Localization of Critical Histidyl Residues Required for Vinblastine-induced Tubulin Polymerization and for Microtubule Assembly*  

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Vinblastine-induced tubulin polymerization is electrostatically regulated and shows pH dependence with a pI ~7.0 suggesting the involvement of histidyl residues. Modification of histidyl residues of tubulin with diethylpyrocarbonate (DEPC) at a mole ratio of 0.74 (DEPC/total His residues) for 3 min at 25 °C completely inhibited vinblastine-induced polymerization with little effect on microtubule assembly. Under these conditions DEPC reacts only with histidyl residues. For complete inhibition two histidyl residues have to be modified. Demodification of the carboxyethyl histidyl derivatives by hydroxylamine led to nearly complete recovery of polymerization competence. Labeling with [14C]DEPC localized both of these histidyl residues on β-tubulin at β227 and β264. Similarly, tubulin modification with DEPC for longer times (8 min) resulted in complete inhibition of microtubule assembly, at which time ~4 histidyl residues had been modified. This inhibition by DEPC was also reversed by hydroxylamine. The third histidyl residue was found on α-tubulin at α88. Thus, two charged histidyl residues are obligatorily involved in vinblastine-induced polymerization, whereas a different histidyl residue on a different tubulin monomer is involved in microtubule assembly. 

Tubulin can polymerize to rings, microtubules, sheets, bundles, and spirals depending on incubation conditions, drugs, etc. We have recently shown that vinblastine-induced tubulin polymerization into spiral structures is a two-step process with a critical concentration and a latent period for polymerization that is shortened by addition of polymer seeds. The process is similar to microtubule assembly in a number of respects, but unlike the latter, it is extremely sensitive to the presence of GTP and other oligoanions (1). This anion sensitivity resides primarily in the extreme C terminus of the β monomer since removal of that portion of the monomer by subtilisin abolishes the anion inhibition (2). We proposed that the β-C terminus had to interact with a positively charged domain of tubulin to form vinblastine-induced spirals, a process that is interrupted with a critical concentration and a latent period for polymerization over the pH range of 6.5–7.3 with a pI near 7.0. This suggested that histidyl residue(s) might be part of the cationic domain required for spiral formation and the oligoanion promoted inhibition. Accordingly, we have studied the effects of modifications of critical His residues of tubulin by diethylpyrocarbonate (DEPC) on vinblastine-induced spiral formation, and we have compared these to changes produced in microtubule assembly. The rat brain tubulin dimer contains 24 histidyl residues. Because the rate of histidyl reaction toward DEPC varies widely between and within proteins (3–7), such an approach would be useful only if the critical residues were also the most reactive ones. This proved to be the case, and two highly reactive histidyl residues were found to be required for spiral formation, whereas 1–2 additional histidyl residues must be modified to block microtubule assembly. 

MATERIALS AND METHODS 

GTP, vinblastine sulfate, DEPC, [14C]DEPC (1.6 mCi/mmol), hydroxylamine hydrochloride, N-acetyl-Met-Leu-Ph, and N-acetyl-t-tyrosine amide were from Sigma. Acetyl-Ser-Gly-OH was from Research Plus Inc., and N-acetyl-Gly-Gly-His-Gly was from Cyclo Chemical Corp. Excision grade trypsin was from Calbiochem, and HPLC grade water and acetonitrile were from Fisher. Rat brain tubulin (>99% pure) was prepared as described previously (8). 

Vinblastine-induced polymerization was carried out in Mes assembly buffer (0.1 mM Mes, pH 6.9; 1 mM MgCl2; and 1 mM EGTA) containing 10% Me2SO at 25 °C in a thermostated Cary 219 double-beam spectrophotometer using 3-mm path length cuvettes at 350 nm. For microtubule assembly 1 mM GTP was added to the Mes assembly buffer. 

For polymerization competence measurements, 0.5 mM DEPC in Mes assembly buffer at 25 °C. The time course of the reaction was monitored by an increase of absorbance at 240 nm due to the formation of the N-carboxyhistidyl derivative. At different times, the reaction was quenched with 3 mM imidazole, and the product was tested for polymerization competence by turbidity measurements at 350 nm at 25 °C in the presence of 45 μM vinblastine. Microtubule assembly competence was measured in Mes assembly buffer containing 10% Me2SO with 1 mM GTP in addition. 

Demodification of the derivatized tubulin by hydroxylamine was carried out as follows: tubulin (5.3 mg/ml) was reacted with 1 mM DEPC for 4 min at 25 °C, and the reaction was quenched with 3 mM imidazole. The mixture was incubated with 92 mM hydroxylamine (adjusted to pH 6.9) for 30 min at 25 °C. The reaction was monitored by a decrease in the absorption at 240 nm. Since imidazole and hydroxylamine interfered with vinblastine-induced polymerization, these compounds were removed by centrifugation (at 1,900 × g for 2 min) in a SPIN-X filter unit packed with Sephadex G-25 preequilibrated for 24 h in Mes assembly buffer. For microtubule assembly this removal step was unnecessary because these concentrations of imidazole or hydroxylamine had no effect on the polymerization of native tubulin. Tubulin concentrations were measured by the biocinchoninic acid methods using bovine serum albumin as standard. The demodified tubulin was tested for polymerization competence as above. 

To ascertain the specificity of the DEPC reaction under our conditions, 0.5 mM His-, Tyr, Ser-, and Met-containing peptides listed above were reacted for 8 min at 25 °C with 0.5 mM DEPC and analyzed on a C-18 reverse phase column (10 × 250 mm) with a 1–94% acetonitrile gradient containing 0.05% trifluoroacetic acid. To identify other reaction products, the same reactions were carried out with 10 mM DEPC, i.e., 20 times our “normal” concentration. 

In order to localize the reactive His residues the [14C]-modified tubulin

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Critical Histidyl Residues for Tubulin Polymerization

RESULTS

The earlier pH titration data for vinblastine-induced polymerization yielded a pK of ~7.0, suggesting involvement of His residues and the possibility that modification of such residues might shed light on their role in spiral formation (1). Accordingly, we reacted tubulin with DEPC, a reasonably specific reagent forming mono- or disubstituted carbethoxyhistidyl derivatives (9). Tubulin (2.8 mg/ml) was reacted with 0.5 mM DEPC for various time intervals at 25 °C, and the reaction was quenched with 3 mM imidazole. The tubulin so modified was polymerized with 45 μM vinblastine at 25 °C as shown in Fig. 1. The numbers on the curves refer to the number of minutes of DEPC treatment. With increasing time of reaction there is a marked decrease in the maximal rate of polymerization, a marked increase in the latent period, and a smaller decrease in the extent of polymerization. Thus, for the 2-min modification, there is a 45-fold decrease in the maximal rate of polymerization, and the extent of polymerization is decreased by 1/3 near the plateau (data not shown). With 3 min of reaction time no polymerization occurred, and the extent of polymerization is decreased by 1/3 near the plateau (data not shown). With 5 min of reaction time no polymerization could be induced under these conditions. These results suggest that histidine residues are involved in the vinblastine-induced polymerization of tubulin.

Formation of Histidine Derivatives and Reversal of the Reaction with Hydroxylamine—DEPC is known to form an N-carbethoxyhistidyl derivative, a reaction that can be followed by measuring the increase in the absorbance at 240 nm (the increase in OD240 (9)). As shown in Fig. 2A (arrow), after addition of 92 mM hydroxylamine, the OD240 decreased by ~90% provided the DEPC reaction had been allowed for only 4 min. With prolonged reaction times, reversal was less complete.

It remained to be determined whether or not the removal of the carbethoxy moiety also restored the capacity of the demodified tubulin to polymerize under the influence of vinblastine. The results are shown in Fig. 2B. Tubulin was reacted with DEPC for 4 min which completely blocks polymerization (curve 1). After 4 min 92 mM hydroxylamine was added, and the OD240 was monitored until a new plateau had been attained (~30 min). The hydroxylamine was removed by centrifugation through a spin column. The extent of polymerization was restored to 2/3 that of unmodified tubulin by 9 min (compare curve 2 with curve 3), although the initial rate was slower, and 78% when the plateau of polymerization had been achieved. It should be noted that the exposure to hydroxylamine and its subsequent removal by spin column had no significant effect on the extent of polymerization (curve 4). Negatively stained preparations of untreated and demodified tubulin yielded similar structures consisting of spirals, rings, and relatively loosely organized aggregates; the demodified preparations contained relatively more of the latter. Thus, polymerization competence closely paralleled the degree of His interaction with DEPC, providing further evidence that His residues of tubulin are likely to be involved in vinblastine-induced tubulin polymerization.

Although DEPC is relatively specific for His residues, it has...
been shown that under appropriate conditions it will also react with Tyr, Ser, and Met (9). In addition, the disubstituted His derivative can be formed when DEPC is present in excess. It is apparent from Fig. 3 that under the reaction conditions used for tubulin, the only product formed from acetyl-Gly-Gly-His-Gly is the mono-N-carbethoxyhistidyl derivative (Fig. 3A, lower panel, where peak 1 is the unsubstituted peptide, and peak 2 is the mono-substituted peptide). With higher DEPC concentrations (10 mM), the unsubstituted peptide has reacted completely, and a new peak 3 of the disubstituted peptide appears (Fig. 3A, upper panel). Similar results were obtained for acetyl-tyrosine amide where a derivative is seen only at high DEPC concentrations (Fig. 3B) but not under our low mole ratio conditions. High DEPC concentrations did not react with seryl (acetyl-Ser-Gly) or methionyl (acetyl-Met-Leu-Phe) peptides at these reaction times (Fig. 3, C and D). Thus, under our standard reaction conditions used for tubulin, DEPC leads only to the formation of the mono-substituted histidyl derivative.

Determining the Number of Histidine Residues Modified—To quantify the number of His residues modified by DEPC under our standard conditions, $OD_{240}$ was monitored as a function of time. N-Carbethoxyhistidine has a molar extinction coefficient at 240 nm of 3200 $M^{-1} cm^{-1}$ (9). Assuming that all singly substituted His residues yield the same absorbance at 240 nm, we calculated the equivalent number of His residues reacted after 3 min of reaction to be 2 (Fig. 4). As shown in Fig. 1, at this time interval there is complete inhibition of vinblastine-promoted tubulin polymerization. This suggests that these two His residues are required in the vinblastine effect (see inset to Fig. 4). Moreover, the bulk of the inhibition has already occurred when only the equivalent of 1 His residue per dimer has been modified. Attempts to identify the more important His residue were unsuccessful. Although very short (1 min) labeling times indicated equal reactivity of the two pertinent His residues, the specific activity of the label was not high enough to get labeling at even shorter times. It should be pointed out that all 24 His residues of the dimer can react provided that higher concentrations (10 mM) of DEPC are permitted to react for 50 min.

Localization of the DEPC-modified His Residues—It remained then to localize the two His residues required for vinblastine-induced spiral formation from tubulin. Tubulin (2.8 mg/ml) was reacted with 0.5 mM $[^{14}C]$DEPC at 25 °C for 3 min. The reaction was stopped with 3 mM imidazole, and tryptic peptides were analyzed as described under "Materials and Methods."
Methods.” Only two peaks (except for non-peptide front-running material) were significantly labeled; Fig. 5 shows a chromatogram of the digest, and the inset shows an expanded portion containing the labeled peaks 1 and 2. The peptides were sequenced by Edman degradation for the first 10 amino acids. Peptide 1 yielded the sequence Leu-X(His)-Phe-Phe-Met-Pro-Gly-Phe-Ala-Pro and identifies tryptic peptide β263–276. The X(His) indicates that this residue was present in very low amount but not absent. The location of the single His residue in this peptide was βHis-264. Peptide 2 yielded the sequence Leu-Thr-Thr-Pro-Thr-Tyr-Gly-Asp-Leu-Asn-X(His)-Leu-Val-Ser, consistent with tryptic peptide β217–241; the single His residue in this peptide is located at βHis-227. Note that these two His residues are not very near the proposed vinblastine-binding site (11), consistent with the apparently normal binding of vinblastine found in this study.

Microtubule Assembly and Histidine Residues—Vinblastine-induced spirals are unusual tubulin polymers whose contacts differ from those of microtubules. It was thus important to know whether a similar His requirement exists for microtubule assembly. Hence, experiments similar to the above were carried out with DEPC, quenching with imidazole, and hydroxylamine treatment, except that polymerization was directed to microtubule assembly. Fig. 6A shows the assembly of modified tubulin at 25 °C in Mes assembly buffer containing 1 mM GTP and 10% Me2SO. The numbers on the curves indicate the duration of DEPC treatment. It is clear that with increasing duration of the reaction, the extent of assembly decreases, there is an increase in the latent period, and a decrease in the maximal rate of polymerization. Half-reduction in the extent of polymerization occurs at less than 3 min, and by 8 min no assembly is detected up to 20 min. Clearly reactive histidines are needed also for microtubule assembly, although equal degrees of inhibition require longer reaction times.

To check whether or not hydroxylamine would reverse this inhibition as well, 28 μM tubulin was reacted with 0.5 mM DEPC for 15 min and then with 92 mM hydroxylamine for 30 min. Removal of the hydroxylamine proved to be unnecessary. As shown in Fig. 6B the complete inhibition of assembly (compare curve 3 with the control, curve 1) was restored ~2/3 upon hydroxylamine exposure (curve 2). Moreover, the restored polymer was still cold-sensitive (arrow), and the cold-disassembled tubulin reassembled upon rewarming (curve 4), a property expected from microtubules.

The longer reaction times required to achieve complete inhibition of microtubule assembly with DEPC suggested that additional His residues may have been modified. Analysis by OD240 as done above for the vinblastine studies revealed that the equivalent of two additional His residues was modified when microtubule assembly was inhibited. A comparison between spiral polymers and microtubule assembly provided in Fig. 7 clearly demonstrates the different His requirements. Loss of the first two reactive histidines leading to complete inhibition of vinblastine-induced polymerization produced only 17% inhibition of the maximal rate of assembly, and modification of one additional His residue led to only 67% inhibition of assembly (see inset to Fig. 7). It required an equivalent of ~4 His modifications to block assembly completely. It is noteworthy that 15 min of DEPC reaction with tubulin at the low mole ratio completely prevents the ability of 10 μM taxol or 1 mM zinc sulfate to promote polymerization (data not shown).

Localization of the Additional His Residues—Tubulin (2.8 mg/ml) was reacted with [14C]DEPC for 8 min and hydrolyzed and chromatographed as above. Fig. 8 shows that, in addition to the two peptides identified in Fig. 5, a third peptide labeled with circled 3 was identified and sequenced. By contrast to the first two peptides, peptide 3 derived from α-tubulin residues 85–96 with the following composition: Glu-Leu-Phe-X(His)-Pro-Glu-Leu-Ile-Thr, where αHis-88 was the modified residue. We were unable to identify a specific fourth His residue that became labeled with [14C]DEPC, and although there were various weakly labeled peaks formed in 8 min, none seemed to predominate.

![Figure 5](image_url)

**Fig. 5. Separation of tryptic peptides of [14C]DEPC-treated tubulin.** A linear gradient of 0.95–47.5% acetonitrile in 0.05% trifluoroacetic acid was run on a C18 reverse phase HPLC column for 150 min at 1 ml/min. Peaks were collected manually and counted for 14C. Peaks 1 and 2 are the labeled peptides containing 12.5 and 11%, respectively, of the total radioactivity added (excluding the non-peptide portion); this was 5.8 and 4.9 times the average background; these were submitted to amino acid sequencing. The inset shows an expanded portion of the chromatogram showing the two labeled peptides.
Critical Histidyl Residues for Tubulin Polymerization

DISCUSSION

Vinblastine has multiple effects on the properties of tubulin as a function of its concentration. At substoichiometric concentrations it reduces the dynamic instability of microtubules (12); at intermediate concentrations it blocks assembly from tubulin dimers (13, 14); and at higher concentrations it promotes the formation of various non-microtubule polymers whose structures are a function of the solvent composition. These vinblastine-induced polymers include dimers of dimers (15), single or double rings, ring-like crystalloids, one or two protofilament spirals of widely varying helical pitch and length, poorly structured aggregates, clusters of spirals formed into rods, rosettes, or dense aggregates, and finally, well ordered paracrystals (16–23). A plausible but unproved explanation has been that vinblastine somehow leads to a weakening of lateral contacts between protofilaments, possibly due to curvature (24), thus preventing lateral interactions between protofilaments but not spiral formation. However, no significant changes in the CD spectra are observed when vinblastine is added to tubulin at a 1:1 mole ratio (15), and the formation of two-filament spirals is difficult to explain with this proposal.

The present study suggests, however, that modifications can be introduced into the tubulin dimer that severely hampers spiral formation (we use this term for all vinblastine-induced polymers unless otherwise specified) while having only a minor effect on microtubule assembly. Thus, the reversible derivatization of βHis-227 and βHis-264 with diethyl pyrocarbonate completely prevents the formation of vinblastine-induced polymers, whereas assembly of such altered dimers into microtubules proceeds at nearly the normal rate and extent. This inhibition is not an effect on the vinblastine-binding site because the binding of a fluorescent vinblastine analogue (11) is unaffected by these modifications, and vinblastine can still block assembly of the modified tubulin into microtubules. Moreover, βHis-227 lies in the colchicine binding domain, yet the binding-dependent enhancement of fluorescence of a colchicine analogue (2-methoxy-5-[2′′,3′′,A′′-trimethoxyphenyl] tropane) occurs normally in the modified tubulin.

At least one, and probably two additional His residues must be modified with diethyl pyrocarbonate in order to block microtubule assembly. In contrast to the modifications required for the vinblastine effect, the modification of the third His residue, leading to substantial assembly inhibition, occurs on β-tubulin at αHis-88. For complete inhibition of assembly, the equivalent of a fourth His residue had to be derivatized, but this appears to be represented by a number of weakly 14C-labeled His residues of similar reactivity toward DEPC, and we were unable to specify its location. Demodification of the polymerization-incompetent tubulin with hydroxylamine restored assembly competence and yielded normal appearing microtubules in negatively stained electron micrographs. What remains to be determined is whether or not the two most reactive His residues on β-tubulin need to be modified as well as αHis-88 to block microtubule assembly. Two earlier studies have dealt with the relation of His residues to microtubule assembly. Lee et al. (25) had previously demonstrated that modification of 3 His residues led to a partially reversible inhibition of microtubule assembly, although location in the modified His residues in the monomers or in the primary sequence was not investigated. In the other study only one His residue was highly reactive toward DEPC despite a longer reaction time, and 60% of the products was not reversible with hydroxylamine treatment (26, 27). By contrast, in our experiments the reactive His residue in α-tubulin was His-88, but the two most reactive His residues resided in β-tubulin. Their modification was fully reversible with hydroxylamine, and they played only a minor role in microtubule assembly.

We had previously postulated a requirement for an interaction between the anionic β-C terminus and a cationic domain in tubulin that would be required for spiral formation and which could be competed for by oligomannos. Titration using turbidity assays indicated that these cation(s) had a pK ~7.0 and might be represented by one or more His residues (2). We now show

FIG. 6. Inhibition of microtubule assembly by DEPC and its reversal by hydroxylamine. A, tubulin (2.8 mg/ml) was reacted with 0.5 mM DEPC in Mes assembly buffer with 10% Me₂SO at 25 °C for different times. The reaction was stopped with 3 mM imidazole followed by 30 min exposure to 92 mM hydroxylamine at 25 °C. 1 mM GTP was added to start microtubule assembly as measured by OD₃₅₀ at 25 °C. Numbers on the curves indicate duration of reaction with DEPC. B, for reversibility 2.8 mg/ml tubulin was reacted as above, stopped after 15 min with 3 mM imidazole, and exposed to 92 mM hydroxylamine for 30 min at 25 °C. Curve 1, unmodified tubulin + hydroxylamine; curve 2, modified/demodified tubulin; curve 3, DEPC treatment only; curve 4, sample from curve 2 was cooled for 30 min on ice and the reincubated at 25 °C.

FIG. 7. Comparison of histidyl residue requirements for vinblastine-induced polymerization and microtubule assembly. The number of His residues modified was obtained from the ΔOD₃₅₀ and is plotted against the maximal polymerization rate (ΔOD₃₅₀ min⁻¹) normalized with respect to the control values. Filled circles indicate spiral formation, and open circles indicate microtubule assembly.
Critical Histidyl Residues for Tubulin Polymerization

FIG. 8. Reverse phase C18 HPLC separation of tryptic peptides of [14C]DEPC-labeled tubulin reacted for 8 min. A 0.95–63% acetonitrile gradient in 0.05% trifluoroacetic acid was run for 120 min at 1 ml/min. Peaks were collected manually and counted for 14C. Peak 3 is the newly labeled peptide; it contained 11.6% of the 14C (5.7 times the average background) and was submitted for amino acid sequencing. Peaks 1 and 2 are the same as in Fig. 5 but were run under different conditions.

that two His residues on β-tubulin are involved in the cationic domain, i.e. βHis-227 and βHis-264. From the published electron crystallographic structure (28) the two His-β residues (227 and 264) are located, respectively, in helix 7 and β-strand 7; whether these are present as ion pairs or unpaired charges cannot be stated in the absence of published coordinates. The increasing fraction of His-227 and -264 bearing a charge when the pH is brought to 6.0 leads to increased rates of turbidity generation, decreased requirements for vinblastine, and decreased sensitivity to oligoanions.2

The ionic nature of the interactions in spiral formation leads to the prediction that the vinblastine-induced spiral formation should be sensitive to the ionic strength of the system. This is indeed the case, and with unmodified tubulin, addition of 65 mM NaCl to our standard incubation mix abolishes spiral formation (data not shown). This salt effect has been seen at similar salt and protein concentrations and has been ascribed to nonspecific shielding (20, 21, 29). The present model offers a plausible explanation. The two types of polymer formed from tubulin are both electrostatically regulated but in opposite directions as follows: spiral formation by electrostatic attraction and microtubule assembly by electrostatic repulsion (see Ref. 10 and references therein). Elevated ionic strength will decrease or abolish both types of interaction and, therefore, inhibit spiral formation and stimulate microtubule assembly. The salt effects are not, however, entirely nonspecific because promotion of microtubule assembly by monovalent salts shows selectivity for different alkali metal chlorides and guanidinium hydrochloride (8, 30).

We conclude that polymerization to spirals (vinblastine) and to microtubules has a number of distinguishing characteristics in addition to the shape of the polymer. These are as follows.

GTP Requirement—Whereas microtubule assembly has a strict requirement for GTP, polymers induced by vinblastine form in the absence of GTP (16, 31), and no GTPase activity can be measured during spiral formation with vinblastine (32). In fact, in the appropriate conditions, GTP (and other oligoanions) will inhibit vinblastine-induced polymerization (1).

Temperature Requirement—Microtubule assembly is very cold-sensitive, and low temperatures will depolymerize preformed microtubules. By contrast, polymerization induced by vinblastine occurs readily at 0 °C, and the preformed polymer is cold-stable (18, 20, 24, 33, 34); we have confirmed this cold stability.

Stability—As noted earlier (21) we have found that vinblastine-induced spirals are far more easily disrupted by dilute glutaraldehyde than are microtubules.

Histidyl Residues—Finally, the His residues involved in vinblastine-induced polymers reside in β-tubulin (His-228 and His-264), whereas both may interact with the β-C terminus, whereas the critical His residue for microtubule assembly is in α-tubulin at position 88. The latter is found in a loop between helix 2 and β-strand 3 of α-tubulin (28). Its modification may affect lateral contacts between protofilaments, but additional structural analysis will be required to test whether or not these locations can account for the effects of His modifications described above.

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