction, was used in all the experiments reported here. The purified tubulin dimers (Figure 2, gel B or gel C) were 96–97% tubulin, with the remaining 3–4% of the stain residing principally in 2–3 bands of molecular weight lower than the α- and β-tubulin subunits, as determined by scans of quantitatively stained gels (Sloboda & Rosenbaum, unpublished results).

Decoration of Intact, Smooth-Walled Microtubules with MAPs. To assay for the association of MAPs with smooth-walled microtubules by SDS–polyacrylamide gel electrophoresis, tubulin dimers from the Bio-Gel column were first concentrated by pressure dialysis to greater than 8 mg/mL so that the tubulin dimers alone would readily self-assemble in the absence of any added associated proteins. Aliquots of the concentrated dimers were then incubated in conical centrifuge tubes for 1 h at 37 °C, and the assembled tubulin was collected by centrifugation. The pellets, containing smooth-walled microtubules, were resuspended in buffer containing colchicine plus increasing concentrations of MAPs. The microtubules were then immediately collected by centrifugation and the supernatants and pellets prepared for gel electrophoresis. The results of this experiment are shown in Figure 4A. It can be seen that as the concentration of MAPs in the resuspension buffer increased the sedimented microtubules had increasing amounts of MAPs associated with them until the MAP/tubulin ratio appeared to reach a saturating

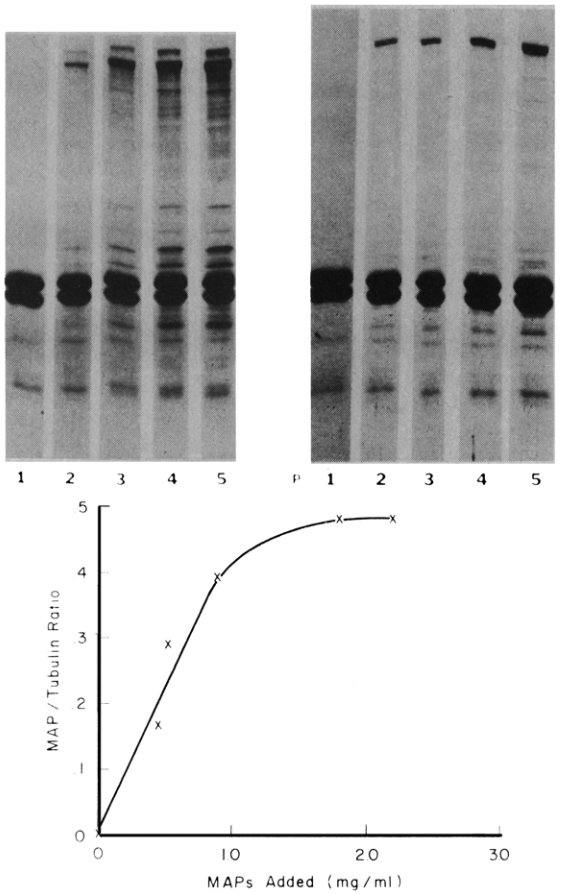

FIGURE 4: (A, top) Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (Laemmli, 1970) of supernatant (S) and pellet (P) samples obtained after intact, smooth-walled microtubules were resuspended in the presence of increasing concentrations of the MAP fraction and then resedimented. MAP fraction concentrations were (1) 0.0 mg/mL, (2) 0.5 mg/mL, (3) 1.1 mg/mL, (4) 1.6 mg/mL, and (5) 2.2 mg/mL. Tubulin concentration was held constant at 4.7 mg/mL in 1–5. Note that as the concentration of MAPs in the resuspending solution increased the sedimented microtubules had an increasing amount of MAPs associated with them while the distribution of tubulin in the supernatants and pellets remained relatively constant. (B, bottom) Increase in the MAP/tubulin ratio of decorated microtubules as a function of the MAP fraction concentration used as the resuspending solution. These data have not been affected by nonspecific aggregates of tubulin which might have formed during the assembly reaction since, during routine examinations of the assembled product by negative-stain electron microscopy, no such aggregates were present.

value; by contrast, the amount of MAPs left in the supernatant continued to increase as the concentration of MAPs in the resuspension buffer increased. However, the distribution of tubulin in both the pellet and supernatant gels remained relatively constant throughout the range of MAP concentrations tested. These observations suggested that although the MAPs were associating with the microtubules the tubulin dimer–polymer equilibrium was not shifted by the presence of the MAPs, an observation verified by turbidity data obtained simultaneously; this provided direct evidence that the colchicine included in the resuspending buffer was acting to block the MAP-stimulated tubulin assembly reaction. This result, then, strongly suggested that increasing amounts of the MAPs were associating with the smooth-walled microtubules as the concentration of MAPs in the suspending solution increased.

To quantitatively determine the stoichiometry of this association, aliquots of smooth-walled microtubules were resuspended in increasing concentrations of MAPs and the

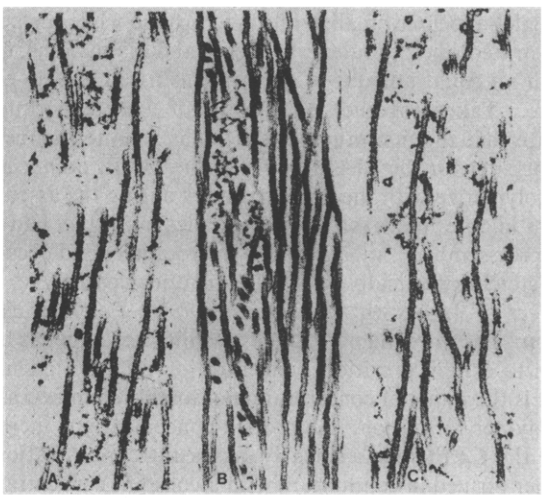

FIGURE 5: Thin sections of microtubules: (A) assembled in the presence of the MAPs; (B) assembled from a high concentration of purified tubulin dimers in the absence of the MAPs; these microtubules, therefore, have comparatively smooth-walls; and (C) decorated microtubules obtained by resuspending a preparation such as shown in B in the presence of an excess (saturating) concentration of the MAPs. The fuzzy, filamentous coating has been restored in C even though tubulin assembly has been inhibited by the presence of 10^{-4} M colchicine.

MAP/tubulin ratio in the resulting microtubules was determined from scans of gels quantitatively stained with Fast Green as shown in Figure 4B. When the concentration of assembled tubulin was held constant while the concentration of MAPs in the suspending buffer was increased, there was a linear increase in the amount of MAPs that sedimented with the assembled tubulin until the tubulin became saturated. Thus, a given amount of smooth-walled microtubules could accept as associated filaments only a finite amount of MAPs. This saturation occurred at an approximate MAP/tubulin ratio of 0.48 which corresponded to a preparation that was approximately 68% tubulin. Therefore, this saturating MAP/tubulin ratio was approximately the same as the MAP/tubulin ratio in microtubules purified by successive cycles of assembly and disassembly in the presence of the MAPs; these microtubules were approximately 70% tubulin, i.e., a MAP/tubulin ratio of 0.43, a value which falls within the range of protein composition reported previously for in vitro assembled brain microtubules (Borisy et al., 1975; Rosenbaum et al., 1975; Sloboda et al., 1975).

When viewed by thin-section electron microscopy, intact microtubules resuspended in the MAPs were identical in morphology with microtubules assembled in the presence of the MAPs. As has been reported by others (Dentler et al., 1975; Murphy & Borisy, 1975), microtubules assembled from tubulin dimers in the presence of the MAPs have a fuzzy filamentous coating as shown in Figure 5A; if preparations of MAP-free tubulin dimers were assembled by using high concentrations of the dimers, the resulting microtubules had smooth walls (Figure 5B). When these smooth-walled microtubules were resuspended in saturating concentrations of MAPs (determined in Figure 4B), they appeared as shown in Figure 5C. The MAP-decorated microtubules (i.e., smooth-walled microtubules resuspended in the presence of the MAPs) had a filamentous coating similar to microtubules assembled in the presence of the MAPs (Figure 5A). It should also be noted that the smooth-walled microtubules packed more tightly at the same centrifugal speeds than the microtubules containing MAPs as a filamentous coating (see also Kim et al., 1979). In fact, the pellet of smooth-walled mi-

crotubules appeared small, white, and opaque while the pellet of decorated microtubules was larger and diaphanous, even though an equal amount of tubulin was assembled in each sample. Taken together, the results of these experiments indicate that the microtubule lattice has a finite number of binding sites for the MAPs and that the MAPs do not have to copolymerize with the tubulin dimers during the assembly process in order to attach to microtubules but rather can add on to preassembled, smooth-walled microtubules and decorate them stoichiometrically with a filamentous coating.

Effect of MAPs on the Tubulin Dimer–Polymer Equilibrium. The in vitro microtubule assembly reaction has been shown to exhibit a critical concentration (C_c) for assembly which is the minimal concentration of total protein necessary for polymer formation. As protein concentrations increase above the C_c, there is a linearly increasing concentration of polymer formed in equilibrium with a constant concentration of monomer. The situation is similar to the condensation of a gas to a liquid, and therefore microtubule polymerization has been termed a condensation equilibrium (Gaskin et al., 1974; Johnson & Borisy, 1975) after the name first applied to actin polymerization by Oosawa & Kasai (1962). The existence of a C_c, which is consistent with a helical polymerization mechanism, was predicted thermodynamically by Oosawa & Higashi (1967) for subunit addition onto a growing helical polymer and demonstrated experimentally by Johnson & Borisy (1975, 1977) for the tubulin assembly reaction. For a helical condensation polymerization of this type these authors have shown that the equilibrium constant for the association reaction is approximately defined by

$$K_{eq} = 1/C_c$$

where K_{eq} is the equilibrium constant for the association reaction and C_c is the critical concentration (Oosawa & Higashi, 1967).

Previously, in experiments concerned with the kinetics of the MAP-stimulated assembly of tubulin dimers, we reported (Sloboda et al., 1976b) that an increasing mass of polymer was formed as the MAP/tubulin ratio was increased. This suggested that the MAPs were stoichiometrically inducing microtubule formation by shifting the tubulin dimer–polymer equilibrium toward the formation of polymer. Therefore, based on the above equation, a factor that noncatalytically favors the association reaction will result in an increase in the equilibrium constant, and this can be measured by noting a corresponding decrease in the C_c.

However, before the effect of the MAPs on the C_c for tubulin assembly was determined, experiments of the following type were performed to establish the C_c of purified tubulin dimers for use as a comparison. To do this, samples of phosphocellulose-purified tubulin dimers were concentrated to greater than 8 mg/mL by pressure dialysis; the protein was then assembled by incubation at 37 °C for 60–90 min. This concentrated stock solution was then diluted with buffer at 37 °C and the total absorbance change was measured after a new equilibrium was established. The approach to equilibrium in an experiment such as this is dependent solely on the rate constant for the depolymerization reaction and the number concentration of microtubules, as shown by Johnson & Borisy (1977), and results in the rapid establishment of a stable equilibrium. Therefore, the final absorbance change at equilibrium, which was proportional to the amount of polymer formed, was plotted as a function of total protein concentration; the x-intercept determined by a least-squares fit of the data defines the critical concentration for the assembly reaction. The data (Figure 6) show that the C_c for

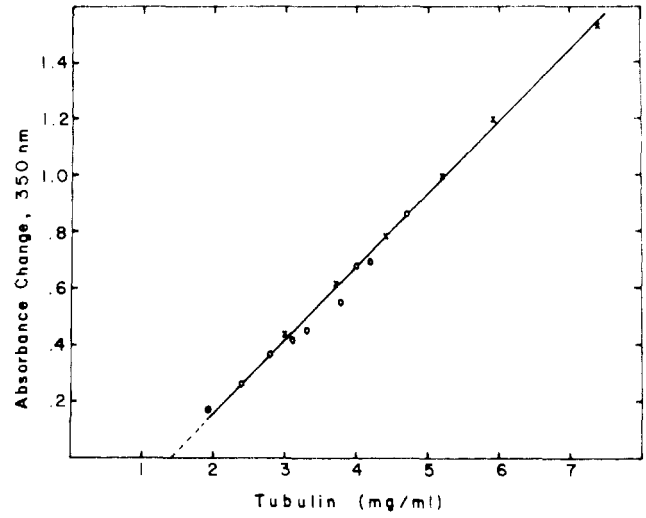

FIGURE 6: Estimation of the C_c for tubulin assembly in the absence of any added microtubule-associated proteins (i.e., at a MAP/tubulin ratio of zero). The total A_{350} was determined by dilution of a concentrated stock solution of assembled, MAP-free microtubules as described in the text. The C_c given by the x-intercept was obtained by linear regression; for these data, which represents the results obtained with two individually prepared samples of phosphocellulose-purified tubulin (x, O), the C_c is calculated to be equal to 1.39 mg/mL ($r^2 = 0.992$).

the assembly of phosphocellulose-purified, MAP-free tubulin is thus 1.39 mg/mL, which is in good agreement with the value (~1.0 mg/mL) predicted by the theoretical analysis of Murphy et al. (1977a).

Next, when the critical concentration for tubulin assembly in the presence of varying concentrations of MAPs was determined in a similar manner, it was found that the C_c decreased as the MAP/tubulin ratio increased. Specifically, as the MAP/tubulin ratio was increased from 0 to 0.48 to 0.55, for example, the C_c in terms of the tubulin concentration decreased from 1.39 to 0.67 to 0.28 mg/mL. Thus the MAP fraction directly affected the assembly of the purified tubulin dimers by decreasing the C_c, which is equivalent to an increase in the K_{eq} of the association reaction.

Direct Demonstration of the Stabilization of Microtubules by MAPs. Since an increase in the MAP/tubulin ratio resulted in an increase in the equilibrium constant for the assembly reaction, it followed that the stability of the resulting polymer formed also increased (here stability is defined as polymer being favored over monomer at increasing MAP/tubulin ratios). This increased stability in the presence of the MAPs could result from either an increase in the relative rate of the forward, association reaction or a decrease in the rate of the reverse, dissociation reaction. Indeed, it has recently been shown by Murphy et al. (1977a) that the high molecular weight MAPs most likely function in the assembly reaction by decreasing the rate of dissociation. To measure directly the effect of MAPs on microtubule stability, the disassembly kinetics in response to cold treatment were measured for microtubules containing various concentrations of MAPs (i.e., conditions where MAPs were absent, where MAPs were present in limiting amounts, or where MAPs were present in great excess). To do this, three aliquots of assembled MAP-free microtubules were collected by centrifugation, and the pellets were then resuspended in equal volumes of buffer alone or buffer containing two different concentrations of MAPs. These samples were then placed in a cuvette at 37 °C and depolymerization was initiated by lowering the temperature to 4 °C. The kinetics of microtubule depo-

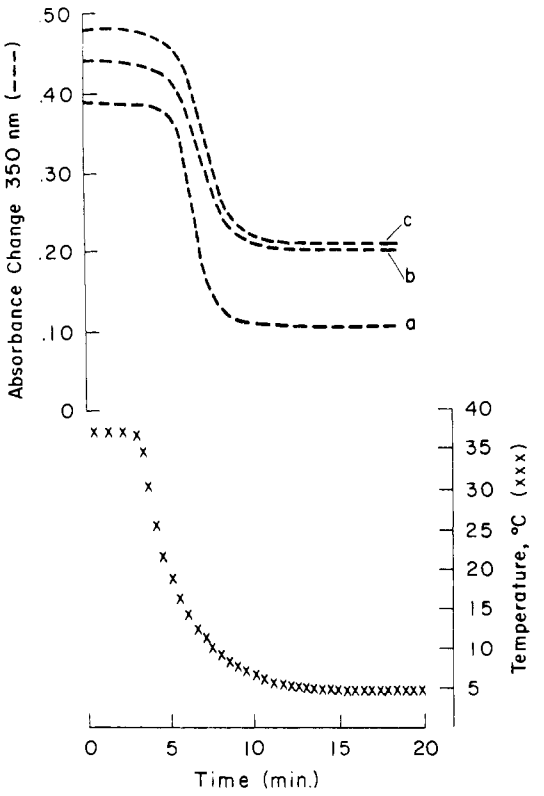

FIGURE 7: Effect of MAPs on the cold stability of assembled tubulin. Upper curves: depolymerization kinetics, measured turbidimetrically, of microtubules suspended in buffer alone (curve a), buffer containing MAPs at 4.0 mg/mL (curve b), and buffer containing MAPs at 1.1 mg/mL (curve c). Note that as the MAP concentration increased from a limiting to a saturating amount in the resuspending buffer the corresponding rate of depolymerization, measured as absorbance change per 5 min, decreased (see text for details and rates). Lower curve: temperature (°C) of the cuvette contents during the course of the depolymerization. Note that loss of turbidity, and therefore depolymerization, begins in all samples at approximately 19 °C.

lymerization for these three different MAP/tubulin ratios are shown in Figure 7. The lower portion of the figure shows the temperature inside the cuvette while the upper curves show loss of turbidity (microtubule depolymerization) as a function of time after the temperature drop. It can be seen from examination of curves a–c that three different rates of disassembly were obtained from the three samples. The fastest rate of disassembly (curve a) was obtained from the sample which was resuspended in buffer alone. The rate of depolymerization for this sample calculated from curve a was $0.650A/5$ min. Those microtubules resuspended in a high concentration of MAPs (curve b), which were expected to be most stable since they had the highest MAP/tubulin ratio, had a correspondingly low rate of depolymerization of $0.350A/5$ min. Finally, the microtubules resuspended in an intermediate concentration of MAPs (curve c) had a rate of disassembly of $0.450A/5$ min, intermediate to samples a and b. Thus, under the conditions employed, MAPs slowed the cold dissociation reaction of in vitro assembled microtubules; these data, in conjunction with the C_c data reported above, provide indirect evidence that is consistent with the observation of Murphy et al. (1977a) that the high molecular weight MAPs function to decrease the reverse rate constant of the assembly reaction.

Discussion

Microtubules assembled in vitro from homogenates of vertebrate brain are composed of several proteins in addition to the α-and β-tubulin subunits. We have named these proteins MAPs (Sloboda et al., 1975) for microtubule-associated proteins to indicate the fact that they are integral components of microtubules assembled in vitro (Sloboda et al., 1975; Berkowitz et al., 1977) and that they play a significant role in the tubulin assembly reaction (Sloboda et al., 1976a,b; Murphy et al., 1977b). When tubulin is assembled in the presence of the MAPs, the microtubules formed have filamentous projections associated with them that are composed of MAPs (Dentler et al., 1975; Murphy & Borisy, 1975; Amos, 1977; Kim et al., 1979). The data presented in this paper have extended these previous studies to show that intact, smooth-walled microtubules, which have been assembled at high tubulin concentrations in the absence of the MAPs and, therefore, lack the surface projections, can be decorated with the high molecular weight microtubule-associated proteins (MAPs 1 and 2) after tubulin assembly has occurred. That the decoration process is not an artifact of the procedure is demonstrated by the following observations. (1) As the concentration of MAPs increased in the resuspending buffer used in the decoration experiments the amount of MAPs that decorated and therefore sedimented with the microtubules increased until a saturating ratio of MAPs to tubulin was reached (Figure 4A,B). (2) The maximum amount of MAPs that could be attached to preformed, smooth-walled microtubules in the absence of microtubule assembly (i.e., in the presence of 10^{-4} M colchicine; see Figure 1) was almost identical with the amount of MAPs associated with microtubules assembled in the presence of the MAPs; the MAP/tubulin ratios in each of these cases were approximately 0.48 and 0.43, respectively. (3) The MAPs lowered the critical concentration for assembly which subsequently increased the equilibrium constant for the assembly reaction. (4) The resulting decorated microtubules were more stable in the cold, and this stability increased as the MAP/tubulin ratio of the decorated microtubules increased (Figure 7).

Previously (Sloboda et al., 1976b) we have demonstrated that the MAPs affected both the initiation and elongation processes of the microtubule assembly reaction. The effect on elongation was shown by experiments in which a greater mass of microtubules was assembled at the apparent equilibrium point of the reaction as the MAP/tubulin ratio was increased until the system became saturated (Sloboda et al., 1976b). Thus, the MAPs affected the tubulin dimer–polymer equilibrium by shifting the equilibrium toward the formation of polymer. That is, the MAPs were capable of stabilizing the formed polymer, and, therefore, the equilibrium constant for the association reaction (K_{eq}) should increase as a function of the MAP fraction concentration. Since it has been demonstrated that the K_{eq} for the assembly of a helical polymer such as a microtubule is the inverse of the critical concentration (C_c) of total protein necessary for assembly (Oosawa & Higashi, 1967; Johnson & Borisy, 1975), the effect of MAPs on K_{eq} was determined by measuring the C_c of assembly for several MAP/tubulin ratios. These results showed directly that the MAPs increased the K_{eq} of the assembly reaction by decreasing the C_c; the net result of this was that the formed polymer was made more stable by the interaction of the MAPs with the tubulin.

However, the MAPs could increase K_{eq} either by increasing the rate at which dimers added on to the polymerizing microtubule or by decreasing the rate at which dimers came off the microtubule. It is not possible from the data reported here to distinguish between these two possibilities, but the results from other laboratories (Murphy et al., 1977a; Engelborghs et al., 1977) indicate that the MAPs decrease the rate of

depolymerization without affecting the rate at which dimers add on to the growing microtubule. Presumably, the MAPs affect the overall microtubule assembly reaction by binding to the tubulin subunit lattice; this MAP binding stabilizes the assembled tubulin and results in a shift of the tubulin dimer–polymer equilibrium toward the formation of polymer.

A direct effect of the MAPs on the stability of microtubules was demonstrated in this report by showing that the rate of depolymerization in response to cold (4 °C) treatment was a function of the MAP/tubulin ratio. When microtubules were resuspended in increasing concentrations of the MAP fraction and induced to disassemble by lowering the temperature from 37 to 4 °C, the rate of depolymerization was greatest in the sample containing the least amount of MAPs. As the MAP fraction concentration increased, the corresponding rate of disassembly in response to cold treatment decreased (Figure 7). This was interpreted as a direct effect of MAPs on the cold stability of assembled microtubules. A similar observation has been reported by Haga & Kurokawa (1975) who showed that microtubules assembled in the presence of the MAPs were more stable to colchicine dissociation than microtubules assembled in the absence of the MAPs; in addition, Murphy et al. (1977a) have shown that nontubulin accessory proteins diminish the rate and amount of depolymerization of microtubules that occurs when a sample of microtubules is diluted.

The preparation used for decoration and stabilization of smooth-walled microtubules in all of the experiments reported here contained primarily the two high molecular weight microtubule-associated proteins MAP 1 and MAP 2 (Figure 2F). This same fraction contained all of the tubulin assembly stimulating activity and differed only by the presence of MAPs 1 and 2 from another phosphocellulose column fraction (0.18 M KCl wash, Figure 2E) that did not have any stimulating activity (Figure 3); this latter fraction was composed of proteins having a molecular mass of 58 000–75 000 daltons. The tubulin dimer fraction used in this study was obtained from either a molecular-sieve column or from a phosphocellulose column; both sources were determined to be greater than 96% by weight α- and β-tubulin (see also Berkowitz et al., 1977). High-resolution acrylamide/urea gradient gels showed that there were no detectable proteins in the area of the gel corresponding to the molecular mass of MAP 1 and MAP 2 (300 000–350 000 daltons; see Sloboda et al., 1975; Borisy et al., 1975) or to Tau (55 000–75 000 daltons; see Penningroth et al., 1976; Cleveland et al., 1977).

Both Bio-Gel and phosphocellulose purified tubulin dimers were capable of self-assembling into intact microtubules in the absence of any added associated proteins provided the tubulin concentration was sufficiently high. Thus, the critical concentration (C_c) for the assembly of purified tubulin dimers was readily calculated to be 1.39 mg/mL (Figure 6), which agreed well with the estimated value ($\sim$1 mg/mL) of the C_c for tubulin assembly in the absence of any added nontubulin proteins predicted by the analysis of Murphy et al. (1977a). Therefore, the data reported here and that reported by Murphy et al. (1977a) contradict the findings of Witman et al. (1976) who reported that a protein factor (called Tau) was required stoichiometrically for phosphocellulose-purified tubulin to assemble onto and thus elongate microtubule seeds. This discrepancy could be explained if a large percentage of the dimers isolated by Witman et al. using phosphocellulose chromatography were inactive, such that the effective concentration of dimers capable of assembly was below the C_c for microtubule polymerization (cf. Murphy et al., 1977a). If a large percentage of the dimers were inactive in these studies, then an investigation of the assembly of phosphocellulose-purified dimers onto seeds as a function of dimer concentration would have indicated this.

It should be noted that in the data reported here the possibility existed, however remote, that trace amounts of contaminants with assembly stimulating activity were present in the tubulin dimer fraction. This seemed unlikely in light of the fact that, on overloaded gels of the tubulin dimer fraction, no bands were detected with molecular weights greater than that of tubulin, especially in the regions of the gel occupied by MAPs 1 and 2 or Tau (Figure 2B,C). A similar conclusion has been reached by Murphy et al. (1977a).

We must conclude from our results and those of other workers in the field (Lee & Timasheff, 1975; Murphy et al., 1977a; Berkowitz et al., 1977; Lee et al., 1978) that, under physiological conditions of pH and ionic strength, the tubulin dimer alone contains sufficient information to specify the structure of a microtubule and that the microtubule-associated proteins function in the in vitro assembly reaction by shifting an otherwise unfavorable equilibrium toward the formation of polymer by stabilizing the assembled tubulin. Experiments must now be designed to relate this in vitro data to actual conditions of microtubule assembly and disassembly in vivo. That MAPs indeed have a significant role in the assembly and function of microtubules in vivo is strengthened by recent findings showing that MAPs can be found associated with microtubules in situ (Sherline & Schiavone, 1977) and that they also appear to be integral components of the mitotic apparatus (Sherline & Schiavone, 1978).

Acknowledgments

We are grateful to Pat Naples, Sharon Nordstrom, and Kay Dickersin for expert technical assistance. Thanks are also extended to Drs. John V. Kilmartin, Robert A. Bloodgood, and Lester I. Binder for helpful discussions during the course of this work.

References

Amos, L. A. (1977) *J. Cell Biol. 72*, 642.
Berkowitz, S. A., Katagiri, J., Binder, H. K., & Williams, R. C., Jr. (1977) *Biochemistry 16*, 5610.
Bloodgood, R. A., & Rosenbaum, J. L. (1976) *J. Cell Biol. 71*, 322.
Borisy, G. G., Marcum, J. M., Olmsted, J. B., Murphy, D. B., & Johnson, K. A. (1975) *Ann. N.Y. Acad. Sci. 253*, 107.
Burton, P. R., & Ferdandez, H. L. (1973) *J. Cell Sci. 12*, 567.
Cleveland, D. W., Hwo, S. Y., & Kirschner, M. W. (1977) *J. Mol. Biol. 116*, 207.
Dentler, W. L., Granett, S., & Rosenbaum, J. L. (1975) *J. Cell Biol. 65*, 237.
Dentler, W. L., Granett, S., Witman, G. B., & Rosenbaum, J. L. (1974) *Proc. Natl. Acad. Sci. U.S.A, 71*, 1710.
Engelborghs, Y., DeMaeyer, L. C. M., & Overbergh, N. (1977) *FEBS Lett. 80*, 81.
Gaskin, F., Cantor, C. R., & Shelanski, M. L. (1974) *J. Mol. Biol. 89*, 737.
Gorovsky, M. A., Carlson, K., & Rosenbaum, J. L. (1970) *Anal. Biochem. 51*, 654.
Haga, T., & Kurokawa, M. (1975) *Biochim. Biophys. Acta 392*, 335.
Johnson, K. A., & Borisy, G. G. (1975) in *Molecules and Cell Movement* (Inoue, S., & Stephens, R. E., Eds.) pp 119–141, Raven Press, New York.
Johnson, K. A., & Borisy, G. G. (1977) *J. Mol. Biol. 117*, 1.

Kim, H., Binder, L. I., & Rosenbaum, J. L. (1979) *J. Cell Biol.* (in press).
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lee, J. C., & Timasheff, S. N. (1975) *Biochemistry 14*, 5183.
Lee, J. C., Tweedy, N., & Timasheff, S. N. (1978) *Biochemistry, 17*, 2783.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Murphy, D. B., & Borisy, G. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2696.
Murphy, D. B., Johnson, K. A., & Borisy, G. G. (1977a) *J. Mol. Biol. 117*, 33.
Murphy, D. B., Vallee, R. B., & Borisy, G. G. (1977b) *Biochemistry 16*, 2598.
Oosawa, F., & Higashi, S. (1967) *Prog. Theor. Biol. 1*, 79.
Oosawa, F., & Kasai, M. (1962) *J. Mol. Biol. 4*, 10.
Penningroth, S. M., Cleveland, D. W., & Kirschner, M. W. (1976) in *Cell Motility* (Goldman, R., Pollard, T., & Rosenbaum, J., Eds.) Vol. 3, Book C, pp 1233–1257, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Rosenbaum, J. L., Binder, L. I., Granett, S., Dentler, W. L., Snell, W., Sloboda, R. D., & Haimo, L. (1975) *Ann. N.Y. Acad. Sci. 253*, 147.
Ross, H., Olmsted, J. B., & Rosenbaum, J. L. (1975) *Tissue Cell 7*, 107.
Schacterle, G. R., & Pollack, R. L. (1973) *Anal. Biochem. 51*, 654.
Sherline, P., & Schiavone, K. (1977) *Science 198*, 1038.
Sherline, P., & Schiavone, K. (1978), *J. Cell Biol. 79*, R9.
Sloboda, R. D., Dentler, W. L., Bloodgood, R. A., Telzer, B. R., Granett, S., & Rosenbaum, J. L. (1976a) in *Cell Motility* (Goldman, R., Pollard, T., & Rosenbaum, J., Eds.) Vol. 3, Book C, pp 1171–1212, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sloboda, R. D., Dentler, W. L., & Rosenbaum, J. L. (1976b) *Biochemistry 15*, 4497.
Sloboda, R. D., & Rosenbaum, J. L. (1977) *J. Cell Biol. 75* (part 2), 286a.
Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L., & Greengard, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 177.
Weisenberg, R. C. (1972) *Science 177*, 1104.
Weingarten, M. D., Lockwood, A. H., Hwo, S. Y., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858.
Witman, G. B., Cleveland, D. W., Weingarten, M. D., & Kirschner, M. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4070.
Yamada, K. M., Spooner, B. S., & Wessells, N. K. (1971) *J. Cell Biol. 49*, 614.

Isolation and Characterization of Ca^{2+}-Dependent Modulator Protein from the Marine Invertebrate *Renilla reniformis*†

Harold P. Jones, John C. Matthews, and Milton J. Cormier*

ABSTRACT: An acidic, low molecular weight (18 400–19 100) protein capable of activating porcine brain phosphodiesterase in the presence of calcium has been purified 2700-fold from the anthozoan coelenterate, *Renilla reniformis*. The protein has physical, spectral, and chemical properties similar to those of modulator proteins isolated from mammalian species. Amino acid composition studies reveal no significant differences between the *Renilla* and mammalian modulator proteins. For example, we observed 1 mol of ϵ-*N*-trimethyllysine per mol of protein, no tryptophan or cysteine, and high levels of glutamic and aspartic acid residues. The protein from *Renilla* complexes with troponin I and T subunits in the presence of calcium and quantitatively replaces porcine brain modulator in the calcium-dependent activation of porcine brain phosphodiesterase. The protein has a high affinity for calcium as judged by the low levels of free calcium required for modulator-dependent activation of phosphodiesterase. The similarities in physical and chemical properties, high affinity for calcium, and identical calcium-dependent activities of this protein from *Renilla* (as compared with modulator protein purified from mammalian systems) suggest that a high degree of structural conservation has been retained in modulator proteins isolated from these diverse evolutionary forms.

During attempts to purify cyclic nucleotide phosphodiesterase from mammalian brain extracts, Cheung (1970, 1971) and Kakiuchi et al. (1970) independently discovered a protein activator for this enzyme, which, in addition to Ca^{2+}, was required for maximal activity. We will use the term modulator protein for this activator because of its multiple Ca^{2+}-dependent functions, as originally suggested by Watterson et al. (1976). Modulator protein was initially isolated to homogeneity from extracts of bovine brain and heart and characterized as an acidic and heat-stable Ca^{2+}-binding protein having a molecular weight in the range of 15 000–20 000 (Teo et al., 1973; Teo & Wang, 1973; Lin et al., 1974; Wolff & Brostrom, 1974).

Additional regulatory functions of modulator protein have recently been described including the activation of soluble preparations of brain adenylate cyclase in the presence of Ca^{2+} (Brostrom et al., 1975; Cheung et al., 1975) and the activation of a Ca^{2+}-dependent protein kinase found in smooth and skeletal muscle which results in the phosphorylation of myosin light chain (Yagi et al., 1978; Dabrowska et al., 1978;

† From the Bioluminescence Laboratory, Department of Biochemistry, University of Georgia, Athens, Georgia 30602. *Received July 25, 1978.* This work was supported in part by the National Science Foundation (BMS 74-06914). This work is Contribution No. 375 from the University of Georgia Marine Institute, Sapelo Island, Georgia.

0006-2960/79/0418-0055$01.00/0