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Ethoxyformylation of Tubulin with [^{3}H]Diethyl Pyrocarbonate: A Reexamination of the Mechanism of Assembly Inhibition†

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ABSTRACT: In this study we reexamined the basis for the profound inhibitory effects of low concentrations of diethyl pyrocarbonate (DEP) on tubulin's ability to assemble into microtubules [cf. Lee, Y. C., Houston, L. I., & Himes, R. H. (1976) *Biochem. Biophys. Res. Commun. 70*, 50–56]. Assembly inhibition at low DEP concentrations can be resolved into two components: a component reversible with hydroxylamine (attributed to monoethoxyformylation of histidyl residues) that contributes ~40% of the inhibition and a hydroxylamine-resistant component (attributed to ethoxyformylation of non-histidyl residues) that contributes ~60% of the inhibition. Comparisons between the extent of assembly inhibition associated with each component and the degree of residue modification argue for the involvement of a small number of highly reactive residues in the inhibition process. To identify these residues, tubulin was reacted with limiting concentrations of [^{3}H]DEP and subjected to tryptic digestion and HPLC analysis. Only one moderately reactive histidyl residue was detected. This residue (~2–3-fold more reactive than the bulk histidyl residues) eluted in an apparently large, hydrophobic fragment. We failed to detect any non-histidyl residues that were exceptionally reactive to [^{3}H]DEP. However, we did observe that the N-terminal methionyl residues in native protein were ethoxyformylated at rates comparable to that of the bulk histidyl residues. In denatured protein these methionyl residues were ethoxyformylated to a much larger extent (~3–4-fold) than the bulk histidyl residues. We suggest that the N-terminal methionyl residues in tubulin are partly buried or are in a salt-bridge interaction in native protein and that ethoxyformylation of these residues disrupts tubulin structure and interferes with microtubule assembly.

Microtubules are cylindical structures several hundred angstroms in diameter and typically several microns long that participate in a variety of diverse functions in eucaryotic cells including mitosis, morphogenesis, and vesicle transport (Soifer, 1986). A molecular understanding of these functions will involve in part detailed knowledge of the properties of the major constituent protein of the microtuble, tubulin, and its two homologous subunits, α- and β-tubulin. These subunits are each ~450 amino acid residues in length (Ponstingl et al., 1981; Krauhs et al., 1981), display ca. 40% sequence homology, and have different functional roles which at present are only poorly understood (Luduena, 1979; Cleveland, 1987).

We have had a longstanding interest in the α-subunit. This subunit contains a highly reactive lysyl residue, Lys 394, whose methylation renders tubulin assembly incompetent (Szasz et al., 1982, 1986). Chemical modifications of this reactive residue have been implicated in the pathophysiology of diabetes (Williams et al., 1982) and in alcohol-induced hepatic necrosis (Jennett et al., 1987, 1989), two disorders that result in impaired microtubule function. Lys 394 is thought to be in a positively charged cluster consisting of Arg 390, His 393, and Lys 394 which renders this residue reactive as a nucleophile (Szasz et al., 1986). The role of this cluster in assembly is not understood, although it has been suggested that the cluster interacts with highly negatively charged regions in the C-terminal domain of α-tubulin to stabilize a conformation essential for microtubule assembly (Blank et al., 1986).

Lee et al. (1976) reported that microtubule assembly is inhibited by low concentrations of DEP[1] and proposed that

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tubulin contains one or more reactive histidyl residues essential for microtubule assembly. We have wondered whether His 393, like Lys 394, is also highly reactive toward electrophiles and was one of the histidyl residues implicated by Lee et al. (Szasz et al., 1986). In this study we directly probed the reactivities of the histidyl residues in tubulin using [^{3}H]DEP. We show that His 393, in contrast to Lys 394, has a diminished reactivity in native tubulin and is therefore not one of the essential histidyl residues implicated by Lee et al.

Our study revealed that the mechanism of assembly inhibition by DEP is more complex than originally perceived. There have been extensive studies of the C-terminal moieties of tubulin, which have been shown to be important for tubulin conformation and function (Serrano et al., 1984; Sullivan & Cleveland, 1986; Vera et al., 1989). Much less is known about the N-terminal moieties of tubulin. Lee et al. (1976) noted that a significant fraction (≥40%) of the assembly inhibition induced by DEP results from the modification of a non-histidyl residue(s). Our study suggests that this non-histidyl component is caused by the ethoxyformylation of the N-terminal methionyl residues of tubulin.

Materials and Methods

Materials. Met-Arg was purchased from U.S. Biochemicals. Leu-Asp-His-Lys was synthesized by a manual procedure developed in this laboratory using fluorenylmethoxycarbonyl (Fmoc) protective group chemistry and confirmed by amino acid composition analysis. *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated trypsin (Sigma) was purified by reverse-phase HPLC according to the method of Titani et al. (1982).

Preparation of Microtubule Protein. Microtubule protein (MTP) was isolated by repetitive cycles of assembly–disassembly from bovine brains following a temperature-based procedure (Sternlicht & Ringel, 1979) modified from the method of Gaskin et al. (1974). MTP was ~85% tubulin and ~15% microtubule associated proteins (MAPs) as determined by gel electrophoresis (Laemmli, 1970). MAP-depeleted tubulin (PC-tubulin) was prepared from MTP by chromatography on precycled Whatman P11 phosphocellulose following the method of Cleveland et al. (1977).

Synthesis of [^{3}H]DEP. [^{3}H]DEP was synthesized by following procedures modified from the methods of Melchior and Fahrney (1970) and Igarishi et al. (1985). Briefly, ethanol of high specific activity was prepared by reducing acetaldehyde with sodium boro[^{3}H]hydride in triethylene glycol dimethyl ether (triglyme). The [^{3}H]ethanol was distilled from this reaction mixture and added to a washed suspension of sodium hydride in diethyl ether. The mixture was then incubated for 6 h at room temperature in the presence of a stream of CO_2 gas. Ethyl chloroformate was added and the reaction maintained at 60 °C for 36 h. The final product, [^{3}H]diethyl pyrocarbonate ([^{3}H]DEP, 1.3 g, ~90 mCi total activity), was collected by distillation and stored at −120 °C in sealed ampules (50-μL portions) or used as 10 mM working stocks in absolute alcohol.

The specific activity of [^{3}H]DEP was estimated at 13.7 ± 1.2 Ci/mol by averaging the value obtained by using the ion-exchange chromatography procedure described by Igarishi et al. (1985) with that calculated from the dpm/μL of the [^{3}H]DEP stock and the specific density of DEP (1.101 g/mL, 25 °C; Aldrich Catalog). In the latter calculation appropriate corrections were made for radioactivity purity, estimated at ca. 60% from HPLC (cf. Figure 5A)], and DEP chemical purity, estimated at 73% by reaction of aliquots from the [^{3}H]DEP stock with N^{α}-acetylhistidine (extinction coefficient for N^{α}-acetyl-N^{im}-(ethoxyformyl)-L-histidine = 3.6×10^3 AU at 240 nm).

N^{im}-Ethoxyformylation of Tubulin. Ethoxyformylation of tubulin was accomplished by procedures modified from that of Szasz et al. (1986). Briefly, GTP-depleted preparations of MTP (~3–4 mg/mL) in PB buffer (pH 6.4) were reacted with limiting concentrations of DEP (≤1 mM, prepared from 100 mM stocks in absolute alcohol) for 12 min at room temperature. The reaction was quenched with 10 mM imidazole (recrystallized) in PB buffer, and the products were centrifuged twice through 10 mL of Sephadex G-25 columns equilibrated in PB+2.5M G (4 °C). In the assembly studies, half of the DEP-modified material was reacted with 0.1 M hydroxylamine for 1 h at room temperature and then dialyzed for 3 h at 4 °C against PB+2.5M G, and 0.1 M hydroxylamine (pH 6.7) and overnight against PB+2.5M G buffer. Hydroxylamine-treated and untreated samples were adjusted to a common protein concentration of ca. 3.8 mg/mL (Lowry et al., 1951), supplemented to 1 mM GTP and assayed for assembly competence on a Gilford 2400-2 multichambered spectrophotometer (350 nm, 37 °C; Berne, 1974; Szasz et al., 1986).

In the tryptic mapping studies, GTP-depleted PC-tubulin (~4 mg/mL) was reacted in PC buffer with 0.1 mM [^{3}H]-DEP ([DEP]:[histidyl residue] = 1:10) for 15 min at 37 °C. The samples were immediately alkylated with 2.5 mM *N*-ethylmaleimide for 15 min at 37 °C and then precipitated with 5 volumes of ice-cold ethanol and centrifuged. The washed pellets were resuspended in 50 mM Hepes, pH 8.0, to a final concentration of ~10 mg/mL and incubated with 5% w/w trypsin at 37 °C for 4 h. Aliquots (70 μL) of the final solution were either analyzed by reverse-phase HPLC or reacted with 0.1 M hydroxylamine for 2 h at room temperature (these conditions reversibly removed >95% of the ethoxyformyl group from acetyl-N^{im}-(ethoxyformyl)histidine controls) and then analyzed. PC-tubulin preparations (~4 mg/mL) denatured with heat (75 °C, 30 min) or with 6 M guanidine hydrochloride (37 °C, 30 min followed by chromatography on a Sephadex G-25 column) were reacted with [^{3}H]DEP and processed for tryptic digestions in a manner identical with that used for native protein.

Radiolabel incorporation into tubulin dimer was estimated by SDS–PAGE (Laemmli, 1970). Briefly, 2–3 mg/mL MTP or PC-tubulin was reacted with [^{3}H]DEP (≤1 mM) for 12 min at room temperature. A portion of the modified preparation was treated with hydroxylamine (0.1 M, 2 h at room temperature). Aliquots from the hydroxylamine-treated and untreated samples were removed, supplemented with BSA, and electrophoresed on 9% acrylamide gels cross-linked with 0.37% *N*,*N*′-diallyltartardiamide (Bio-Rad Labs). The gels were lightly stained with Coomassie brilliant blue R-250, and protein mass in the α- and β-tubulin bands were determined by comparisons of the integrated intensity of these bands with that of the BSA standards. The tubulin bands were then excised and dissolved in 3% periodic acid for scintillation counting (Beckman LS7500, 20% counting efficiency). The dpm values were averaged, normalized to total number (23) of histidyl residues in tubulin, and expressed as equivalents per tubulin

[1] Abbreviations: PB, a microtuble protein stabilizing buffer (pH 6.7) consisting of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), 2 mM EGTA, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, and 0.5 mM $MgCl_2$; DEP, diethyl pyrocarbonate; MTP, microtubule protein; MAPs, microtubule associated proteins; PC buffer, a pH 6.7 buffer consisting of 25 mM MES, 2 mM EGTA, and 1 mM 2-mercaptoethanol.

dimer (1 equiv = 23 ethoxyformyl groups) (cf. Table I).

N^{im}-Ethoxyformylation of the Synthetic Peptides. The synthetic peptides Leu-Asp-His-Lys (LDHK) or Met-Arg (MR) (prepared as 10 mM stocks in water) were reacted at 1–3 mM with 0.5 equiv of either [^{3}H]DEP or DEP (15 min at room temperature in 30 mM, pH 7.0, phosphate buffer). Aliquots were treated with hydroxylamine hydrochloride (0.10 M final concentration, 1 h at room temperature), and all samples were then characterized by reverse-phase HPLC. N^{im}-(Ethoxyformyl)-LDHK was identified by its selective reversal to LDHK following hydroxylamine treatment.

HPLC Analysis. The N^{im}-ethoxyformyl derivative of histidine has a half-life of ~50 h at pH 7 and 25 °C (Means & Feeney, 1971). Igarishi et al. (1985) have shown that fast performance chromatography can be successfully used to identify these moderately labile sites in DEP-treated proteins. In this study tryptic digests of [^{3}H]DEP-modified tubulin were typically chromatographed on a SynChrom SynCropak RP-P C18 column (250 × 4.1 mm; 300-Å pore, 6.5-μm particle diameter resin) in a modified Waters HPLC system. Some of the studies (e.g., Figure 7) were done on a Vydac 218TP54 C18 column (250 × 4.1 mm; 300-Å pore, 5.0-μm particle diameter resin) using a 7125 Rehodyne injector and a 1.8-mL Altex dynamic mixer. The latter modifications improved buffer mixing and chromatogram reproducibility. Gradient conditions for tryptic mapping were 1 mL/min flow rate, buffer A (water plus 0.1% TFA), and then a 2-h linear gradient at 0.5% buffer B (acetonitrile plus 0.1% TFA)/min. Effluents were monitored at 210 nm at 1 AUFS and collected at 1-min intervals. DPM recovered (Figures 2–6) were uncorrected for column retention losses (estimated at ~40–60%). Gradient conditions for modified peptides (Figures 5 and 7A) were the same except the linear gradient was at 1% B/min.

Results

Synthesis and Characterization of [^{3}H]DEP. [^{3}H]DEP (13.7 ± 1.2 Ci/mol) was synthesized from [^{3}H]ethanol by a method improved from that of Igarishi et al. (1985) using NaH instead of sodium metal to facilitate sodium ethoxide formation. The final product was ca. 60% pure by HPLC reverse-phase chromatography, with [^{3}H]ethanol as the major radioactive contaminant (cf. Figure 5A, upper panel), and ca. 73% pure by reaction with N^{α}-acetyl-L-histidine (Materials and Methods). DEP elutes later than ethanol on C18 reverse-phase columns because of hydrophobic interactions of its ethoxyformyl groups with the column (Biscoglio de Jimenez Bonino et al., 1986). In this study we exploited the hydrophobicity of the ethoxyformyl group to assign ethoxyformylated tryptic fragments from [^{3}H]DEP-modified tubulin.

Assembly Inhibition. GTP-depleted preparations of MTP (~3 mg/mL) were reacted with limiting concentrations of DEP (≤1 mM) for 12 min at room temperature. Aliquots were assayed for assembly competence before and after treatment with 0.1 NH_2OH (Figure 1). Microtubule assembly was totally inhibited at 1 mM DEP and partially inhibited at lower DEP concentrations. Lee et al. (1976) have shown by electron microscopy that short, "normal"-appearing microtubules are produced in this concentration range. The assembly inhibition was separable into contributions from hydroxylamine-sensitive and hydroxylamine-resistant components (Figure 1; Table I). Hydroxylamine-treated samples typically displayed ~40% lower levels of inhibition than the corresponding untreated samples (Figure 1; Table I). Since hydroxylamine rapidly and completely reverses the monoethoxyformylation of histidyl residues by DEP, ~40% of the assembly inhibition could be unambiguously assigned to this type of histidine modification (Table I). The hydroxylamine-resistant component, which contributed ~60% of the inhibition, was attributed mainly to non-histidyl residue modifications (see below and Discussion). Table I also includes a data point (~0.7 mM DEP) from Lee et al.'s (1976), study, in which these authors demonstrated a similar 40% reversal of the original assembly competence of DEP-modified tubulin by treatment with hydroxylamine.

Ethoxyformyl incorporation was estimated in a separate study using [^{3}H]DEP (≤ 1 mM) (Materials and Methods). SDS–PAGE analysis of DEP-modified proteins indicated that the α- and β-subunits were modified to similar extents (~1:1)

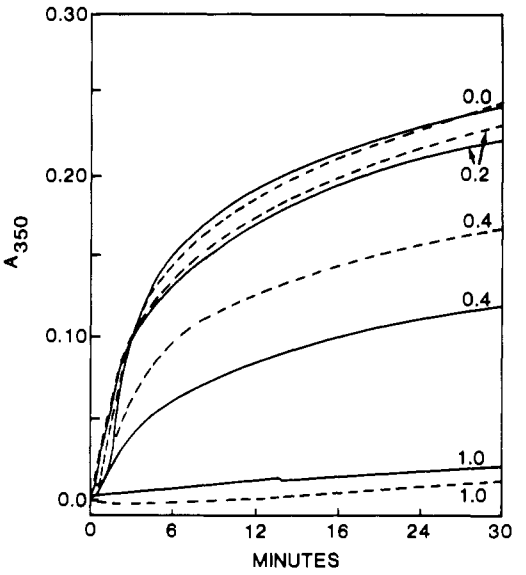

FIGURE 1: Effects of DEP on microtubule assembly. Microtubule protein (~3 mg/mL) was reacted with varying concentrations of DEP (0, 0.2, 0.4, and 1.0 mM) for 12 min at room temperature. A portion of the material at each DEP was treated with NH_2OH. Both the treated (---) and untreated materials (—) were simultaneously assayed for assembly competence on the basis of solution turbidity at 350 nm. This study also revealed small amounts of nonspecific aggregation of microtubule protein evident as a continuous increase in the A_{350} absorbance at "end-state" (t > 20 min). This background aggregation, which was common to all samples and therefore independent of DEP concentration, had no significant effect on estimates of assembly inhibition (Table I).

Table I: Contributions of the Histidyl and Non-Histidyl Modifications to Assembly Inhibition[a]

DEP (mM)	NH_2OH	% inhibition	% of inhibition contributed by: His mod[b]	% of inhibition contributed by: non-His mod[b]	ethoxyformyl equivalents per dimer[c]
0.2	–	9			0.06
	+	5	44	56	0.02
0.4	–	55			0.11
	+	34	38	62	0.04
0.7[d]	–	100			0.16[e]
	+	57	43	57	0.05
1.0	–	100			0.2
	+	100	?	?	0.07

[a] Assembly inhibition data from Figure 1. [b] Percent contribution from non-histidyl modifications defined as (% inhibition following NH_2OH treatment)/(% inhibition before NH_2OH treatment). Percent contribution from histidyl modifications defined as 100% minus percent contribution from non-histidyl modifications. [c] 1 equiv per tubulin dimer = 23 ethoxyformyl groups. [d] From Lee et al. (1976), where 1.6 mg/mL MTP in assembly (PB) buffer was reacted with 0.8 mM DEP for 10 min at room temperature. Reexpressed in terms of the conditions used in the present study, this corresponds to a reaction with 0.7 mM DEP for 12 min at room temperature. [e] Not reported by Lee et al. Values shown are our estimated values.

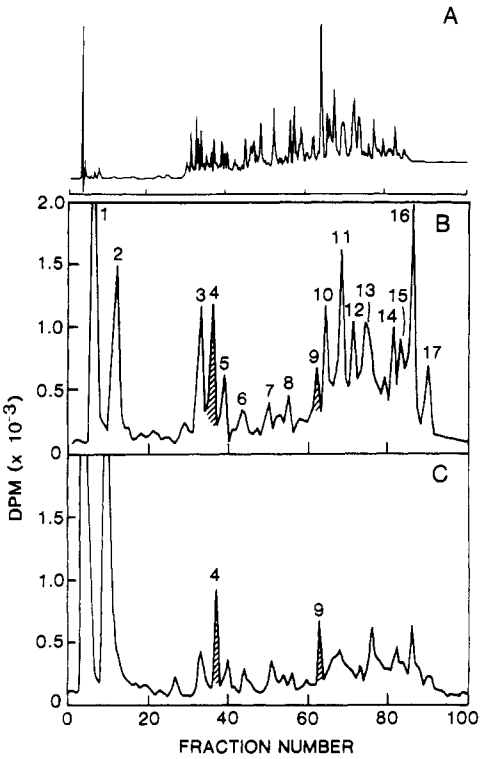

FIGURE 2: [^{3}H]DEP modification of native tubulin. PC-tubulin was reacted with 0.1 mM [^{3}H]DEP and tryptic-digested. Half of the digest (4.5 nmol of protein) was chromatographed: (A) UV trace; (B) radiolabel trace. The remaining portion was treated with hydroxylamine and then chromatographed: (C) radiolabel trace. Total dpm in (B) (35 000) and (C) (30 000) were identical within 13%. Note the loss of radiolabel from the peptide region in (C) and its accumulation in peak 2 as [^{3}H]ethanol. In (B), peaks 3–17 contained ca. 27 000 dpm; peak 4, 1050 dpm; and peak 16, 1850 dpm. In (C), peaks 3–17 contained 10 300 dpm (~56% reversal); peak 4, 775 dpm; and peak 16, 200 dpm. An average histidyl residue in (B) incorporated 650 dpm (=dpm in peaks 3–17 × 0.56/23).

in approximate agreement with their histidyl content (1.3:1). These studies also demonstrated that ethoxyfomyl incorporation increased linearly with DEP concentration and established that low-level modifications were responsible for the assembly inhibition (Table I). Analysis of the hydroxylamine-reversible component indicated a disproportionate relationship between the inhibition of microtubule assembly and the ethoxyformylation of histidyl residues. We estimated from a comparison of the "+" and "−" hydroxylamine data (Table I, [DEP] = 0.4 and 0.7 mM) that introduction of ~0.07 and 0.11 ethoxyformyl equiv into the histidyl residues of tubulin inhibited microtubule assembly by ca. 21 and 43%, respectively. If assembly inhibition proceeded through a strict 1:1 relationship with ethoxyformylation, one would predict ~7 and 11% inhibitions, respectively. This apparent discrepancy between predicted and observed inhibitions can be resolved if tubulin contains one or more moderately reactive histidyl residues essential for microtubule assembly. Our data suggested that if these essential histidyl residues exist, they would have to be at least ~21/7- to 43/11-fold more reactive than the bulk histidyl residues to account for assembly inhibition.

Analysis of the hydroxylamine-irreversible component also implicated one or more highly reactive non-histidyl residues in the inhibition process. We estimated that reaction of tubulin dimer with 0.4 and 0.7 mM DEP introduced ~0.9 and 1.2 ethoxyformyl groups, respectively, into non-histidyl residues, causing ca. 34 and 57% inhibition of microtubule assembly (Table I). On the basis of a comparison with histidyl residue modifications, the nonreversible component appeared to involve

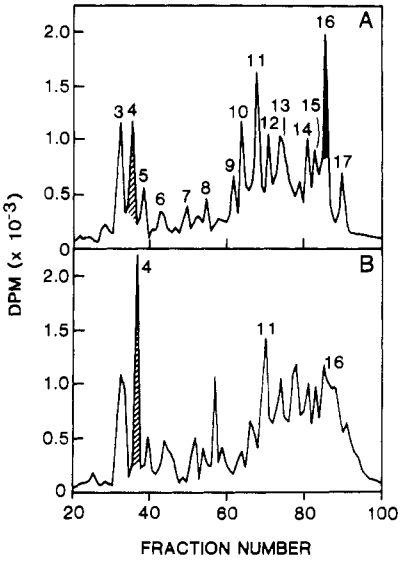

FIGURE 3: Demonstration of a reactive histidyl residue in native tubulin dimer. Panels A and B display, respectively, the tryptic digests from native and heat-denatured PC-tubulins reacted with 0.1 mM [^{3}H]DEP. Peak 16 (black), which arose from a histidyl residue (Figure 2), displayed an enhanced reactivity in the native state [compare (A) and (B)]. In contrast, peak 4 (stripe) displayed a reduced reactivity.

one or more essential non-histidyl residue(s) that were at least ~34/7- to 57/11-fold more reactive than the bulk histidyl residues. Studies were undertaken to identify the reactive histidyl and non-histidyl residues.

Ethoxyformylation of Tubulin Dimer. MTP and PC-tubulin were reacted with limiting concentrations of [^{3}H]DEP (0.1 mM), tryptic-digested, and analyzed by HPLC. The two preparations gave similar chromatograms; however, chromatograms from the PC-tubulin digests that lacked MAPs were better resolved. HPLC analysis revealed a complex UV fragmentation pattern at 210 nm that was unchanged by ethoxyformylation (Figure 2A). Seventeen partially resolved radiolabeled peaks were detected (Figure 2B). Peak 2 was assigned to the major breakdown product, [^{3}H]ethanol. Peak 1, which was not identified, appears to represent additional breakdown products. Peaks 3–17 were assigned to [^{3}H]-DEP-modified peptide fragments. This region contained several prominent peaks, e.g., peaks 11 and 16, that were likely candidates for the reactive residues. Most (~60%) of the radiolabel in peaks 3–17 was labile toward hydroxylamine (Figure 2C). This labile fraction was attributed to histidyl residue modifications. Peaks 4 and 9 were present both in the untreated (Figure 2B) and in the hydroxylamine-treated (Figure 2C) samples and were assigned to non-histidyl modifications. The residual radiolabel background seen in Figure 2C was also assigned to non-histidyl modifications. Surprisingly, the radiolabel remaining after hydroxylamine treatment was devoid of any intense peaks and gave no indication of an exceptionally reactive non-histidyl residues (Discussion).

To further probe for reactive residues, PC-tubulin was subjected to heat or guanidine-hydrochloride denaturation, then DEP-modified tryptic-digested, and analyzed. Comparisons of chromatograms from native and denatured digests are shown in Figures 3 and 4. A general broadening of peaks 3–17 was observed upon denaturation which may represents labeling of previously "buried" histidyl residues (Szasz et al., 1986). A moderately reactive histidyl residue (~2–3-fold more reactive than bulk histidyl residues) was consistently detected in peak 16 from native digests (Figure 3, Table II). Our attempts to identify this residue or unambiguously assign

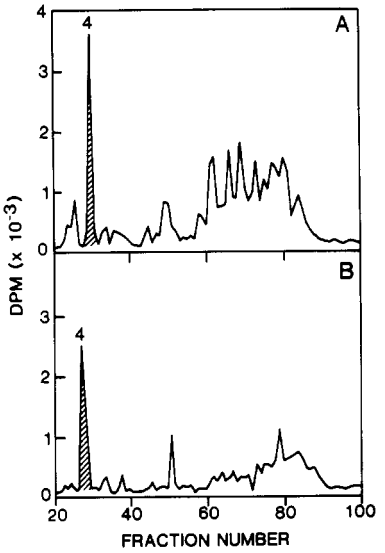

FIGURE 4: [^{3}H]DEP modification of denatured tubulin. PC-tubulin was denatured in 6 M guanidine hydrochloride and then reacted with 0.1 mM [^{3}H]DEP and tryptic-digested. Half of the digest (ca. 3.5 nmol) was directly chromatographed (A); the remaining portion was reacted with hydroxylamine and then chromatographed (B). Retention times differ slightly from those of Figures 2 and 3 because of column aging. Total dpm in (A) (~35 000) and (B) (33 700) were identical within 5%. In (A), peaks 3–17 contained ca. 29 700 dpm; peak 4, 3100 dpm; and peak 16, 1100 dpm. In (B), peaks 3–17 contained 12 900 dpm (~56% reversal); peak 4, 2500 dpm; and peak 16, 380 dpm. An average histidyl residue in (A) incorporated 740 dpm (=dpm in peaks 3–17 × 0.56/23).

Table II: Relative Reactivities of Selected Peaks[a]

	relative reactivity in	
	native protein	denaturated protein[b]
peak 4		
hydroxylamine-sensitive component[c]	0.42[d]	0.81[d]
hydroxylamine-insensitive component[e]	1.2[f]	3.4[f]
peak 16	2.5[d]	0.97[d]
	2.3[g]	

[a] Data based on Figures 2 and 4. Reactivities relative to bulk histidyl residues. [b] Denatured with 6 M guanidine hydrochloride. [c] Assigned to His 393 (see text). [d] From dpm ratio: (minus NH_2OH – plus NH_2OH)/average histidyl residues. [e] Assigned to N-terminal methionyl residues (see text). [f] From dpm ratio: plus NH_2OH/average histidyl residue. [g] From dpm ratio: (minus NH_2OH – plus $NH_2OH)_{native}$/(minus NH_2OH – plus $NH_2OH)_{denatured}$.

it to the α- or β-subunits were frustrated by the lability of the ethoxyformyl group and were unsuccessful. A moderately reactive non-histidyl residue was detected in peak 4 from denatured digests (Figures 3 and 4). A comparison of native and denatured digests suggested that the reactivity of this residue had increased ~3-fold with denaturation (Figures 2B and 4A; Table II). Although hydroxylamine had little effect on peak 4, analyses of the dpm content in peak 4 before and after treatment with hydroxylamine did reveal a small hydroxylamine-sensitive component (~20–30% of total dpm) (Table II). This component was assigned to an overlapping histidyl modification. Further studies suggested that this component arose from a coeluting tryptic fragment that contained His 393 from α-tubulin.

His 393 Has a Reduced Reactivity in the Native State. Since DEP-modified peptides were minor products in the tryptic digests (Figure 2), our attempts to identify the radiolabeled peaks by sequencing were unsuccessful. We consequently explored the use of synthetically prepared ethoxyformylated peptides as HPLC *retention time standards* to assign a number of fragments in the digest.

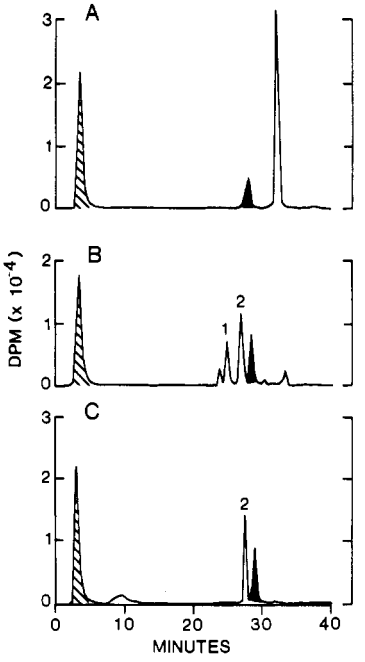

FIGURE 5: [^{3}H]DEP modification of synthetic LDHK. 1 mM LDHK was reacted with 0.5 mM [^{3}H]DEP for 15 min at room temperature and then analyzed by reverse-phase HPLC (1% buffer B/min using a Synchrom C18 column) (Materials and Methods): (A) [^{3}H]DEP; (B) [^{3}H]DEP + LDHK; (C) [^{3}H]DEP + LDHK + NH_2OH. Radiolabel contaminants and/or non-peptide products are shown in black. The hatched peak at ca. 3 min corresponds to [^{3}H]ethanol.

Region 390–394 in α-tubulin contains a cluster of positively charged residues consisting of Arg 390, His 393, and Lys 394. Tryptic digestion of tubulin generates a tetrapeptide, LDHK, containing two positively charged residues from this region, His 393 and Lys 394. In the native protein Lys 394 is highly reactive to nucleophiles such as formaldehyde and acetaldehyde (Szasz et al., 1986; Jennett et al., 1987, 1988). If His 393 were also highly reactive, relatively large amounts of the N^{im}-(ethoxyformyl)-LDHK derivative should be present in the tryptic digests. However, no exceptionally reactive peptide corresponding to this peptide was detected. The moderately reactive peptide that was detected, peak 16 (Figures 3 and 4), eluted at an acetonitrile concentration (ca. 45%) characteristic of hydrophobic peptides (Figure 3A) and was unlikely to be the N^{im}-ethoxyformyl derivative of LDHK, a relatively hydrophilic fragment. This suspicion was confirmed by direct DEP modification of synthesized LDHK. Two major radiolabel products were observed following treatment of LDHK with [^{3}H]DEP (peaks 1 and 2, Figure 5B). These products emerged later than LDHK (cf. Figure 7A), which lacks an ethoxyformyl group, but earlier than DEP (Figure 5A), which contains two ethoxyformyl groups, and were consequently assigned to monoethoxyformylated derivatives. Peak 1 was sensitive to hydroxylamine and was further assigned to the N^{im}-ethoxyformyl derivative of the histidyl residue; peak 2 was unaffected by hydroxylamine and was assigned to the N^{α}-ethoxyformyl derivative of the N-terminal leucyl residue[2] (Figure 5C). Several minor peaks eluted near DEP. These peaks were attributed to disubstituted LDHK (possible candidates being the N^{α}-, $N^{im'}$-, or $N,N^{im'}$-diethoxyformylated products). When significantly higher concentrations of

[2] LDHK has free α- and ϵ-amino groups. We assigned the modification to the α-amino group since this group is known to react much more readily with DEP at physiological pH (Miles, 1977).

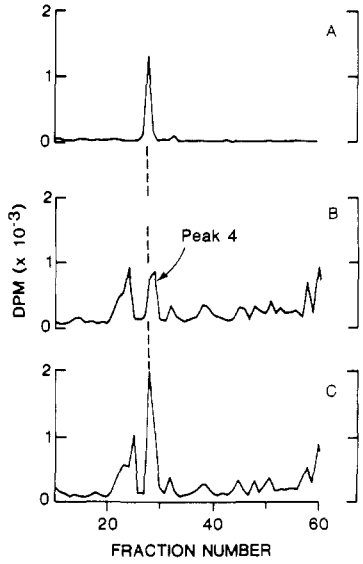

FIGURE 6: Assignment of N^{im}-(ethoxyformyl)-LD^{393}HK in tryptic digests. (A) [^{3}H]-N^{im}-(ethoxyformyl)-LDHK ("spike"); (B) peak 4 region from tryptic digests of [^{3}H]DEP-modified tubulin; (C) spike + digest. The superposition of peak 4 and spike demonstrates that a component of peak 4 is N^{im}-(ethoxyformyl)-LD^{393}HK (see text and Table II).

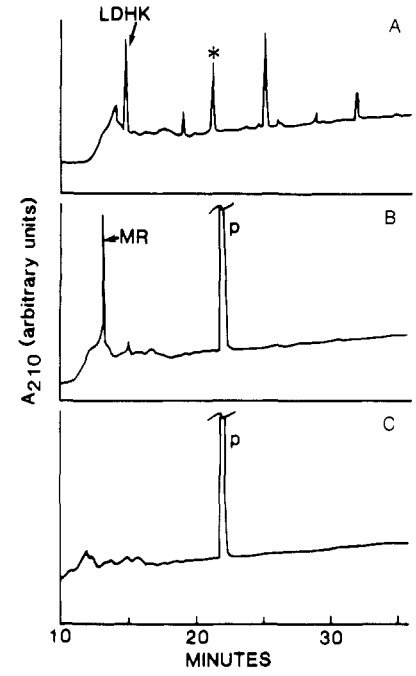

FIGURE 7: Model compound studies show that N$^{\alpha}$-ethoxyformylated N-terminal fragments coelute with N^{im}-(ethoxyformyl)-LDHK: (A) LDHK (7 mM) + DEP (3 mM); (B) MR (5 mM) + DEP (5 mM). MR was quantitatively converted to a single product ("P") with the same retention time (22 min) as N^{im}-(ethoxyformyl)-LDHK [* in (A)]. P was isolated and treated with hydroxylamine. As expected, this product was resistant to hydroxylamine consistent with its assignment to N^{α}-(ethoxyformyl)-MR (C). Chromatograms in this figure were obtained by using a Vydac C18 RP column.

reactants were used (Figure 7A), the minor peaks increased in intensity but still remained relatively insignificant.

To further ascertain the level of His 393 reactivity in native protein, synthetic N^{im}-(ethoxyformyl)-LDHK was used to "spike" tryptic digests of [^{3}H]DEP-modified tubulin. Surprisingly, the spike eluted coincident with peak 4 (Figure 6). Peak 4 arises primarily from a non-histidyl modification but does contain a small component (~30% of total dpm) that is sensitive to hydroxylamine (Figure 2; Table II). The spiking experiment suggests that this component corresponds to the N^{im}-(ethoxyformyl)-LD^{393}HK fragment. On the basis of this assignment and a careful comparison of the dpm in the hydroxylamine-sensitive component with total dpm in the tryptic digests, we concluded that in the native protein His 393 has a *reduced* reactivity relative to the average histidyl residue (Table II).

Assignment of Peak 4. α- and ϵ-amino groups are possible sites for DEP modification (Lundbald & Noyes, 1984). Previous studies using reductive methylation showed that the N$^{\alpha}$-amino groups at the N-termini of tubulin have a *reduced* reactivity in the native state relative to denatured protein (Szasz et al., 1986). This suggested that peak 4 corresponded to the ethoxyformylated N-termini tryptic fragments, N^{α}-(ethoxyformyl)-Met-Arg, from α- and β-tubulin. To test this possibility, Met-Arg was reacted with DEP and analyzed by HPLC. A single product was obtained (Figure 7B) which eluted at the peak 4 position coincident with N^{im}-(ethoxyformyl)-LDHK (Figure 7A). Further studies confirmed that the product was highly resistant to hydroxylamine consistent with its assignment to N^{α}-(ethoxyformyl)-Met-Arg (Figure 7C).

Discussion

In this study we directly probed the reactivities of histidyl residues in tubulin by analysis of tryptic digests of the [^{3}H]-DEP-treated protein (Figures 2–4). The identification of DEP-modified residues can be complicated (Lundbald & Noyes, 1984). Ethoxyformylation increases peptide hydrophobicity and alters retention times on reverse-phase columns (Figure 5; Biscoglio de Jimenez Bonino et al., 1986). Furthermore, the requirement that DEP concentrations be kept low to minimize side reactions (Miles, 1977) causes the ethoxyformylated fragments to be minor species in proteolytic digests and renders their isolation and characterization difficult (Igarishi et al., 1985). In addition, the DEP reaction is only partially specific for histidyl residues, and labeling at other residues can occur (Miles, 1977). To circumvent these problems, we have used synthetically prepared ethoxyformylated peptides as authentic HPLC retention time standards to identify modified fragments from specific regions in tubulin dimer. We focused on the Lys 394 region of the α-subunit and on the N-termini of tubulin (Figures 6 and 7), two regions previously implicated in microtubule assembly (Szasz et al., 1986; Yaffe et al., 1988).

Lys 394 in the native α-subunit is ca. 15-fold more reactive toward HCHO and $NaCNH_3$ than bulk lysyl residues are. One might similarly expect His 393 to show an enhanced reactivity toward electrophiles. However, His 393 was found to have a *reduced* reactivity toward DEP (Figure 6; Table II). Reactivity difference at the His 393 and Lys 394 sites could reflect steric constraints in the folded protein that limit the accessibility of the larger DEP molecule to His 393 while permitting access of the smaller HCHO and $CNBH_3^{-1}$ molecules to Lys 394. Alternatively, His 393 may be involved in a specific interaction such as a salt bridge which restricts its reaction with DEP. Our data do not distinguish between these two interpretations. The latter interpretation, however, is consistent with our earlier model for the Lys 394 region which hypothesized that this region stabilizes a conformation important for microtubules assembly via salt-bridge interactions with adjacent negatively charged residues (Blank et al., 1986; Szasz et al., 1986).

Lee et al. (1976) proposed that tubulin has several reactive histidyl residues essential for microtubule assembly. Our study supports this suggestion, although we detected only one such

possible residue by HPLC analysis (peak 16, Figure 3). This residue was ~2–3-fold more reactive than the bulk histidyl residues (Table II). While this level of reactivity is modest, it is not atypical for reactive/essential histidyl residues (Igarishi et al., 1985; Lundbald et al., 1984) and falls within the range expected for the essential histidyl residue(s) implicated in the assembly inhibition studies (Figure 1; Table I). We have not as yet identified the reactive residue. This residue elutes in a hydrophobic, probably large, fragment that emerges late in the chromatograms of tryptic-digested tubulin. We anticipate that future studies using synthetic ethoxyformyl peptides as HPLC standards will help establish its identity.

The assembly inhibitions at all DEP concentrations in this study showed major contributions (~60%) from an "irreversible", i.e., hydroxylamine-insensitive, component. This component was attributed to monoethoxyformyl modification of a non-histidyl residue rather than to $N,N^{im\prime}$-bis(ethoxyformyl) modifications of histidyl residues that are also resistant to reversal.[3] This assignment was supported by our observation of insignificant amounts of a $N,N^{im\prime}$-bis(ethoxyformyl) product in the modification studies in LDHK (Figures 5 and 7A). We presumed that the histidyl residue in LDHK is a good model for the bulk histidyl residues in tubulin. Although it is possible that the reactive histidyl residue detected in tubulin undergoes much more extensive diethoxyformylation than LDHK and contributes to the irreversible component, we think this possibility unlikely since this residue is monoethoxylated at rates only 2–3 times faster than bulk histidyl residues are (Figure 3).

Our assignment of the irreversible component to modifications of non-histidyl residue(s) left us with a dilemma. In our analysis (Table I) we assumed a ~1:1 relationship between the extent of assembly inhibition and the extent to which essential residues in tubulin are modified by DEP. On this basis, the inhibition studies appear to implicate at least one highly reactive *non-histidyl* residue in the assembly process. However, we found no evidence for such reactive residues in our HPLC study of digested DEP-modified tubulin. This failure may have resulted from retention of the reactive residues on the HPLC column or, possibly, from loss of the modifications due to chemical lability during the analysis. However, when the gradients were run to ca. 100% acetonitrile, we failed to detect any late-eluting radiolabeled peptides. At present we cannot completely exclude the possibility that the highly reactive modifications were lost due to chemical lability. If so, these modifications would have to be at least an order of magnitude more labile than the N^{im}-ethoxyformyl modification of histidine. To the best of our knowledge there is no precedent for such labile modifications in protein reacted with DEP.[4] We also do not believe that the hydroxylamine-resistant component represents nonspecific denaturation. Lee et al. (1976) have shown, for example, that colchicine binding is largely unaffected by DEP treatment. Further, the major portion of the radiolabel retained in the protein following hydroxylamine treatment is probably contained in the bulk lysyl residues. Methylation of these lysyl residues has little effect on microtubule assembly (Szasz et al., 1982, 1986). It is consequently difficult to understand why ethoxyformylation of these residues should significantly affect assembly.

We have been forced to consider the possibility that the irreversible component results from *substoichiometric* modifications of an essential non-histidyl residue. Antimicrotubule drugs, such as colchicine and podophyllotoxin, form drug–tubulin complexes that coassemble with tubulin and potently inhibit microtubule assembly at substoichiometric ratios (<1:10) of complex to tubulin (Sternlicht & Ringel, 1979; Andreu & Timasheff, 1986). The basis for this process is not understood but may involve long-range distortions of the microtubule lattice. Thus, the essential non-histidyl residue(s) implicated in our DEP inhibition studies need not be more reactive than bulk histidyl residues. Rather, they need to play a functional role whose disruption by DEP causes unusually profound effects on assembly analogous to that observed with drug–tubulin complexes. Further studies are clearly required to test this hypothesis.

We proposed from our analysis (Figures 2–4 and 7) of peak 4 that DEP modified the N-terminal methionine(s) of native tubulin and believe that this N-terminal modification contributes to the assembly inhibition.[5] The N-terminal modifications occurred at rates comparable to that observed for bulk histidyl residues (Figure 2) but less than that observed for N-termini in denatured protein (Figures 3 and 4). Sherman et al. (1983) noted a similar enhancement of N-termini reactivity with tubulin denaturation in their reductive methylation study. These observations strongly suggest that the N-terminal methionines are partially buried or salt-bridged in native protein. Yaffe et al. (1988) showed that α-tubulin expressed in *Escherichia coli* lysates is assembly *incompetent*, whereas this subunit is fully assembly competent when translated from α-tubulin message by use of rabbit reticulocyte lysates. They attributed this loss of assembly competence to formylation of the N-terminal methionine in the *E. coli* lysates. We suggest that the ethoxyformylation of the N-termini noted in this study also generates tubulin subunits/dimers with altered assembly properties. Whether this modification causes substoichiometric inhibition remains to be determined.

Our present study complements the recent studies of Cleveland and co-workers. The N-terminal regions of the nascent tubulin chains play an important role in the autoregulation of tubulin synthesis (Yen et al., 1988; Cleveland, 1988). Cleveland and co-workers have proposed that a regulatory protein (free tubulin dimer?) binds cotranslationally to the exposed first four amino acid residues of the nascent tubulin chains and causes degradation of polysomal-bound tubulin mRNA. We have argued that the N-termini of the native dimer, in contrast to that of the nascent chains, are partly buried or possibly in salt-bridge interactions. This

[3] $N,N^{im\prime}$-Bis(ethoxyformyl)histidyl products undergo rapid ring opening in the presence of hydroxylamine with retention of the ethoxyformyl groups (Avaeva & Krasnova, 1975; Miles, 1977).

[4] Cysteinyl or tyrosyl residues also undergo ethoxyformylation in proteins (Miles, 1977; Lundblad et al., 1984). There is no evidence that these modifications are significantly more labile than histidyl modification, nor is there evidence that such modifications occur in tubulin. The number of free sulfhydryl groups in tubulin as assayed by DTNB appears to be unchanged by DEP (Lee et al., 1976), while spectrophotometric analyses in this laboratory have failed to reveal significant levels of tyrosyl residue modifications by DEP.

[5] We cannot at this time completely exclude the possibility that peak 4 also contained significant, perhaps dominant, contributions from an unidentified fragment bearing a N^{ϵ}-ethoxyformyl modification at a *lysyl* residue. Such a fragment, like the Met-Arg N-terminal derivative, would also be resistant to hydroxylamine. However, we think this alternative assignment to be unlikely. The relative reactivity of the hydroxylamine-resistant component in native peak 4, for example, is about one-third the reactivity of the hydroxylamine-resistant component in denatured peak 4 (cf., Table II). This difference in reactivities is virtually *identical* with that observed for the native denatured *N-termini* of tubulin in reductive methylation experiments (Sherman et al., 1983). The most straightforward interpretion of our modification data and those of Sherman et al. (1983) is that the N-terminal methionines are *partly buried or salt-bridged* in native protein yet capable of reaction with reagents such as DEP and HCHO.

structural feature is apparently important for tubulin function since modification of the N-termini renders tubulin assembly incompetent. Tubulin dimers are present in cells at much higher concentration than the nascent chains. We suggest that this N-terminal structure could also provide a mechanism for sequestering the N-terminal regions, thus ensuring that the dimer does not compete with the nascent chains for the regulatory proteins postulated by Cleveland and co-workers. Futher studies will be required to test this hypothesis.

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