

Colchicine-binding Activity Distinguishes Sea Urchin Egg and Outer Doublet Tubulins

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ABSTRACT The colchicine-binding activity of tubulin has been utilized to distinguish the tubulins from two distinct microtubule systems of the same species, the sea urchin *Strongylocentrotus purpuratus*. We have analyzed the colchicine-binding affinities of highly purified tubulins from the unfertilized eggs and from the flagellar outer doublet microtubules by van't Hoff analysis, and have found significant differences in the free energy, enthalpy, and entropy changes characterizing the binding of colchicine to the two tubulins. The data indicate that significant chemical differences in the tubulins from the two functionally distinct microtubule systems exist, and that the differences are expressed in the native forms of the tubulins. Our findings are discussed in terms of the possibility that the colchicine-binding site may be an important regulatory site on the tubulin molecule.

Microtubules participate in various fundamental processes in eucaryotic cells. For example, mitotic chromosome movement, ciliary and flagellar beating, and the structural organization of the cytoplasm are based, at least in part, on organized arrays of microtubules (see references 1 and 2 for current reviews). Functionally different microtubule systems often display differential stability and organization. These observations have led to the hypothesis that chemically distinct tubulins may be employed to form functionally different microtubules (3).

Tubulin, the basic building block of all microtubules, is a heterodimeric protein composed of two nonidentical chains, alpha and beta, of molecular weight 50,000 (4, 5). Early protein sequencing studies indicated that the alpha- and beta-tubulins were highly conserved proteins (6). Recently, the nucleotide and corresponding amino acid sequences encoded by beta-tubulin mRNA and by 90% of alpha-tubulin mRNA from embryonic chick brain have been established (7), and the amino acid sequences of porcine brain alpha- and beta-tubulins have been deduced by traditional protein sequencing methods (8, 9). Similarly, cDNA clones for alpha-tubulin from rat brain (10, 11) and a single-copy DNA fragment containing the beta-tubulin gene from yeast (12) have been sequenced. These studies support the earlier suggestion that at least some of the alpha- and beta-tubulins are highly conserved proteins. For example, the amino acid sequences of chick brain alpha- and beta-tubulins and of one alpha- and

one beta-tubulin variant of porcine brain were found to differ in only three and four amino acid residues, respectively (7-9).

However, a lower amino acid sequence homology in beta-tubulins between the chicken and yeast (~70%) indicates that a moderate degree of sequence diversity exists among the tubulins (12). Further, colchicine inhibits the polymerization of yeast tubulin in vitro ~1,000 times less effectively than it inhibits the assembly of brain tubulin (13), suggesting that the colchicine binding sites of the tubulins from the two sources are significantly different (see Discussion).

Recent evidence indicates that both the alpha- and beta-tubulins from several tissues are heterogeneous (14-17). For example, the tubulins from calf and chick brain have been resolved into ~17 chemically distinct subspecies by high resolution isoelectric focusing (16, 17). A significant proportion of the heterogeneity in the chick brain tubulin must be due to post-translational modification of at least some of the tubulin gene products, because only four alpha- and four beta-tubulin genes exist in the chicken genome (see reference 17). It has also been shown that tubulins from different tissues of the same species can differ chemically (18-22). For example, Murphy and Wallis (18) have found that the beta-tubulin subunits from brain tissue and from erythrocytes of the chicken have different electrophoretic mobilities and isoelectric points, and two dimensional peptide mapping has indicated that the differences may be due to alterations in the

primary structure of the chains. Further in a preliminary report, Sullivan et al. (19) have found that two beta-tubulin genes in the chicken code for beta-tubulins with significantly different amino acid sequences.

The sea urchin represents a unique developmental system for study of the synthesis, modification, and assembly of tubulins destined for different microtubule organelles. Evidence for the existence of chemical heterogeneity in the tubulins has also been observed in sea urchins. Bibring et al. (20) found that the alpha-tubulin from mitotic spindles of the sea urchin *Strongylocentrotus purpuratus* could be resolved into two distinct species by polyacrylamide gel electrophoresis, whereas flagella alpha-tubulin from sperm of this organism ran as a single species. Further, Stephens (21) observed significant differences between the alpha- and beta-tubulin chains from egg cytoplasmic tubulin and from ciliary and flagella outer doublet tubulins of the sea urchin *Strongylocentrotus droebachiensis* by a thin layer peptide mapping procedure. Stephens' data suggests that there are primary structural differences among cytoplasmic, ciliary, and flagellar microtubules in the sea urchin.

Colchicine, a drug that inhibits microtubule polymerization by altering the kinetics of tubulin addition and loss at microtubule ends (23–30), binds to a unique site on the native tubulin dimer (reviewed in reference 2). In the present study, we have used the colchicine-binding site of tubulin as a probe for chemically distinct tubulins from the purple sea urchin, *Strongylocentrotus purpuratus*. Thermodynamic parameters calculated from van't Hoff plots of binding data indicate that the colchicine-binding sites of tubulins purified from the cytoplasm of unfertilized eggs and from the outer doublet microtubules of sperm flagella are substantially different. Our experiments support the results of previous investigators indicating that chemical differences exist between the tubulins from two different microtubule systems of the same species, and they demonstrate that the differences are reflected in the biochemical properties of the native tubulin dimers. We suggest that colchicine may be a useful probe for an important regulatory site on the tubulin molecule.

MATERIALS AND METHODS

Sea Urchin Tubulin Preparations: All tubulins were obtained from gametes of the purple sea urchin *Strongylocentrotus purpuratus*. Eggs and sperm were obtained from adult urchins after injection of 0.52 M KCl into the body cavities (31, 32). Experiments with egg tubulin were performed with crude 150,000 g supernatant fractions of unfertilized egg homogenates (5% tubulin) prepared as described by Pfeffer et al. (33), and with highly purified tubulin (>98% pure) prepared from unfertilized eggs by the method of Detrich and Wilson (32). Briefly, the crude supernatant fraction was prepared by homogenization of washed, de-jellied eggs at low speed with a motor-driven Teflon-glass tissue homogenizer in 20 mM sodium phosphate, 100 mM sodium glutamate, pH 6.75, followed by centrifugation for 1 h at 4°C, and was freshly prepared for use in colchicine-binding experiments. Purified and 100% assembly-competent egg tubulin was prepared by chromatography of egg supernatant fractions on DEAE-Sephacel followed by two cycles of temperature-dependent microtubule assembly and disassembly *in vitro*. Twice-cycled egg tubulin (C₂S, nomenclature is that of Borisov et al. [34]) was equilibrated with 20 mM sodium phosphate, 100 mM sodium glutamate, 0.5 mM MgCl₂, 0.02% sodium azide, pH 6.75 (L-GNPM buffer) containing 0.1 mM GTP by passage of the tubulin solution through a 1 × 5-cm column of Bio-Gel P-2 equilibrated with the same buffer. The tubulin solution was prepared for storage by dropwise freezing in liquid nitrogen (35). Frozen beads were stored at -80°C.

Experiments with sperm flagella tubulin were performed with protein solubilized from a purified outer-doublet microtubule suspension using a French pressure cell (36) (78% tubulin), and also with 100% assembly-competent tubulin purified by sonication followed by two cycles of microtubule assembly and disassembly *in vitro* by the method of Farrell and Wilson (31) (>95%

tubulin). Outer doublet tubulin was equilibrated in L-GNPM buffer plus 0.1 mM GTP and stored as frozen beads as described previously for purified egg tubulin. Protein was determined by the method of Lowry et al. (37) with BSA as the standard.

Colchicine-binding Assay Procedures: The binding of colchicine to tubulin was determined as described previously by Pfeffer et al. (33). Binding of colchicine (ring C, [³H]methoxy, New England Nuclear, Boston, MA final specific activity, 0.1 Ci/mmol) was carried out in phosphate-glutamate buffer (crude supernatant fractions of egg tubulin, and outer doublet tubulin solubilized in the French pressure cell) or in L-GNPM buffer plus 0.1 mM GTP (cycled forms of tubulin) for the desired times and at the desired temperatures (see Fig. legends). All binding samples contained 0.1 mM vinblastine sulfate to decrease the rate of decay of colchicine-binding activity. Bound colchicine was separated from free colchicine by gel filtration with 1 × 18-cm columns of Bio-Gel P-10 equilibrated in L-GNPM buffer plus 0.1 mM GTP at 4°C. All binding experiments were carried out with tubulin solutions at a concentration of ~0.1 mg/ml tubulin.

RESULTS

Affinity Constants for Colchicine Binding to Sea Urchin Egg and Sperm Tail Tubulins

Affinity constants were determined at four different temperatures between 13° and 37°C for the binding of colchicine to sperm tail outer doublet tubulin solubilized by the French pressure cell (36), by incubating aliquots of the solubilized tubulin solution with different concentrations of labeled colchicine. Values obtained for bound colchicine have not been corrected for decay of binding activity that occurred during incubation, which only corrects the maximum stoichiometry and does not affect the affinity constant values (33, 38). The data for each temperature is shown in the form of a Scatchard plot (39) in Fig. 1. The slope of each line, from which the binding constants (K_A) were obtained, was calculated by linear regression analysis.

Affinity constants were also determined at four temperatures between 8° and 30°C for highly purified egg tubulin (33). The binding data at 30°C only, not corrected for decay, is shown in Fig. 1 for comparison with the data obtained with sperm tail tubulin. The affinities of the two tubulins for colchicine were very different. The affinity constant for the sperm tail tubulin at 30°C was 17.0×10^5 liter/mol, while the affinity constant for the cycled egg tubulin was 3.48×10^5 liter/mol.

The affinity constants for all temperatures studied, both with the purified egg and sperm tail tubulins, are shown in the form of van't Hoff plots (Fig. 2) from which the thermodynamic parameters were calculated (40). Also shown in Fig. 2 for direct comparison are data published previously for the affinity of colchicine binding to egg tubulin contained in crude 150,000 g supernatant extracts of egg homogenates (33). The association constants obtained for the highly purified egg tubulin were reasonably similar to those for tubulin in the egg supernatants, indicating that the nontubulin components at the concentrations present in the egg supernatant did not appreciably affect the colchicine-binding affinity of the tubulin. The association constants of the egg tubulin for colchicine at all temperatures studied were 3–5-fold smaller than the association constants determined for flagellar outer doublet tubulin under identical conditions.

Also shown in Fig. 2 are affinity constants determined at 30° and 37°C with sperm tail outer doublet tubulin purified by sonication followed by two cycles of microtubule assembly and disassembly *in vitro*. The data indicate that the outer doublet tubulin purified by sonication and microtubule assembly and disassembly, which contains no detectable con-

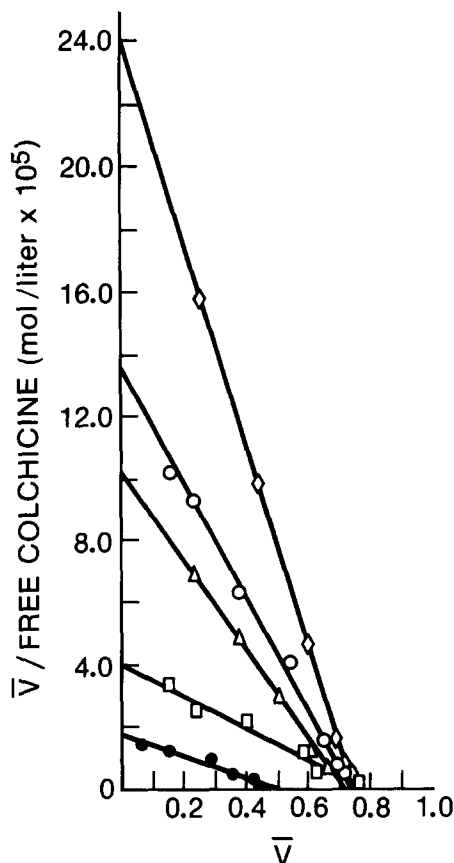


FIGURE 1 Scatchard analyses of the binding of colchicine to sea urchin outer doublet and egg tubulins. Aliquots of outer doublet and egg tubulin solutions (1.0 ml, 0.1 mg/ml tubulin) were incubated with six different concentrations of radioactive colchicine as described in Materials and Methods (2 to 20 μ M colchicine for outer doublet tubulin, and 0.5 to 50 μ M colchicine for egg tubulin). Incubation of outer doublet tubulin was carried out for 8 h at 37°C (\diamond), 20 h at 30°C (O), 48 h at 20°C (Δ), and 56 h at 13°C (\square). Egg tubulin was incubated for 2.5 h at 30°C (\bullet). Equilibrium constants were obtained from the slopes of the lines, which were calculated by linear regression analysis. \bar{V} , moles of colchicine bound per mole of tubulin.

taminating proteins, binds colchicine in an identical fashion with that solubilized by the French pressure cell. These data indicate that the nontubulin proteins present in the outer doublet tubulin prepared by solubilization with the French pressure cell do not affect the colchicine-binding affinity of the tubulin.

The thermodynamic parameters governing the binding of colchicine to tubulin were derived from the van't Hoff plots shown in Fig. 2, and are summarized in Table I. The values for ΔH° and ΔS° of the highly purified egg tubulin and the tubulin in crude egg supernatants were very similar to each other, and both were very different than the values obtained with flagellar outer doublet tubulin. These data are consistent with the conclusion that chemical differences exist between the tubulins from two different microtubule systems of this species.

DISCUSSION

We have determined the thermodynamic parameters for the binding of colchicine to highly purified tubulins from two distinct microtubule systems from the same species, the sea

urchin *Strongylocentrotus purpuratus*. We found that the affinity of purified egg tubulin for colchicine at all temperatures between 8° and 30°C was ~3–5-fold weaker than the affinity of sperm flagellar outer doublet tubulin for the drug. Calculation of thermodynamic parameters from van't Hoff plots of the binding data revealed significant differences in the free energy, enthalpy, and entropy changes characterizing the binding reaction between the two tubulins.

Several lines of evidence support the conclusion that the differences in colchicine-binding activity between the egg and outer doublet tubulins actually reflect differences in the colchicine binding sites of the tubulins. First, the binding data were obtained with highly purified preparations of proteins.

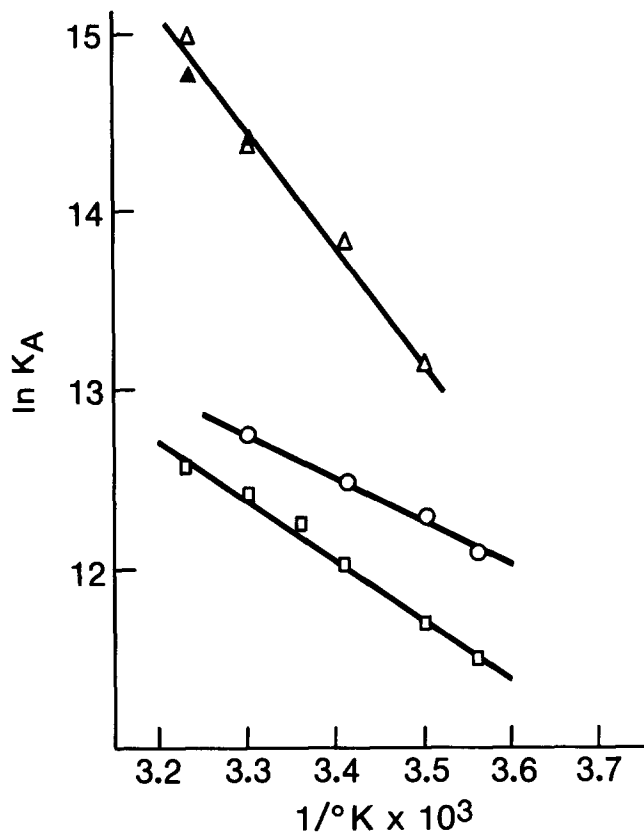


FIGURE 2 Van't Hoff analysis. Binding constants for purified (cycled) egg tubulin (O); tubulin contained in 150,000 g supernatant fractions of egg homogenates (\square); and outer doublet tubulin solubilized with the French pressure cell (Δ) were plotted in the form of van't Hoff plots, from which the thermodynamic parameters of the colchicine binding reaction were calculated. Equilibrium constants for the purified egg tubulin were obtained by incubation for 28 h at 8°C, 24 h at 13°C, 8 h at 20°C, and 2.5 h at 30°C (see Materials and Methods). Binding constants at 30° and 37°C for outer doublet tubulin purified by sonication and two cycles of in vitro assembly and disassembly are also shown (\blacktriangle).

TABLE I
Thermodynamic Parameters for Colchicine Binding to Sea Urchin Tubulins

Tubulin preparation	ΔH_0 kcal/mol	ΔS_0 eu	$\Delta G_{13^\circ\text{C}}$ kcal/mol
Egg tubulin in supernatant fraction	6.6	46.5	-6.7
Egg tubulin-purified	4.6	40.4	-6.9
Outer doublet tubulin	13.6	73.4	-7.4

Second, two different methods for preparing the outer doublet tubulin were employed (French pressure cell and sonication/assembly and disassembly). Identical binding results were obtained with both preparations, suggesting that the differences observed between the egg and outer doublet tubulins did not result from differences in preparative variables. Thus, we conclude that the binding differences reveal the existence of chemical differences between the egg tubulin and the outer doublet tubulin. We have attempted to confirm the differences in egg and outer doublet tubulins by multicomponent Scatchard analysis of mixtures of the two tubulin preparations. However, due to differences in the kinetics of colchicine binding and in the rate of decay of the colchicine-binding site of the two tubulins, these studies were unsuccessful (data not shown). The inability to find appropriate conditions for simultaneous determination of the affinities of the two tubulins in mixtures of the tubulins underscores the marked differences in the colchicine-binding activity of the two proteins.

It is not known whether the variation in the tubulin composition from the two sources, and the differences in the colchicine-binding sites of the two tubulins, are due to expression of different tubulin genes in the two cell types, or due to post-translational modification of one or a few tubulin gene products. Large numbers of tubulin genes have been found in the sea urchin genome (41), but it is not yet known what proportion of the genes is expressed.

Microtubules from different species or from different microtubule systems within a single species show differential sensitivity to colchicine (1, 2). Inhibition of mitosis in plant cells by colchicine usually requires very high drug concentrations as compared with the colchicine concentrations required to inhibit mitosis in mammalian cells. For example, mitosis in *Hemanthus katharinae* endosperm cells is unaffected by a colchicine concentration below ~2.5 mM (42), whereas complete inhibition of mitosis in human cells, strain K.B., occurs at 0.05 μ M colchicine (43). The affinity of tubulin for colchicine in extracts of the yeast *Saccharomyces cerevisiae* has been estimated to be 4×10^2 liters/mol at 37°C (44), and as previously noted, inhibition of yeast tubulin assembly by 50% requires 2 mM colchicine (13). Similarly, no colchicine-binding activity could be detected in extracts of *Tetrahymena thermophila* (at 0.5 μ M colchicine; [45]), and high colchicine concentrations are required to inhibit cilia regeneration in this species; a process that depends in part upon assembly of microtubules (46). However, tubulin from mammalian brain binds colchicine at 37°C with an affinity of approximately 2×10^6 liters/mol, and half-maximal inhibition of tubulin addition to the ends of bovine brain microtubules at steady state in vitro occurs at 0.13 μ M colchicine (24). These data indicate that the affinities of tubulins from lower eucaryotic organisms and from higher plants for colchicine are generally much weaker than the affinities of tubulins from higher eucaryotic organisms in the animal kingdom for the drug. This is the first report demonstrating that significant differences exist in the affinities of tubulins from the same species for colchicine.

Differential sensitivity of microtubules in cells to the action of colchicine could occur in two general ways; (a) different microtubule systems could be composed of tubulins with different affinities for colchicine and (b) different microtubule systems could have different assembly/disassembly or stability characteristics. In the mitotic spindles of Chinese hamster ovary cells, the interpolar microtubules are significantly more

sensitive to the action of colcemid (a structural analog of colchicine) than are the kinetochore microtubules (47). Interpolar microtubules have also been found to be more sensitive to cold temperatures than kinetochore microtubules (48). It is conceivable that the differential sensitivity of kinetochore and interpolar microtubules to colcemid is due, in part, to differences in the tubulin composition of the two classes of spindle microtubules, with the kinetochore microtubules having a somewhat lower affinity for the drug than the interpolar spindle fibers. If correct, it might mean that differences in the assembly and disassembly dynamics of specific microtubule classes may be associated with chemical changes in the vicinity of the colchicine-binding site.

In support of the hypothesis that the colchicine site of tubulin may be an important regulatory site for microtubule assembly and disassembly, Farrell and Wilson (30) have found that the kinetics of tubulin addition and loss at the two ends of bovine brain microtubules in vitro are differentially sensitive to the action of colchicine, with the kinetics of tubulin addition and loss at the steady-state assembly ends being far more sensitive to inhibition by the drug than the kinetics at the disassembly ends. Thus, colchicine substantially augments the kinetic differences between the two ends of the polymers in this microtubule system. A molecule with affinity for the colchicine site of tubulin which has the ability to differentially affect the kinetics of tubulin addition and loss at the two microtubule ends in a colchicine-like manner would be a versatile regulatory molecule for modulation of microtubule assembly and the polarity of microtubule growth in cells. The existence of different microtubule systems composed of tubulins with different affinities for such a colchicine-like regulatory model would provide the cell with the ability to differentially regulate the assembly and growth polarity of microtubules destined for different cell functions.

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