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INVOLVEMENT OF TRYPTOPHAN RESIDUES IN COLCHICINE BINDING AND THE ASSEMBLY OF TUBULIN

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Chemical modification of tubulin with 2-hydroxy-5-nitrobenzyl bromide, a reagent selective for tryptophan, inhibits tubulin's colchicine binding and in vitro assembly activities. Loss of colchicine binding shows a linear relationship with the modification of tryptophan residues, and is complete when not more than five residues are modified. GTP affords partial protection against this loss of colchicine binding. The in vitro assembly of tubulin is somewhat less sensitive, since microtubules are formed from tubulin dimers possessing 3-4 but not five modified residues. Furthermore, two of the eight tryptophans per dimer are reactive when tubulin is assembled into microtubules.

Tubulin's ability to self-assemble and the inhibition of this process by the alkaloid drug colchicine are two of the most striking biochemical features of the tubulin-microtubule system. Although there have been numerous studies on the assembly mechanism and the binding of various ligands (divalent cation and nucleotides) and drugs (for reviews 1-4) to tubulin, there is still a paucity of information on the role of specific tubulin domains and amino acid residues involved in these assembly and binding activities. Sulfhydryl groups (5-7), histidine residues (8) and basic amino acids (9-11) have been implicated in the assembly process, while only sulfhydryl groups have been suggested to function in colchicine binding (6).

The slowness and nearly irreversible nature of colchicine binding have been explained by a ligand induced conformational change in tubulin (12,13), which follows colchicine's initial attachment to a tropolone site and brings the trimethoxyphenyl binding site into proper position to establish a very stable binary complex (14). The aromatic structure of these colchicine domains suggests that their interaction with tubulin may be mediated by aromatic amino acid residues.

Abbreviations: HNBB: 2-hydroxy-5-nitrobenzyl bromide

PC-Tubulin: Tubulin purified by phosphocellulose chromatography

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To further understand the structural aspects underlying tubulin's assembly and colchicine binding activities, the selective modification of tryptophan residues with the Koshland reagent, 2-hydroxy-5-nitrobenzyl bromide (15,16) has been performed. The characteristics of this chemically modified tubulin are described in this report.

MATERIALS and METHODS

Lamb brain tubulin was purified by temperature dependent cycles of assembly-disassembly following the procedure of Shelanski et al. (17). Microtubule pellets were stored at -70°C, resuspended in 0.1M MES (pH 6.8), 0.5 mM EGTA and 1.5 mM $MgCl_2$ prior to their use and a third cycle of assembly-disassembly was performed. Tubulin was freed of remaining MAPs (5-10%) by a phosphocellulose chromatography (PC-tubulin) as described by Weingarten et al. (18). The remaining unbound nucleotide and the exchangeable guanine nucleotide were removed by charcoal extraction in the presence of 1.5 mM EDTA as described previously (19). Charcoal extracted tubulin contained 1.0 ± 0.1 mole of guanine nucleotide per mole of tubulin dimer.

The treatment of tubulin with 2-hydroxy-5-nitrobenzyl-bromide (HNBB) dissolved in DMSO was performed according to the following steps: charcoal extracted PC-tubulin in 0.1 M MES buffer (pH 6.8), 1.5 mM Mg^{+2} (Modification buffer), was incubated at either 19°C or 25°C with the modifying reagent at the concentrations indicated and a final [DMSO] <9%. Aliquots (25-50 ul) of the incubation medium were removed at different time intervals, diluted 7 to 10 fold and assayed for colchicine binding activity or microtubule assembly. Control samples were incubated under identical conditions but in the absence of the Koshland reagent as indicated above. Microtubules collected by centrifugation were resuspended in warm Modification buffer with 10% DMSO, 1 mM EGTA, 1 mM GTP and incubated at 25°C with 26 mM HNBB and collected by warm (25°C) centrifugation. Determination of the number of modified tryptophan residues in tubulin and microtubules was performed as described by Koshland et al. (15) using a molar extinction coefficient of 1.8×10^4. Total tyrptophan was assessed on denatured samples boiled in 8M urea.

The colchicine binding assay was performed by measuring the radioactivity of [^{3}H]-colchicine bound to tubulin and retained on Whatman DE-81 filter discs as described by Weisenberg et al. (20)

Tubulin aliquots were diluted with Modification buffer and adjusted to 0.5 mM EGTA, 1 mM GTP and phosphocellulose derived MAPs at a final concentration of 0.55 mg/ml. Tubulin assembly was assessed by the sedimentation assay (21) or turbidity increase at 550 nm, where the modifying reagent had insignificant absorbance. Electron microscopic observations of microtubules assembled from modified or native tubulin were performed on glutaraldehyde fixed, uranyl acetate stained grids as described elsewhere (19). Protein concentrations were determined by the procedure of Lowry et al. (22) or by $A_{280} = 1.15$ per mg/ml (23).

The number of cysteine residues in lamb brain tubulin was determined by p-chloromercuribenzoate titration as described by Means and Feeney (24).

Results:

Incubation of PC-tubulin with HNBB resulted in a marked decrease in tubulin's colchicine binding ability (Figure 1A). HNBB (26 mM) produced a time dependent loss of colchicine binding activity that displayed biphasic inactivation kinetics. A fast initial decay ($k'_{fast} = 0.153$ min $^{-1}$) in colchicine binding was followed by a slower rate of inactivation ($k'_{slow} = 0.033$ min $^{-1}$), when compared to control

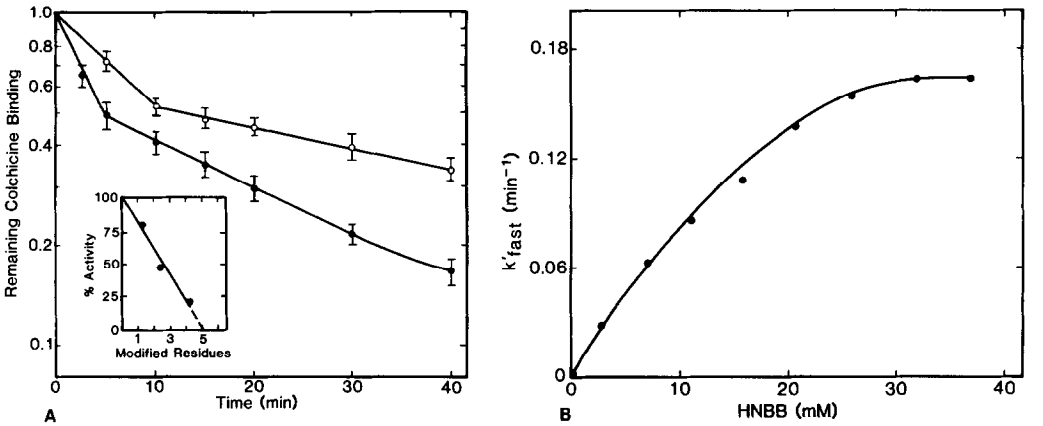

Fig. 1A Time dependent inactivation of tubulin's colchicine binding by HNBB treatment in the presence or the absence of GTP.
Tubulin samples purified by phosphocellulose chromatography (11 mg/ml) were incubated at 25°C with 26 mM HNBB. Aliquots of 25 ul were withdrawn at time intervals indicated, diluted 1:10 with Modification buffer and fractions of 50 ul were assayed for colchicine binding (0-0) relative to an untreated control sample. Another sample at the same tubulin concentration was preincubated at 4°C for 10 min. with 2 mM GTP, 2.5 mM Mg^{2+}, treated as above and assayed for colchicine binding (0-0). Inset shows the relation of the loss in colchicine binding activity to the number of modified tryptophan residues per tubulin dimer.

Fig. 1B Dependence of the apparent first order rate constants (k'_{fast}) for the tubulin colchicine binding inactivation on the HNBB concentration.
Different tubulin samples (11 mg/ml) were incubated at 25°C in Modification buffer with HNBB at the concentrations indicated in the figure. The apparent first order rate constants were calculated from the semilogarithmic plots of the kinetic data using a linear regression program. Experimental procedures are as described in Methods.

samples that compensated for tubulin's well-known spontaneous decay in colchicine binding activity (25). Interestingly, the addition of 2 mM GTP to the reaction mixture reduced the initial inactivation rate to $k'_{fast} = 0.068\ min^{-1}$ (Figure 1). The possibility that GTP reacted directly with HNBB and lowered its effective concentration was ruled out by chromatographic analysis of GTP and HNBB mixtures. This partial protection by GTP may be a consequence of nucleotide binding to tubulin's exchangeable GTP site and thereby stabilizing the colchicine binding site as suggested previously (26).

The inactivation kinetics of colchicine binding were examined at increasing concentrations of HNBB (0-37 mM). The relationship between the fast inactivation rate constant (k'_{fast}) and HNBB concentrations was not linear, but showed saturation kinetics (Figure 1B). This concentration dependence curve suggests the formation of a tubulin HNBB intermediate prior to the irreversible coupling of the modifying reagent to the tubulin molecule (27).

TABLE 1

Determination of Modified Tryptophan Residues in Tubulin and Microtubules

	Incubation	% Colchicine Binding		% Assembly	# Modified Tryptophans*	
		+ GTP†	- GTP		+ GTP	- GTP
A Tubulin	30 min	40%	22%	-----	3.0	4.2
					Tubulin (Supernt.)	MTs (pellet)
B Tubulin	30 min.	-------		29%	4.5	3.5
	90 min.	-------		5%	4.6	3.8
C Microtubules‡	30 min.	-------		100%	---	2.1

Tubulin or microtubules were incubated with 26 mM HNBB at 25°C for the time indicated. Colchicine binding, tubulin assembly and the number of modified Trp- residues were determined as described in Methods.
*Moles of Trp- per mole of tubulin dimer (110,000 Mr)
†GTP (2 mM) was present during the incubation with HNBB
‡Stabilized microtubules were incubated with HNBB and resedimented.

The total number of tryptophan residues in denatured lamb brain tubulin was determined to be 8.0 $\pm$ 0.1 per 110,000 Mr dimer, and is identical to the eight tryptophans found in chick and pig brain tubulins (28,29). The number of tryptophan residues modified under the reaction conditions of Figures 1A and B was determined (Inset Figure 1). The loss in colchicine binding activity shows a linear relationship with the number of modified tryptophan residues and extrapolated to a maximum number of five modified tryptophans coinciding with the complete loss of colchicine binding activity. The partial protective effect of GTP on colchicine binding during HNBB treatment was also reflected in a reduced number of modified tryptophan residues, 3.0 mole/mole dimer, in the presence of nucleotide compared to 4.2 modified residues in its absence (Table 1A).

The assembly properties of HNBB modified tubulin were also examined. Interestingly, HNBB concentrations below 12 mM had no detectable effect on microtubule formation; however, higher concentrations of HNBB led to a sigmoidal loss of assembly activity (Figure 2). Neither GTP (2 mM) nor the nucleotide

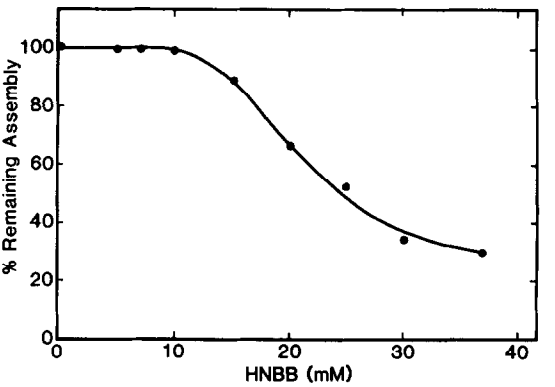

Fig. 2 Relation between extent of tubulin assembly and HNBB concentration traction.
Individual PC-tubulin samples (11 mg/ml) were incubated at 25°C for 30 min. in Modification buffer with increasing concentrations of HNBB. Aliquots of 50 ul were obtained from each sample, diluted 1:7 and adjusted to 1 mM GTP, 0.5 mM EGTA, 0.55 mg/ml MAP and the extent of assembly determined by the sedimentation assay.

analog, GMPPNP, afforded tubulin protection against assembly inactivation by HNBB. Following an incubation with 30 mM HNBB assembly activity was only 34% of the untreated control sample when assessed by the sedimentation assay (21) and 20-25% by the turbidity assay. The latter assay is known to be more sensitive to microtubule length (30), suggesting that shorter microtubules may be formed by the modified tubulin preparation.

Microtubules formed from the modified tubulin preparation were visually compared to those assembled from unmodified tubulin. Electron micrographs show that both tubulin preparations form microtubules of similar structure; however, there is a shift in the size distribution toward shorter microtubules when modified tubulin is used (Figure 3).

The microtubules assembled from preparations of HNBB-modified tubulin were directly examined for the number of modified tryptophan residues (Table IB). When the assembled microtubules (29%) and the unassembled tubulin fraction (71%) were compared, tubulin in microtubules averaged one less mole of modified tryptophan than its unassembled counterpart, 3.5 and 4.5 moles respectively. The extent of tubulin modification by this HNBB concentration (26 mM) did not change significantly with increased incubation time, although the efficiency of assembly decreased. The insensitivity of tubulin assembly to low concentrations (0-10 mM) of HNBB (Figure 3) which modify 1-2 typtophan residues per dimer (data not shown)

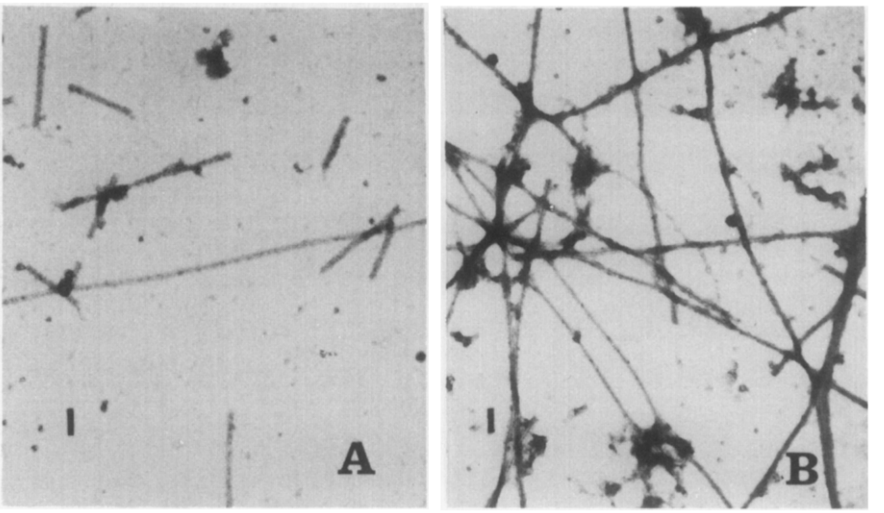

Fig. 3 Electron microscopy of microtubules obtained from HNBB reacted and unreacted tubulin samples.
A. Tubulin purified by phosphocellulose chromatography (11 mg/ml) was treated with 26 mM HNBB at 25°C for 30 min. and processed for electron microscopy as indicated in Methods.
B. Tubulin sample incubated in the absence of HNBB. The bars represent 1000A°.

was surprising; therefore, we determined the number of exposed tryptophan residues on intact microtubules (Table I-C). Microtubules were collected by centrifugation from an assembly reaction and stabilized by resuspension in warm buffer containing 10% DMSO; following their incubation with HNBB at 25°C the microtubules were collected by centrifugation (21) and separated from any free HNBB by molecular sieve chromatography. Approximately two moles of tryptophan per tubulin dimer in microtubules are readily exposed to the environment, and their modification does not promote the breakdown of microtubules.

Discussion:

These studies demonstrate that a highly reactive colored reagent with selectivity for tryptophan residues, 2-hydroxy-5-nitrobenzyl bromide leads to a loss of both colchicine binding and self-assembly activities in tubulin. Colchicine binding activity decreases linearly with increasing modification of tryptophan residues resulting in a complete loss of activity when five of the eight tryptophan moieties are modified. The biphasic inacativation kinetics (Figure 1) can be explained by a rapid reaction of tubulin with HNBB leading to

a partially active tubulin which is followed by a slower reaction producing the inactive molecule (27). Alternatively, the kinetics could be explained by two different molecular forms of tubulin that are modified at different rates (two parallel first order reactions) to produce inactive tubulin.

The protective effect of GTP may be related to a stabilization of the colchicine binding site as suggested by McClure and Paulson (26). However, GTP binding to the exchangeable site does not produce any significant change in tubulin structure as analyzed by circular dichroism (19).

Tubulin assembly is somewhat less sensitive to modification of tryptophan residues, since two moles per dimer can be modified without loss of assembly activity; furthermore, two moles are readily exposed to the solvent when assembled and their modification does not appear to effect microtubule structure. It is not known whether these are the same two tryptophan residues that react in the dimer and the polymer. The modification of 3-4 tryptophans still allows tubulin to assemble; however, modification of 4-5 residues essentially inactivates tubulin (Table I).

In addition to tryptophan, cysteine can be modified by HNBB but its reactivity is very much slower than tryptophan and occurs at alkaline pH (16). The increased sensitivity of colchicine binding to low levels of modifying reagent when compared to assembly activity is opposite what one might expect if -SH groups were being modified, since tubulin assembly has been shown to be inhibited by the modification of only one or two cysteines per dimer (5-7) while inactivation of colchicine binding requires modification of six or more sulfhydryl groups (6). A direct determination of the number of cysteine residues in tubulin before and after HNBB (26 mM) modification was carried out by p-chloromercuribenzoate titration (24). The number of cysteines was calculated to be 20 $\pm$ 0.2 mole per mole tubulin dimer and showed no significant decrease ($\leq$2%) following HNBB treatment.

In tubulins of known amino acid sequence (28,29) the positions of tryptophan are constant and their neighboring sequences are highly conserved suggesting that these tryptophan domains within the tubulin molecule are important for structure

and function relationships. Future studies may identify which specific tryptophan residues are modified under conditions where there is no loss or complete inactivation of colchicine binding and tubulin assembly.

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