Characterization of the in Vitro Reassembly of Tubulin Derived from Stable *Strongylocentrotus purpuratus* Outer Doublet Microtubules†

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ABSTRACT: We have characterized the in vitro reassembly properties of tubulin derived from stable outer doublet microtubules of *Strongylocentrotus purpuratus* sperm tails. These data are of special interest, since previous studies have employed tubulins derived from labile microtubules whose physiological properties are strikingly different from those of stable outer doublets. Reassembly occurred optimally in 5 mM 2-(*N*-morpholino)ethanesulfonic acid, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, 10 mM $MgSO_4$, 150 mM KCl, pH 6.7. In contrast to the physiological growth temperature of 11–15 °C for *S. purpuratus*, in vitro polymerization of outer doublet tubulin was optimal at 37 °C. The critical tubulin concentration for assembly was 0.55 mg mL^{-1} at this temperature. Assembly was dependent on GTP (2 mM), and neither GDP nor GMP would substitute. ATP, CTP, and UTP all promoted microtubule formation owing to the presence of a nucleoside diphosphokinase activity. Podophyllotoxin poisoned microtubule formation substoichiometrically: only 2–4% of the total number of tubulin dimers were drug-bound when assembly was inhibited by 50%. These data show that the overall similarities between outer doublet and brain tubulin reassembly are remarkable and suggest that the in vitro polymerization properties of tubulins are highly conserved. Since outer doublet tubulin reassembles to give microtubules with very different properties from outer doublet microtubules, it seems unlikely that the intrinsic in vitro polymerization properties of this tubulin determine either microtubule stability or doublet formation. These data suggest that microtubule-associated proteins are of greater importance in determining certain microtubule properties and functions than the tubulin backbone.

Flagellar microtubules classically show an arrangement of nine outer doublet microtubules encircling a pair of central singlet microtubules (e.g., Warner, 1972). A variety of chemical, physical, and enzymatic treatments selectively solubilize the A-subfiber, B-subfiber, and central pair microtubules (Gibbons, 1965; Stephens, 1970; Shay, 1972; Witman et al., 1972a,b; Behnke & Forer, 1967). An analysis of the A- and B-subfiber and central pair microtubules of sea urchin sperm by tryptic peptide mapping has revealed distinct chemical differences between the tubulins and has indicated that each class of flagellar microtubules may possess a unique tubulin composition (Stephens, 1970, 1976a,b, 1978). In addition, sea urchin flagellar and ciliary microtubules differ in the composition of their α tubulin chains (Bibring et al., 1976), and *Chlamydomonas* outer doublet tubulin has been resolved into five isoelectric variants (Witman et al., 1972b). Thus, in addition to obvious morphological differences, flagellar microtubules differ both with respect to their relative stabilities and to the chemical composition of the constituent tubulin molecules.

In spite of this evidence for heterogeneity, the functional importance of these chemical differences for assembled microtubules is obscure. A major obstacle to investigating this question has been the problem of obtaining native tubulin derived from microtubules exhibiting functional or morphological differences in sufficiently large quantities for biochemical analysis. Outer doublet microtubules offer a model system for examining the question of the relationship between tubulin heterogeneity and microtubule function, since the A- and B-subfiber microtubules may be distinguished both chemically and ultrastructurally from each other, as well as from cytoplasmic microtubules (Stephens, 1970, 1978; Olmsted et al., 1971; Amos & Klug, 1974). However, the inability to solubilize outer doublet microtubules to obtain native tubulin had meant that it was not possible to exploit this system fully. This has recently been accomplished by Kuriyama (1976) and subsequently by other investigators (Binder & Rosenbaum, 1977; Farrell & Wilson, 1977, 1978), using sonication as the method of solubilization. Outer doublet tubulin prepared in this manner is both native by colchicine binding assays and competent to reassemble in vitro to give bona fide singlet microtubules. Consequently, it should now be possible to investigate the relationships between the chemical composition of tubulin subunits and the properties of the assembled microtubules.

As a first step toward achieving this goal, we have examined the reassembly properties of outer doublet tubulin. The data demonstrate that the reassembly characteristics of outer doublet tubulin are very similar to those of brain tubulin and suggest that the polymerization properties of the two tubulins have been highly conserved. Since outer doublet and cytoplasmic microtubule proteins appear to be chemically distinct (Olmsted et al., 1971; Stephens, 1978), it is unlikely that these chemical differences are relevant for the assembly capabilities of the tubulins. Consequently, the in vitro reassembly properties of tubulins generally may not reveal differences in tubulin chemistry which are functionally important in the assembled microtubule.

Materials and Methods

Preparation of Outer Doublet Microtubules and Tubulin. *Strongylocentrotus purpuratus* were induced to spawn by injection of 0.52 M KCl into the body cavity. The methods of collection and storage of sperm and purification of outer doublet microtubules have been described previously (Farrell & Wilson, 1978). Purified outer doublet microtubules were routinely stored overnight as pellets at 4 °C prior to sonication. Tubulin was solubilized by sonication of the purified doublets suspended in 5 mM Mes,[1] 1 mM EGTA, 1 mM $MgSO_4$, pH 6.7 (sonication buffer), at 0 °C. KCl was added immediately

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PLN, podophyllotoxin; MAPs, microtubule associated proteins; CLC, colchicine.

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following sonication to a final concentration of 150 mM, and the suspension was centrifuged at 200000*g* at 4 °C to prepare a supernatant containing assembly-competent tubulin (Farrell & Wilson, 1978).

Turbidimetric Assay of Microtubule Polymerization. Microtubule reassembly was monitored by light scattering at 350 nm by use of a Gilford Model 2400 spectrophotometer and 300-μL microcuvettes with 1-cm path lengths. The cuvette assembly was maintained at 37 ± 0.1 °C with a circulating water bath. Microtubule polymerization was initiated by transferring the cuvettes from 0 °C to the cuvette assembly and adjusting the tubulin solutions either to 2 mM GTP or to the requisite nucleotide concentration. All reassembly experiments were carried out with 200000*g* supernatants in reassembly buffer (5 mM Mes, 1 mM EGTA, 1 mM $MgSO_4$, 150 mM KCl, pH 6.7) unless indicated otherwise.

The presence of microtubules was verified in all cases by negative-stain electron microscopy. Negatively stained samples were prepared by the method of Olmsted & Borisy (1973) and observed with a Philips EM 300 operating at 80 kV. Protein assays were carried out by the method of Lowry et al. (1951) by using bovine serum albumin as a standard.

Determination of the Number of Tubulin Molecules Complexed with Podophyllotoxin (PLN) to Give Half-Maximal Inhibition of Microtubule Reassembly. The number of tubulin molecules complexed with PLN which gives half-maximal inhibition of outer doublet tubulin reassembly can be calculated if both the apparent inhibition constant (K_i) for the ability of PLN to inhibit microtubule assembly and the dissociation constant (K_D) for the binding of PLN to outer doublet tubulin are known. The K_D for the binding of PLN to outer doublet tubulin at 37 °C has been obtained previously by determining the inhibition constant for the ability of PLN to prevent the binding of [^{3}H]colchicine to outer doublet tubulin (1.1×10^{-6} M, Pfeffer et al., 1976; 1.3×10^{-6} M, Wilson & Meza, 1973).

To determine the PLN concentration which half-maximally inhibits outer doublet tubulin reassembly (apparent K_i for the reassembly reaction), aliquots of 200000*g* supernatants were incubated at 37 °C with varying concentrations of PLN, and the initial rates of turbidity development determined relative to untreated controls. Inhibition of the initial rate of reassembly for each drug concentration was plotted in the form of a double-reciprocal plot, from which we obtained a value for the apparent K_i for the reassembly reaction (see Figure 4).

Determination of the number of tubulin molecules complexed with PLN under conditions of half-maximal inhibition of microtubule reassembly proceeds as follows. From the law of mass action we have

$$K_D = \frac{[PLN]_f[T]}{[PLN\text{–}T]} \quad (1)$$

where $[PLN]_f$ is the concentration of free PLN, [T] is the concentration of uncomplexed tubulin, and [PLN–T] is the concentration of drug-bound tubulin. Since the concentration of free PLN can be expressed in terms of the total PLN concentration, $[PLN]_t$, and drug–tubulin complex concentration, [PLN–T], as

$$[PLN]_f = [PLN]_t - [PLN\text{–}T]$$

equation 1 can be rewritten as

$$K_D = \frac{([PLN]_t - [PLN\text{–}T])[T]}{[PLN\text{–}T]}$$

which upon rearrangement gives

$$[PLN\text{–}T] = \frac{[T][PLN]_t}{K_D + [T]} \quad (2)$$

The apparent K_i for the ability of PLN to inhibit microtubule reassembly half-maximally is, by definition, the concentration of total PLN added, $[PLN]_t$, which inhibits reassembly by 50%. Thus, with both the apparent K_i and the K_D known, eq 2 permits the calculation of the number of tubulin molecules complexed with PLN under conditions of half-maximal assembly inhibition.

Results

Solution Conditions for Optimal Reassembly. We have examined the effects of different concentrations of KCl and Mg^{2+} and different pH values on the polymerization of solubilized outer doublet tubulin in order to define the solution conditions optimal for reassembly.

At KCl concentrations below 100 mM no microtubule formation was detected by light scattering, and only occasional microtubules were visible by electron microscopy. Increasing the KCl concentration from 100 to 150 mM stimulated both the initial rate and the maximal extent of polymerization (Figure 1a). Additional increase in KCl concentration to 200 mM failed to stimulate further either the initial rate or final extent of assembly, and above 200 mM KCl the maximal extent of assembly may have decreased slightly.

The addition of Mg^{2+} stimulated outer doublet tubulin reassembly (Figure 1b). In the absence of added Mg^{2+}, a low level of turbidity development occurred, and microtubules were only infrequently observed by negative-stain electron microscopy. Both the initial rate and maximal extent of reassembly increased with added Mg^{2+} to a concentration of 10 mM (Figure 1b). Above this concentration both the rate and extent of assembly were inhibited (data not shown).

The rate and extent of microtubule polymerization at different pH values are shown in Figure 1c. The optimal pH for maximal extent of reassembly was pH 6.6–6.7, whereas that for the initial rate of assembly was pH 6.6–6.8. Both initial rate and final extent of polymerization decreased rapidly on either side of these pH ranges, and below pH 6.2 the protein precipitated into amorphous aggregates.

Effect of Protein Concentration on Reassembly. The formation of microtubules as a function of protein concentration was assayed both by sedimentation (Figure 1d) and by light scattering (data not shown). An abrupt transition was observed at a protein concentration of 0.6 mg/mL, below which no microtubule formation occurred (no sedimentable protein) (Figure 1d). Above this critical protein concentration, the amount of polymerized protein increased linearly with increasing protein concentration, while the amount of protein in the supernatants remained constant and independent of initial protein concentration. Quantitatively identical results were obtained when the amount of assembled protein was assayed by the development of turbidity at 350 nm (data not shown). Since tubulin comprises 75–85% of the total protein in 200000*g* supernatants of sonicated outer doublet microtubules (Farrell & Wilson, 1978), and since nonspecific protein aggregation accounts for up to 15% of the optical density changes, the critical protein concentration of 0.6 mg/mL corresponds to a critical tubulin concentration of 0.55 mg/mL.

Temperature Dependence of Outer Doublet Tubulin Reassembly. The optimal temperature for reassembly of vertebrate brain microtubules in vitro is similar to the physiological temperature for growth (Olmsted & Borisy, 1973). Since the physiological growth temperature for *S. purpuratus* is 11–15 °C, it was of interest to characterize the

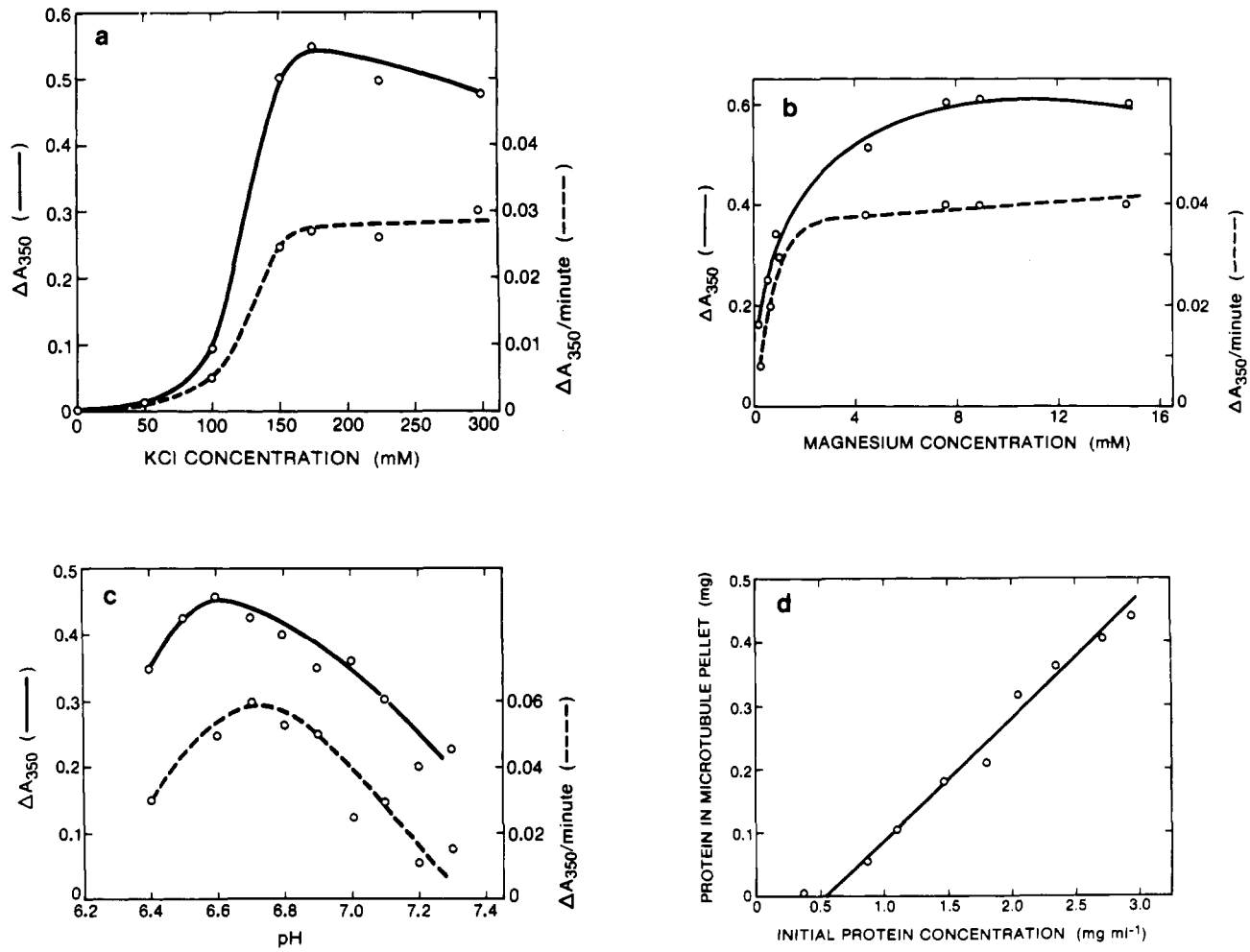


FIGURE 1: (a) Effect of KCl concentration on the reassembly of outer doublet tubulin. Suspensions of outer doublets were sonicated in sonication buffer. Aliquots were adjusted to varying concentrations of KCl and centrifuged at 200000*g* for 1 h at 4 °C. Polymerization in the resulting supernatants was initiated by addition of 2 mM GTP and warming to 37 °C. Maximum extents of polymerization (–); initial rates (- - -). (b) Effect of magnesium concentration on the reassembly of outer doublet tubulin. Suspensions of outer doublets were sonicated in 5 mM Mes, 1 mM EGTA, 150 mM KCl, pH 6.7, adjusted to the requisite magnesium concentration, and centrifuged at 200000*g* for 1 h at 4 °C. Polymerization was initiated by addition of 2 mM GTP to the supernatants and warming to 37 °C. Maximum extents of polymerization (–); initial rates (- - -). (c) Effect of pH on the maximum extents (–) and initial rates (- - -) of polymerization of outer doublet tubulin. Outer doublets were sonicated in 50 mM Mes, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.7, and 150 mM KCl was added immediately following sonication. Aliquots of 200000*g* supernatants were then adjusted to varying pH values, and polymerization initiated by addition of 2 mM GTP and warming to 37 °C. The pH values given on the ordinate are correct for 37 °C. (d) Determination of the critical protein concentration for reassembly of outer doublet tubulin. Varying concentrations of outer doublet 200000*g* supernatant in sonication buffer, 150 mM KCl, pH 6.7, were incubated for 1 h at 37 °C with 2 mM GTP to promote assembly. Assembled microtubules were pelleted by centrifugation at 40000*g* for 45 min and 30 °C, and the protein in the pellets was determined.

temperature requirements for outer doublet tubulin reassembly and to determine whether the optimal temperature coincided with the physiological growth temperature for the whole animal.

In our initial experiments we found that some protein aggregation occurred at temperatures in excess of 30 °C, giving rise to spuriously high optical density values at these temperatures. To correct for this aggregate formation, two types of experiments were carried out. In the first, optical density (350 nm) was determined in 200000*g* supernatants after 1-h incubation at various temperatures. Samples were then chilled to 4 °C for 30 min to depolymerize the microtubules. The optical densities were again determined for each sample, and the values obtained were subtracted from the initial values. We reasoned that if the aggregates were not cold labile, the difference between the optical density values prior to and after chilling would be a valid estimate of microtubule formation (Figure 2).

Since it was possible that some aggregation was cold labile, a second type of experiment was performed in which duplicate samples of 200000*g* supernatants were incubated at the various temperatures in the presence and absence of PLN. In the presence of PLN (5×10^{-5} M) any optical density changes which occur must be due to aggregate formation, since the drug prevents assembly of microtubules. Subtraction of the optical density values obtained in the presence of PLN from those obtained in the absence of PLN should, therefore, reflect microtubule assembly. The results of this experiment were essentially the same as those obtained for the experiment shown in Figure 2.

The results in Figure 2 indicate that the optimal temperature for outer doublet tubulin reassembly is 37 °C. This is considerably higher than the optimal growth temperature for *S. purpuratus*. It is also evident that a sharp transition occurred at approximately 25 °C, with little polymerization occurring below this temperature and extensive polymerization occurring at 27–30 °C.

Microtubule formation was not observed in the above experiments at physiological temperatures (up to 15 °C), even by electron microscopy. However, the protein concentration

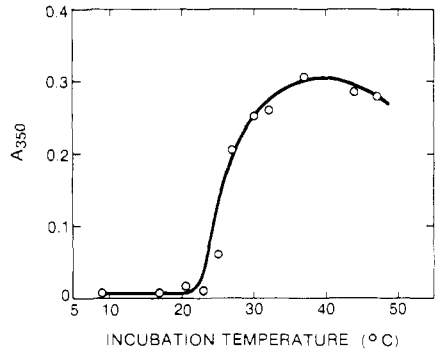


FIGURE 2: Determination of the optimal temperature for reassembly of outer doublet tubulin. Aliquots of 200000*g* supernatants in sonication buffer, 150 mM KCl, 2 mM GTP, pH 6.7, were incubated at varying temperatures for 1 h. Following incubation, the absorbancy at 350 nm of each aliquot was determined and then corrected for aggregate formation by subtracting from this initial value the absorbancy of each aliquot remaining after the aliquots were chilled to 4 °C.

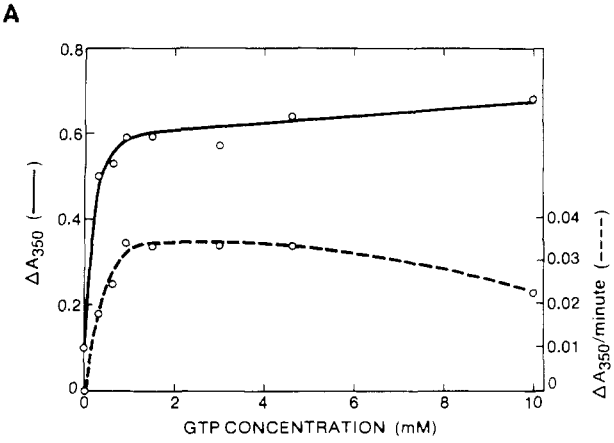
of the 200000*g* supernatants never exceeded 2.5 mg/mL. At higher protein concentrations (4.55 mg/mL) a low level of microtubule polymerization was detectable at 15 °C by electron microscopy but not by light scattering (data not shown).

Nucleotide Requirements for Outer Doublet Tubulin Reassembly. We examined the ability of various nucleotides to promote the reassembly of outer doublet tubulin by adjusting aliquots of 200000*g* supernatants to the requisite concentration of nucleotide and following turbidity development at 37 °C. Increasing the concentration of added GTP to 1.5 mM stimulated both the initial rate and maximal extent of polymerization. Further increasing the GTP concentration to 10 mM resulted in little additional stimulation of the maximal extent of polymerization and appeared to decrease the initial rate (Figure 3A). In the absence of added GTP low levels of polymerization occurred. Therefore, it is possible that sufficient GTP was associated with the solubilized tubulin to promote some microtubule formation (see Stephens et al., 1967; Yanagisawa et al., 1968).

Other guanine nucleotides were ineffective in promoting outer doublet tubulin reassembly; neither GDP nor GMP (2 mM) stimulated turbidity development above that obtained in the absence of added GTP (Figure 3B). In contrast, ATP, CTP, and UTP each at 2 mM stimulated reassembly of outer doublet tubulin to the same extent as 2 mM GTP (Figure 3B).

There seems to be a nucleoside diphosphokinase activity in the 200000*g* supernatants since the other nucleoside triphosphates can support assembly without directly interacting with the tubulin dimer. When [^{3}H]ATP and [^{3}H]GTP were substituted for unlabeled ATP and GTP, respectively, in the experiment shown in Figure 3B, no label was incorporated into the microtubules during polymerization when [^{3}H]ATP was used, whereas [^{3}H]GTP was incorporated into the microtubules with a stoichiometry of 0.5 mol of guanine nucleotide/mol of tubulin dimer (data not shown). Furthermore, addition of ATP during assembly with [^{3}H]GTP did not prevent label incorporation into the microtubules, even when the ATP was present at a 20-fold molar excess to the [^{3}H]GTP (data not shown).

Substoichiometric Inhibition of Microtubule Assembly by Podophyllotoxin. From the data shown in Figure 4, we obtained an apparent K_i of 2.11×10^{-6} mol L^{-1} for PLN poisoning of outer doublet tubulin reassembly. Since the K_D for PLN had been determined previously (Wilson & Meza, 1973; Pfeffer et al., 1976), it was possible to calculate the ratio



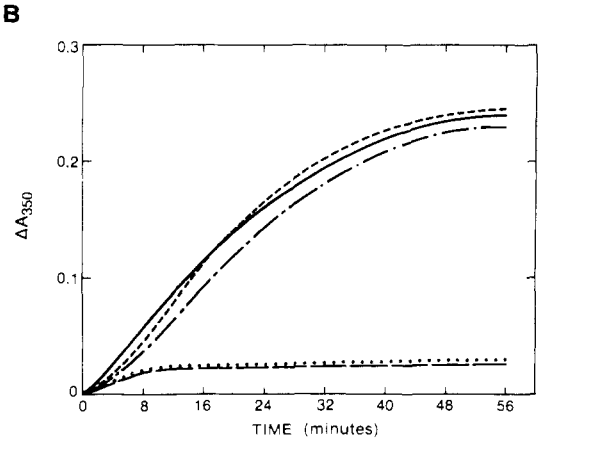


FIGURE 3: (A) Effect of GTP concentration on the reassembly of outer doublet tubulin. Aliquots of 200000*g* supernatants in sonication buffer, 150 mM KCl, pH 6.7, were adjusted to varying GTP concentrations and incubated at 37 °C. Maximum extents of polymerization (–); initial rates (---). (B) Ability of nucleotides to promote the reassembly of outer doublet tubulin. Various nucleotides, each at a final concentration of 2 mM, were added to aliquots of 200000*g* supernatants in sonication buffer, 150 mM KCl, pH 6.7, and incubated at 37 °C to promote assembly. (---) GTP; (–) ATP; (-·-·) UTP and CTP; (···) GDP; (– – –) GMP.

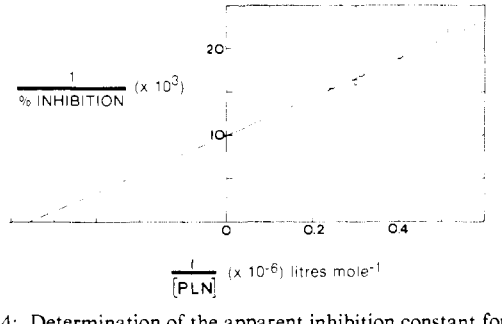


FIGURE 4: Determination of the apparent inhibition constant for PLN poisoning of outer doublet tubulin reassembly. Aliquots of 200000*g* supernatants of outer doublet tubulin in sonication buffer, 150 mM KCl, 2 mM GTP, pH 6.7, were incubated at 37 °C with varying concentrations of PLN. The degree of inhibition of the initial rates of turbidity development, relative to untreated controls, was plotted for each PLN concentration in the form of a double-reciprocal plot, from which we obtained an apparent K_i of 2.11×10^{-6} mol L^{-1}.

of tubulin molecules complexed with PLN relative to the total number of free tubulin molecules to give half-maximal inhibition of assembly (see Materials and Methods). Only 2–4% of the tubulin molecules require to be drug-bound to inhibit

reassembly by 50%. Consequently, PLN poisons the reassembly of outer doublet tubulin substoichiometrically, probably by a mechanism comparable with that observed for PLN poisoning of beef-brain microtubule reassembly (3.6%; R. L. Margolis, C. Rauch, and L. Wilson, unpublished experiments).

Discussion

Outer Doublet Tubulin Reassembly. (*1*) *Temperature and Solution Conditions.* The solution conditions optimal for reassembly of outer doublet tubulin in vitro are similar to those optimal for the reassembly of vertebrate brain microtubules (cf. Olmsted & Borisy, 1973; Gaskin et al., 1974; Haga et al., 1974). Both require high monovalent-ion concentration, the optimum for outer doublet tubulin lying between 150 and 200 mM K^+ (Figure 1a), as compared with an optimum of 140–150 mM for purified porcine-brain tubulin (Olmsted & Borisy, 1975). Similarly, magnesium ions are a common requirement for assembly in both systems, with outer doublet microtubule formation being promoted by added concentrations of up to approximately 10 mM (Figure 1b).

The optimal pH for outer doublet tubulin reassembly was 6.6–6.7 for extent and 6.6–6.8 for initial rate (Figure 1c). These values correspond very closely with the values obtained for the extent (pH 6.6–6.8) and rate (pH 6.6) of porcine brain tubulin assembly (Olmsted & Borisy, 1975). It is of interest that the pH-range optimal for reassembly is also optimal for colchicine binding activity of outer doublet tubulin (Wilson & Meza, 1973), and it has been suggested that the colchicine binding site and the bonding sites involved in microtubule formation may overlap spatially (Olmsted & Borisy, 1973). The observation that the colchicine binding site is masked in intact outer doublet microtubules (Wilson & Meza, 1973) supports this argument.

The optimum temperature for outer doublet tubulin reassembly was 37 °C and no assembly occurred below 25 °C, except at very high tubulin concentrations (Figure 2; Results). Even under these conditions of high protein concentration (4.55 mg/mL) microtubule formation was only detectable by electron microscopy at the lower temperatures. These observations are curious since the normal growth temperature for *S. purpuratus* is 11–15 °C, and the organism cannot survive at 37 °C. Precedents for this apparent paradox have been noted previously. For example, the equilibrium constants for colchicine binding to purified *S. purpuratus* outer doublet tubulin at 37 and 13 °C are 3.2×10^6 and 0.51×10^6 L mol^{-1}, respectively (T. A. Pfeffer and L. Wilson, unpublished experiments). Similarly, the equilibrium constants for tubulin in sea urchin egg supernatants are 2.9×10^5 and 1.2×10^5 L mol^{-1}, respectively. Consequently, it is unlikely that the high optimum temperature for outer doublet tubulin reassembly is an artifact and that the true optimum is closer to physiological temperatures. Since 37 °C is also the optimum temperature for vertebrate brain tubulin reassembly in vitro (Olmsted & Borisy, 1973; Gaskin et al., 1974), the data argue that the tubulin-dimer bonding sites involved in microtubule formation have been highly conserved.

The reason why extensive microtubule formation does not occur in 200000*g* supernatants of solubilized outer doublet microtubules at physiological temperatures is unclear. Perhaps a regulatory component may function to expedite microtubule assembly at the lower temperature during spermatogenesis. Since outer doublet microtubules, once formed, are stable, this component would no longer be synthesized and would therefore be absent from the 200000*g* supernatants. Alternatively, the regulatory component may be lost or inactivated during preparation of the outer doublet microtubules.

Another possibility is that microtubule formation during spermatogenesis might require high localized concentrations of tubulin. The experimental concentrations of tubulin we have employed may therefore have been too low for extensive assembly at these temperatures.

(*2*) *Nucleotide Requirements.* It was of interest that other nucleoside triphosphates stimulated microtubule formation to the same extent as GTP (Figure 3). This does not appear to result from the ability of outer doublet tubulin to bind nucleotides other than GTP, since [^{3}H]ATP was not incorporated into microtubules during ATP-promoted assembly and did not compete with [^{3}H]GTP for binding to the dimer even when present in a 20-fold molar excess. Rather, the efficacy of the other nucleotides seems to result from a nucleoside diphosphokinase activity. The inability of GDP or GMP to promote the reassembly of outer doublet tubulin (Figure 3A) suggests that there are insufficient nucleoside triphosphates endogenous to the 200000*g* supernatants to act as phosphate donors for the guanine nucleotides.

(*3*) *Mechanism of Assembly.* The abrupt monomer–polymer transition observed with increasing protein concentration (Figure 1d) is indicative of a condensation–polymerization reaction of the type described by Oosawa & Kasai (1962) for actin and is similar to that observed with brain tubulin (Olmsted & Borisy, 1973; Olmsted et al., 1974; Gaskin et al., 1974; Weisenberg & Deery, 1976). The critical protein concentration above which microtubule formation proceeds in vitro was noticeably higher for outer doublet tubulin than for vertebrate brain tubulin (0.55 mg/mL in contrast to 0.22 mg/mL). The reason for this is unclear; however, it may be related to the quantity or composition of the microtubule-associated proteins. For example, brain tubulin has a substantially higher critical protein concentration for assembly in the absence of MAPs than in their presence (Herzog & Weber, 1977; Sloboda & Rosenbaum, 1977). The critical protein concentration required for reassembly of outer doublet tubulin, which is 95% pure and lacks the high molecular weight MAPs of brain microtubules (Farrell & Wilson, 1978), is significantly lower than that required for the reassembly of MAP-free brain tubulin (cf. Herzog & Weber, 1977). This suggests that the outer doublet 200000*g* supernatants contain some accessory protein(s), either of low molecular weight or in trace amounts, concerned with expediting microtubule formation. Alternatively, MAPs are not required for the polymerization of outer doublet tubulin.

Drugs such as colchicine, vinblastine, and podophyllotoxin inhibit the assembly of brain microtubules by a potent substoichiometric poisoning mechanism (Olmsted & Borisy, 1973; Wilson et al., 1976). This is now known to occur by the addition of drug or drug–tubulin complex(es) to microtubule ends (Margolis & Wilson, 1977). The success of the substoichiometric mechanism is due to the fact that the assembly and disassembly of bovine-brain microtubules in vitro is not a true equilibrium but is really a steady-state summation of two different reactions which occur at opposite ends of the microtubule. Assembly occurs predominantly at one end, while disassembly occurs predominantly at the other end at steady state (Margolis & Wilson, 1978).

Podophyllotoxin inhibits outer doublet tubulin reassembly substoichiometrically in a manner similar to that described for brain microtubule assembly. Therefore, this indicates that the assembly and disassembly of outer doublet tubulin must occur by a similar opposite-end steady-state mechanism.

We conclude that the overall picture for the polymerization of outer doublet tubulin shows close homology with that for

brain tubulin polymerization and is especially remarkable in view of the diverse properties and functions of the microtubules from which the tubulins were derived.

Control of Microtubule Stability and Structure. The data presented above raise some fundamental questions about microtubule structure and function. For example, how is the stability of microtubules determined, and what determines whether microtubules will be singlets or doublets? The question of microtubule stability is complex because different degrees of stability exist in different populations of microtubules. In addition, microtubules may be defined as "labile" on the basis of their sensitivity to one treatment, yet may be defined as "stable" based upon their response to a second treatment. For example, the central pair microtubules of sea urchin sperm flagellar axonemes are singlet microtubules but are not depolymerized by cold or drug treatments which depolymerize labile cytoplasmic microtubules. However, they are solubilized by high concentrations of salt which do not solubilize stable outer doublet microtubules. Moreover, the extent to which microtubule stability and structure are interrelated is unclear. Doublet microtubules may be stable in part because of their structure. However, the doublet configuration is not essential for stability, at least to antimitotic drugs and cold treatment, since certain singlet microtubules also exhibit these properties (Burgess & Northcote, 1969; Roth, 1967). Doublets may be stable, not because of their structure per se, but because their structure allows the binding of accessory proteins which confer stability. It seems probable, therefore, that the stability of microtubules may involve several factors, and the different stability of individual classes of microtubules may result from variation in the relative prominence of these factors within each microtubule class.

The properties of the microtubules reassembled in vitro from outer doublet tubulin were very different from those of the outer doublet microtubules from which the tubulin was derived. For example, the reassembled microtubules were singlets and were depolymerized by exposure to low temperature and by podophyllotoxin (Farrell & Wilson, 1978). In addition, these microtubules appeared to be in the same steady-state assembly–disassembly condition as in vitro reassembled brain microtubules, even though sperm-tail outer doublets do not appear to be undergoing significant assembly–disassembly in vivo. It is probable, therefore, that neither the stability of outer doublets nor their doublet structure is determined solely by the intrinsic assembly capabilities of the constituent tubulin molecules.

We must consider what other factors might determine microtubule stability in vivo. One possibility is through the number or type of secondary proteins which can associate with the surface of the microtubules. Supportive of this mechanism is the observation that the high molecular weight MAPs of brain microtubules physically associate with brain microtubules and can stabilize them against low temperatures (4 °C) (Sloboda & Rosenbaum, 1977).

It is possible that conformational differences within the tubulin molecules exist because of subtle differences in the chemistry of the tubulins. These differences could cause variation in microtubule surface chemistry which then *determines* the binding, to the surface, of proteins which control microtubule stability.

Another major question posed by the results in this work is that of how the cell regulates formation of doublet microtubules. We consider three possibilities. First, there might be a specific template required for doublet formation. One obvious candidate is the basal body. However, neither isolated basal bodies nor even outer doublet fragments induce doublet formation in vitro from outer doublet tubulin (Binder & Rosenbaum, 1977; Farrell and Wilson, unpublished experiments). Therefore, either the basal body is not the correct candidate or it has been inactivated during isolation.

Conceivably, the blueprint for doublet-microtubule construction is intrinsic to the tubulin subunits. Chemically distinct tubulin molecules with specific surface properties may be required at precise spatial or temporal locations during outer doublet formation. This would require that the cell be able to distinguish between the various tubulin species and finely regulate their synthesis and/or assembly. Finally, the assembly of doublet microtubules may entail a precisely orchestrated interaction between the tubulin subunits and a host of accessory proteins.

In summary, we have found that tubulin solubilized from outer doublet microtubules reassembles in vitro to form microtubules with properties which are very different from the parent microtubules. The striking similarities between outer doublet and brain tubulin reassembly in vitro lead us to suggest that the intrinsic in vitro polymerization properties of these tubulins are very strongly conserved. It seems unlikely that the intrinsic in vitro polymerization properties of outer doublet tubulin determine either microtubule stability or doublet formation.

Since it appears that chemically distinct tubulins can exist within the same cell, it seems evident that the in vitro reassembly properties of tubulins do not reveal all of the differences in tubulin chemistry which are functionally important in assembled microtubules.

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Partial Purification and Characterization of a Human 3-Methyladenine–DNA Glycosylase†

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ABSTRACT: A DNA glycosylase was purified about 30-fold from cultured human lymphoblasts (CCRF-CEM line) and was found to cleave 3-methyladenine from DNA alkylated with methyl methanesulfonate. The enzyme did not promote the release of 1-methyladenine, 7-methyladenine, or 7-methylguanine from DNA nor did it act on denatured methylated DNA. It produced apurinic sites in DNA alkylated with *N*-methyl-*N*-nitrosourea and ethyl methanesulfonate as well as methyl methanesulfonate but not in untreated DNA or in DNA alkylated with nitrogen mustard or irradiated with ultraviolet light or X-rays. The glycosylase was free of detectable endonuclease activity in experiments with untreated DNA or DNA exposed to ultraviolet light; low levels of endonuclease activity, obtained when X-irradiated, alkylated, or depurinated DNA was the substrate, were attributed to contaminant apurinic endonuclease activity. This 3-methyladenine–DNA glycosylase has an estimated molecular weight of 34 000, is not dependent on divalent metal ions, and shows optimal activity at pH 7.5–8.5.

The initial enzymatic step in the excision repair of damaged DNA was long believed to be mediated by an endonuclease. However, a recently discovered class of enzymes, termed DNA glycosylases, can recognize an abnormal base in DNA and cleave it from the deoxyribose moiety, leaving an apurinic or apyrimidinic (AP)[1] site. The glycosylases identified thus far act either on uracil in DNA or on alkylated DNA. Enzymes with the former activity have been isolated from *Escherichia coli* (Lindahl, 1974), *Bacillus subtilis* (Friedberg et al., 1975), and human cells (Sekiguchi et al., 1976; Teebor et al., 1978), while those with the latter have been isolated only from *E. coli* (Kirtikar & Goldthwait, 1974; Riazuddin & Lindahl, 1978) and *Micrococcus luteus* (Laval, 1977). Thus, the initial event in excision repair may be the recognition and cleavage of an abnormal base by DNA glycosylase, with incision of the DNA strand by an AP-endonuclease constituting the second step.

During efforts to characterize an endonuclease activity isolated from human lymphoblasts that acted on either ultraviolet (UV)- or X-irradiated DNA, a new activity specific for methylated DNA became apparent (Brent, 1977). The enzyme was evidently not endonucleolytic and, since it produced AP sites in methylated DNA, it was inferred to be a glycosylase. This paper describes the preliminary purification and characterization of this enzyme from cultured human lymphoblasts; the results confirm that it does indeed catalyze the release of methylated bases from DNA.

Materials and Methods

(*1*) *Cell Growth.* Human lymphoblasts of the CEM-CCRF line were grown in magnetically stirred suspensions in Eagle's minimum essential medium that contained Spinner salts and was supplemented with 10% fetal calf serum. The cultures were maintained in logarithmic growth phase until the time of harvest.

(*2*) *Enzyme Purification.* (a) Extract Preparation. About 1×10^{10} cells were harvested by low-speed centrifugation (200*g* for 5 min) before being washed with phosphate-buffered saline, pH 7.2, at 4 °C. All subsequent steps were carried out at 0 or 4 °C.

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[1] Abbreviations used: AP, apurinic or apyrimidinic; UV, ultraviolet; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DEAE, diethylaminoethyl; MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; MNU, *N*-methyl-*N*-nitrosourea.

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